ROLE OF THE 2'.5'-OLIGOADENYLATE SYSTEM DURING BOVINE MATERNAL RECOGNITION OF PREGNANCY

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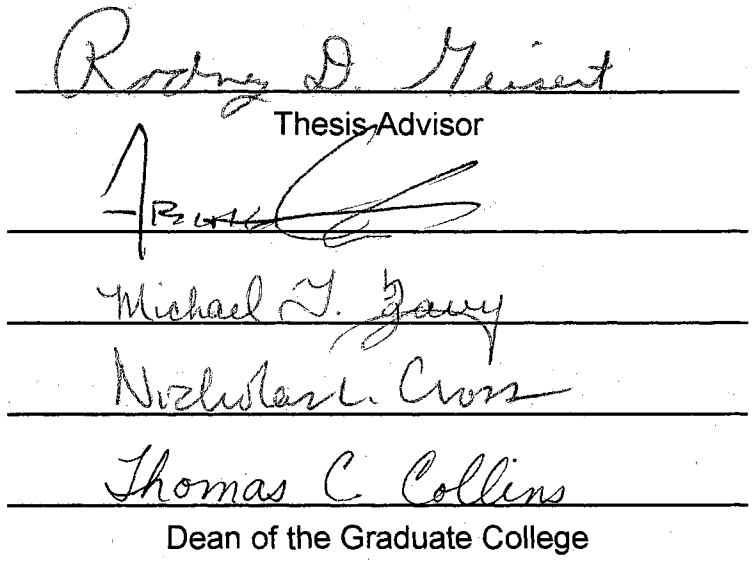

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TABLE OF CONTENTS

Chapter Page

I. INTRODUCTION 1

II. REVIEW OF LITERATURE 3

Histology of the ruminant uterus 3
Introduction 3
Uterine histology 5
Embryonic/Neonatal Development 5
Estrous cycle 9
Pregnancy 11

Control of luteolysis in ruminants 14
Estrous Cycle 14
Role of Progesterone 18
Role of Estrogen 21
Role of $PGF_{2\alpha}$ and Oxytocin 24
The Mechanism of Functional and Structural Luteolysis 28
Maternal Recognition of Pregnancy 33
Ovine Trophoblast Interferon-τ 34
Bovine Trophoblast Interferon-τ 37
Biological Actions of Trophoblast Interferons 38
Interferon-α/β Receptors 41
Biological Actions of Trophoblast Interferons 45
Trophoblast Interferon Endometrial Receptors 48
Trophoblast Interferons and Maternal Recognition of Preg 50
2',5'-Oligoadenylate System 58

III. SECRETORY PROTEIN PROFILES OF PRIMARY AND SUBCULTURED BOVINE ENDOMETRIAL STROMAL CELLS GROWN ON TYPE I COLLAGEN GELS AND PLASTIC 67

Introduction 67

Materials and Methods 70
In vitro Culture of Bovine Endometrial Cells on Different Extracellular Matrices 70
Animals 70
Preparation of Type 1 Collagen-Coated Culture Plates 70
Uterine Cell Isolation 71
Results 72
^{35}S-labeled Secretory Protein Profiles of Stromal Cells Grown on Type I Collagen 72
^{35}S-labeled Secretory Protein Profiles of Stromal Cells Grown on Plastic 77
Discussion 77

IV. THE ROLE OF THE 2',5'-OLIGOADENYLATE SYSTEM DURING BOVINE MATERNAL RECOGNITION OF PREGNANCY 83

Introduction 83
Materials and Methods 86
Animals 86
Uterine Cell Separation 87
Experiment 1a. Preliminary Study (Lipofectin Optimization) 88
Experiment 1b. Intratcellular Protein Profiles-Separated Endometrial Cells 90
Experment 2. Endometrial ^{35}S-labeled Secretory Protein Profiles 92
Experiment 3. Endometrial Prostaglandin $F_{2\alpha}$ Levels in Response to Oxytocin Treatment 94
Prostaglandin $F_{2\alpha}$ Radioimmunoassay 95
Statistical Analysis 96
Results 97
Endometrial ^{14}C-labeled Intracellular Protein Synthesis 97
Endometrial ^{35}S-labeled Secretory Protein Profiles 100
Endometrial Prostaglandin $F_{2\alpha}$ Levels in Response to Oxytocin Treatment 104
Discussion 105

V. GENERAL DISCUSSION 117

REFERENCES CITED 127

APPENDIX A 178
Gross Preparation of Bovine Reproduction Tracts 178
Epithelial Enzyme Incubations 179
Separation of Epithelium 181
Stromal Enzymatic Incubations 184

Isolation of SE, GE and ST 186
Surface Epithelial Rafts 186
Glandular Epithelium 187
Differential Plating (to remove stromal contamination) 188
Stroma 189
Preparation of Enzymes 190
Epithelial Enzyme Wash 191
Stromal Enzyme Wash 192

LIST OF TABLES

Table ..... Page

I. Endometrial Stromal Cells: Matrix Type and Time in Culture. 74

II. Endometrial Stromal Cells Cultured Through 3rd Subculture: Cells Grown on Type I Collagen 76

III. Endometrial Stromal Cells Cultured Through 3rd Subculture: Cells Grown on Plastic 78

IV. Preliminary Study: Lipofectin Optimization 91

LIST OF FIGURES

Figure ..... Page

1. Diagram of proposed mechanism of intracellular events following the binding of IFN-α to the Type 1 IFN cell surface receptor and the subsequent assocation of the 113, 91, 84, 48 kDa subunits to forms interferon-stimulated gene factor 3. (From Schindler et al., 1992)..... 43

2. Diagram of the proposed mechanism of inhibition of luteolysis by conceptus secretion of trophoblast IFN in domestic ruminants during maternal recognition of pregnancy..... 57

3. The mechanism of dsRNA-catalyszed, interferon-induced 2',5'-oligoadenylate [2-5(A)] system in the inhibition to protein synthesis in response to interferon (From Johnstron and Torrence, 1984)..... 59

4. Diagram depicting endometrial stromal cells originally plated on Type 1 collagen or plastic (primary culture) and subsequent subcultivation scheme (numbers correspond to Dish # in Table I). 73

5. Representative 1-D PAGE depicting maximal ^{35}S-labeled secretory protein profile of endometrial stromal cells. Note the 23 secretory proteins indicative of cultured stromal cells..... 75

6. Fig. 6. Inhibition of ^{14}C-labeled intracellular protein synthesis, as a percent of control, in separated bovine endometrial cells, treated with 2-5(A) trimer liposomes and cultured for 6 hours *in vitro*. 98

7. Inhibition of ^{14}C-labeled intracellular protein synthesis, as a percent of control, in separated bovine endometrial cells, treated with 2-5(A) trimer liposomes and cultured for 24 hours *in vitro*. 99

8. Representative autoradiographs of ^{35}S-labeled secretory protein profiles of bovine endometrial explants treated with: (A). $1X10^{4}$ M 2-5(A) oligonucleotide trimer + 1:20 lipofectin (LO3). (B). $1.6X10^{6}$ AVU/ml roIFN-τ (IFN)..... 101

9. Representative autroradiographs of ^{35}S-labeled secretory protein profiles of bovine endometrial explants treated with: (A). $3X10^{-4}$ M AMP + 1:20 lipofectin (LOAMP). (B). DMEM(C). 102

10. Average endometrial $PGF_{2\alpha}$ release (% control) from bovine endometrial explants treated with 2-5(A) liposomes and subsequently treated with oxytocin (OT) *in vitro*. 103

CHAPTER I

INTRODUCTION

Maternal recognition of pregnancy is defined as the period, during early gestation, when the conceptus signals its presence to the mother and inhibits luteolysis to establish pregnancy (Short et al., 1969). Species have evolved different pathways and signals to accomplish maternal recognition of pregnancy (for review see Bazer et al., 1994). In the pig, estradiol secreted from the conceptus on days 11-16 of gestation causes a shift in movement of the luteolysin, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Prostaglandin $F_{2\alpha}$ is secreted toward the uterine vasculature during the estrous cycle. However, following maternal recognition of pregnancy, $PGF_{2\alpha}$ secretion is shifted into the uterine lumen which prevents luteolysis (Bazer and Thatcher, 1977). In the mare, intrauterine migration of the conceptus (10-15X/day) between days 8-16 of gestation allows the conceptus to deliver its signal, which appears to be a small molecular weight protein, throughout both uterine horns (Bazer et al., 1986).

In domestic ruminants, maternal recognition of pregnancy occurs through conceptus secretion of Type I trophoblast interferons (IFN-τ) between days 10-21 of gestation (for review see Bazer et al., 1984, 1989; 1994; Roberts et al., 1989a,b, 1990). Conceptus synthesis and release of trophoblast IFNs activate

biochemical pathways that result in the inhibition of endometrial $PGF_{2\alpha}$ synthesis thus maintaining CL function (Godkin et al., 1982; Helmer et al., 1989b; Salamonsen et al., 1989). Although the signals for maternal recognition of pregnancy are established for ruminants, the cellular mechanism by which they effect the synthesis and secretion of endometrial $PGF_{2\alpha}$ are just now becoming known. The literature review which follows will describe the research which determined the signal for maternal recognition of pregnancy and will provide the information currently available on the cellular mechanism by which luteolysis is inhibited in sheep and cattle.

CHAPTER II

REVIEW OF LITERATURE

Histology of the Ruminant Uterus

Introduction

The uterus is one of the most fascinating and dynamic organs in biology. It is an organ that has been modified, by evolution, to provide an environment conducive for prenatal growth and development in eutherian mammals. The uterus consists of two regions: the endometrium, which is lined with a serous-type of mucosa capable of differentiation, under steroid stimulation, during the estrous cycle and pregnancy; and the myometrium, which consists of smooth muscle and is involved in sperm/conceptus transport, and expulsion of the fetus at parturition (see Mossman, 1977).

The uterus has many functions essential for normal reproductive function during the estrous cycle and pregnancy (see Reynolds, 1965 for review). Contractions of the myometrium, during mating, transport sperm from the site of ejaculation (vagina) to the site of fertilization (oviduct). In nonpregnant domestic animals, the uterus regulates the function of the corpus luteum (CL) by releasing the endometrial luteolysin prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) during the late stages of the

estrous cycle, which kills the CL and allows the animal to return to estrus (McCracken et al., 1972). During pregnancy, the uterus is adapted for the growth and nourishment of the conceptus (Munro et al., 1983; Ferrell, 1991a,b), and during parturition, strong myometrial contractions are responsible for fetal expulsion. The uterus then returns to almost its original shape and condition by the process of involution (see Reynolds, 1965 for review).

In cross-section, the uterus consists of the following layers. The outer layer, termed the perimetrium, consists of coelomic epithelium that is continuous with the mesometrium. Underneath the perimetrium there is a thin layer of connective tissue which encloses the muscle layer of the uterus called the myometrium. The myometrium consists of a layer of longitudinal muscle which overlays a layer of circular muscle. The two myometrial muscle layers are partially separated by the uterine blood vessels. The myometrium encloses the endometrium, which consists of the stratum spongiosum, a loosely organized basal stromal layer and a more compressed layer of connective tissue, the stratum compactum. The stratum compactum is covered by a basement membrane and the luminal epithelium. The endometrial surface epithelium is a pseudostratified tall columnar layer of cells that are continuous with branching uterine glands that extend almost to the myometrium (Marion and Gier, 1971).

In order to understand how embryo-induced changes in the endometrium function to establish pregnancy, it is necessary to review the microanatomy of the

uterus as it is formed during embryogenesis and how it differentiates during the estrous cycle and pregnancy.

Uterine Histology (Embryonic/Neonatal Development)

In ruminants, uterine morphogenesis begins with the fusion of the Mullerian ducts during the first two months of gestation (Marion and Gier, 1971). The Mullerian ducts originate, in the embryo, as an invagination of the coelomic epithelium at the anterior end of the mesonephros immediately lateral to the mesonephric duct (Marion and Gier, 1971). In the bovine, on approximately day 48 of gestation, the Mullerian ducts fuse to form a single tube that penetrates into the urogenital sinus. On approximately day 60 of gestation, there is a breakdown of the single layer of epithelium that separates the two lumens of the fused Mullerian ducts, and forms the common lumen of the uterine body, cervix and vagina. By day 64 of gestation, there is a thickening of the Mullerian duct which differentiate the uterine horn from the oviduct (Marion and Gier, 1971). Morphological differentiation continues to occur rapidly and by day 135 of gestation the characteristic form of the adult uterus is obtained. The characteristic "rams-horned" shape of the adult bovine uterus is due to the rapid growth of the uterus, during gestation, within the limiting confines of the broad ligament (Marion and Gier, 1971).

During fetal development, the luminal surface of the endometrium is organized into irregular rows of nodules consisting of relatively uniform columnar

epithelium (Atkinson et al., 1984; Wiley et al., 1987; Bartol et al., 1988b). In cattle, endometrial caruncles (localized rounded elevations) first appear at approximately 5 months of gestation and apical portion of each endometrial nodule forms the caruncle (Atkinson et al., 1984). The surface epithelium changes from a nodular configuration to a flatter configuration with slight risings formed by caruncles. This change in configuration is due to proliferation of stroma cells and the concomitant increase in connective tissue. This expansion of connective tissue allows the uterus to double in length with very little increase in epithelial cells (Atkinson et al., 1984).

There are no uterine glands present, in the fetus, during the majority of gestation. Uterine glands begin to form in the internodal regions and on the sides of the nodules on approximately day 265 of gestation in the bovine fetus (Atkinson et al., 1984) and during the first two weeks postpartum in the ovine fetus (Wiley et al., 1987; Bartol et al., 1988a,b). During the interval between birth and 3 months post-partum, there is a tremendous increase in bovine gland formation which extend almost to the myometrium (Atkinson et al., 1984). By one year of age, glands are present throughout the endometrial connective tissue except for the region immediately under the caruncular epithelium (Atkinson et al., 1984).

Uterine gland development is an ovary-independent event, in that endometrial glandular development of bilateral and unilateral ovariectomized

neonatal lambs was indistinguishable from that of intact ewes (Bartol et al. 1988a). However, uterine gland development is inhibited by chronic administration of progesterone to neonatal lambs and can be restored through cessation of progesterone treatment (Bartol et al., 1988b). A recent study (Bartol et al., 1995) demonstrated that neonatal exposure to sex steroids has a detrimental effect on later bovine uterine development. These researchers determined that chronic exposure of heifer calves to progesterone and estrogen on or before day 45 postpartum resulted in decreased uterocervical wet weights and altered uterine wall histology, both due to a reduction in the cross-sectional area of the endometrium and myometrium as well as reduced endometrial gland density. It is possible that these changes in endometrial morphology could result in reproductive inefficiency later in life. Further studies must be done to determine if this is the case.

In the sheep, embryonic endometrial organogenesis involves endometrial remodeling characterized by mesenchymal degradation of proteoglycans and glycosaminoglycan (GAG) turnover (Bartol et al., 1988a,b). Glycosaminoglycans are mucopolysaccharides produced by the developing embryonic epithelium of folded (i.e. endometrium), branched (i.e. salivary gland) or lobular (i.e. lung) organs as part of the basal lamina (Bartol et al., 1988b; Bernfield et al., 1973). Studies have implicated GAGs in the normal morphological development of these epithelio-mesenchymal tissues (Bartol et al., 1988b; Bernfield et al., 1973).

Epithelio-mesenchymal organ morphogenesis is characterized by cleft formation, branching and folding of the tissue. These processes require extensive interaction between the epithelium and underlying mesenchyme and involve alterations in the epithelio-mesenchymal interface (Bernfield et al., 1973; Cohn et al., 1977). It has been demonstrated, in embryonic salivary glands, that the areas in the epithelio-mesenchymal interface that are morphogenetically active, are associated with areas where GAG synthesis and degradation are the greatest (Bernfield et al., 1973). Likewise, the epithelio-mesenchymal interface of the ovine fetal endometrium, on day 60 of gestation, is enriched in heavily-sulfated carboxylated GAGs (Bartol et al., 1988a). With the onset of endometrial morphogenetic activity, characterized by cleft formation and development, the quality of the epithelio-mesenchymal interface changes dramatically in that there is a reduction in GAGs. Lateral expansion of primary luminal clefts was also associated with the presence of polyanionic GAGs (Bartol et al., 1988a). During later stages of fetal uterine development, endometrial remodeling, nodular growth, and comparatively rapid lateral cleft expansion is also associated with GAG synthesis and degradation (Bartol et al., 1988a).

The histological differentiation of the uterus coincides with the morphological changes. By day 60 of gestation, the surface epithelium is composed of tall simple columnar cells that are occasionally pseudostratified (Marion and Gier, 1971). Several layers of mesenchymal cells are concentrally

arranged around the epithelium. By day 70 of gestation, three basic histological zones are distinguishable: endometrium - pseudostratified surface epithelium; myometrium - dense unorganized mesenchyme and smooth muscle cells; perimetrium - circularly arranged fibroblasts covered by a cuboidal layer of mesothelium. In the ewe, two subepithelial cell layers are visible during day 55-60 of gestation. These subepithelial cell layers begin differentiate into endometrial stroma and myometrium on approximately day 95 of gestation and is completely differentiated by 4-8 weeks postpartum (Wiley et al., 1987).

Uterine Histology (Estrous Cycle)

The endometrium is made up of three primary cell types: luminal surface epithelium, glandular epithelium and the underlying stromal connective tissue (Findlay et al., 1990). In mature, nonpregnant domestic ruminants, the endometrial epithelial cells are typically simple columnar and are similar between the caruncular and intercaruncular regions (Casida and McKenzie, 1932; Wathes and Wooding, 1980; Atkinson et al., 1984). Branched, coiled endometrial glands descend into the underlying stromal tissue but only in the intercaruncular area (Casida and McKenzie, 1932).

During the estrous cycle there are changes in the appearance of the endometrial epithelium. Early in the ovine estrous cycle (days 1-3), the endometrial surface epithelium is a simple columnar tissue. On day 3-8 of the estrous cycle, the endometrium forms a pseudostratified epithelium which returns

to a simple columnar morphology late in the estrous cycle (days 8-11) (Casida and McKenzie, 1932; Cloud and Casida, 1969).

The endometrial glands show similar morphological changes during the estrous cycle. During proestrus and estrus, the endometrial glands are mitotically active (Ohtani et al., 1993) and during metestrus the glands show increased growth and coiling (Cole and Miller, 1935). There are no progressive changes in the endometrium during the late luteal phase. (Ohtani et al., 1993).

The underlying endometrial stroma cells also undergo morphological change during the estrous cycle (Brinsfield et al. 1974; Ohtani et al., 1993). Utilizing electron microscopy, Brinsfield et al. (1974) determined that the most noticeable change in the ovine endometrium was in the rough endoplasmic reticulum (RER) of the stromal cells. The stromal RER is abundant and extensively dilated on day 0 (estrus) of the estrous cycle. On day 10 to 13 postestrus, the RER decrease in quantity, but are again abundant by day 17 of the estrous cycle. This is contrasted to day 14 of gestation on which the endometrial stromal RER do not increase but there is an increase in stroma mitosis (Ohtani et al., 1993).

It is generally thought that increased quantity and abundance of RER is indicative of increased protein synthesis (Brinsfield et al., 1974; Fawcett. 1981). It appears that the increase in protein synthesis, as indicated by stromal RER appearance during the late estrous cycle, is prevented during similar period of

early pregnancy. Day 14 of gestation is the period of maternal recognition of pregnancy in the ewe, when the conceptus inhibits the endometrial luteolytic mechanism to maintain the CL and establish pregnancy (see Bazer et al., 1994 for review). Through tissue recombination experiments, Cunha et al. (1980) demonstrated that the mesenchyme determines the type of differentiation of the overlying epithelium. It is possible that the increase of RER activity in endometrial stroma cells results in the production of local protein/peptide factors that are responsible for the differentiation of the overlying endometrial epithelium and results in luteolysis. The presence of the conceptus, during the same time of early pregnancy, could prevent this increase in stromal RER activity indirectly by acting though the surface epithelium. There is evidence that the endometrial epithelium can have profound effects on uterine stromal function. In a subsequent tissue recombination study, Cunha et al., (1989) demonstrated that uterine epithelium is involved with the differentiation of uterine mesenchyme into myometrial smooth muscle cells and the associated stromal cells. Research is needed to be done to determine if the inhibition of stromal RER activity, in ruminant species, is involved in the transition of the uterus from cyclicity to pregnancy.

Uterine Histology (Pregnancy)

Intercommunication between the fetal and maternal systems is of paramount importance for the establishment and maintenance of pregnancy in

eutherian mammals. The close association between the maternal and fetal tissues makes this intercommunication possible. Ruminants have a cotyledonary adeciduate type of placenta (Wimsatt, 1950). On the surface of the placenta, the chorionic villi are collected into tufted patches called cotyledons, which are separated from each other by the intercotyledonary (membranous) chorion (Wimsatt, 1950). As described above, the surface of the endometrium is covered by discrete, local rounded elevations called caruncles (Atkinson et al., 1984). During gestation, the cotyledons insert chorionic villi into the caruncle, the combination of the maternal caruncle and the fetal cotyledon is termed the placentome; a single ewe may have 60-100 placentomes in her uterus (see Wimsatt, 1950). Placentomes are the sites of intimate contact between the maternal and fetal circulatory systems, and serve as the sites for the transfer of diffusable substances. Histotroph, produced by the uterine endometrial glands and desquamated epithelium, is taken up by the intercotyledonary areas of the chorion (see Wimsatt, 1950).

Wathes and Wooding (1980) proposed the following timeline for the attachment of the chorion to the endometrium during early pregnancy in the cow. On day 18 of gestation, the chorion and the uterine endometrium come into close proximity of each other. On day 20, attachment has occurred near the fetus, but there is no interdigitation of microvilli of the placental and endometrial epithelium (Wathes and Wooding 1980; King et al., 1980; King et al., 1981). The cuboidal

epithelial cells, present on day 28, are the predominant luminal epithelial cell type characteristic of the rest of pregnancy (Wathes and Wooding, 1980).

An interesting feature of ruminant placentation is the formation of chorionic binucleate cells which migrate from the chorion and fuse with maternal endometrial cells to form a syncytial tissue (Wimsatt, 1950; 1951). Migration of the binucleate cells are more common in the placentome than in the intercotyledonary chorion (Wimsatt, 1950). In the ewe, the binucleate cells migrate into the endometrium and fuse with maternal endometrial cells to form a syncytium within the crypts of the placentome.

By day 28 of gestation there is complete interdigitation between maternal endometrial epithelial cells and fetal chorion cells and cuboidal epithelial cells replace the binucleate cells (Wathes and Wooding 1980; King et al., 1980; King et al., 1981). The binucleate cells die and are phagocytized by the chorion. In the cow, fetal binucleate cells are produced throughout gestation, migrate into the maternal endometrium, but do not form a syncytial layer. This is in contrast to the ewe, where fetal binucleate cells are produced throughout pregnancy, migrate across the microvillar junction, and fuse with and become part of the syncytium throughout pregnancy (Wooding, 1980).

The reproductive system is a highly complex biological system. The uterus plays a central role in reproductive function during the estrous cycle and pregnancy. There are highly regulated interactions between all of the

components of the reproductive system that allows for normal cyclicity and pregnancy.

Control of Luteolysis in Ruminants

Estrous Cycle

Before reviewing the mechanism by which the embryo inhibits luteolysis to establish pregnancy, it is necessary to understand how luteolysis is regulated during the estrous cycle of domestic ruminants. The length of the ovine estrous cycle, during the breeding season, is approximately 16-17 days, with expression of estrus lasting for 24-36 hours. Ovulation usually occurs within 24-30 hours after the onset of estrus (Hansel and McEntee, 1970). The resulting corpus luteum (CL), which forms from the ovulated follicle, increases in weight and secretion of progesterone from day 2-8 which remains constant until day 14 of the estrous cycle. After day 14, CL weight and progesterone secretion rapidly decrease during luteolysis which is usually complete by day 16 of the estrous cycle (Hansel and Echternkamp, 1972).

The length of the bovine estrous cycle is approximately 21-22 days, with expression of estrus occurring over an 18 hour period. Ovulation in the cow usually occurs within 10 hours following the end of estrus (Hansel and McEntee, 1970). As in the ewe, CL weight and progesterone secretion rise rapidly from day 4-12 and remain relatively constant until day 16 postestrus. Luteolysis, in the

cow, is initiated on approximately day 17-18 of the estrous cycle, with females returning to estrus by day 21. Considerable animal to animal variation occurs in the time that luteolysis in initiated, ranging from day 15-23 postestrus (Hansel and Echternkamp, 1972).

In cyclic females, the interestrous interval is tightly controlled by endocrine interactions within the hypothalamic-pituitary-ovarian-uterine axis. Several hormonal mechanisms must act in concert for luteolysis to take place. The current model of luteolysis in domestic ruminants, as proposed by McCracken et al. (1984), involves interactions between progesterone, estrogens, oxytocin and $PGF_{2\alpha}$ In brief, luteal progesterone initially (day 3-10 of the estrous cycle) inhibits endometrial estrogen and oxytocin receptors. After approximately 10 days of progesterone exposure, endometrial progesterone receptors are down-regulated, removing the inhibition for the synthesis of oxytocin and estrogen receptors. At the same time, estrogen from the mid-cycle wave of follicular growth stimulates oxytocin release from the posterior pituitary gland and also increases PG synthetase levels in the endometrium. The neurohypophyseal oxytocin binds to endometrial oxytocin receptors and stimulates release of $PGF_{2\alpha}$ from the endometrium into the uterine vasculature. Through counter-current exchange at the uterine-ovarian pedicle, $PGF_{2\alpha}$ crosses over into the ovarian artery and stimulates release of luteal oxytocin. The endometrial $PGF_{2\alpha}$ and luteal oxytocin form a positive feedback loop that result in luteolysis. After circulating

progesterone levels have declined, as a result of the effects of $PGF_{2\alpha}$, follicular estrogens are needed to complete luteolysis.

Recent studies have confirmed this model of luteolysis in the ewe. Spencer and Bazer (1995) demonstrated, using *in situ* hybridization, that endometrial progesterone receptor (PR) mRNA levels decreased markedly on day 15 of the estrous cycle compared to day 6. A decrease in endometrial PR is associated with increased endometrial estradiol receptor between day 11 and 15 of the estrous cycle, which corresponds with the development of the luteolytic mechanism in ewes (Hansel and Echternkamp, 1972). The decrease in endometrial PR and increase in endometrial estrogen receptor (ER) precedes the induction of oxytocin receptor (OTR) gene expression in the endometrial epithelium (Spencer et al., 1995b). These data collectively suggest, in the ewe, that the down-regulation of the endometrial epithelial PR is required for the up-regulation of endometrial epithelial ER and OTR, that then sets the stage for the positive feedback loop between luteal oxytocin and endometrial $PGF_{2\alpha}$ that results in luteolysis.

Recently, in the cow, the role of luteal oxytocin in the mechanism of luteolysis has become controversial. As stated before, the positive feedback loop between luteal oxytocin and endometrial $PGF_{2\alpha}$ that results in demise of the CL has been established in the ewe (Flint et al., 1990). Historically, these results have been extrapolated to the cow, since both species are domestic ruminants

and have many common reproductive traits. It has been suggested that the cow and ewe may actually differ regarding the role that OT plays during luteolysis (Howard et al. 1990). While it has been demonstrated that administration of oxytocin early in the estrous cycle stimulates the release of $PGF_{2\alpha}$ into the uterine vein, the actual amount of endometrial $PGF_{2\alpha}$ that actually reaches the CL is debatable because veno-arterial transfer of $PGF_{2\alpha}$ could not be demonstrated in these experiments (Milvae and Hansel, 1980). Also, it is debatable whether or not luteal oxytocin is present in the bovine ovary at the time of luteolysis (W. Hansel, personal communication). Kruip et al. (1985) could not immunocytochemically localize oxytocin in the CL during the time of luteal regression; and luteal oxytocin mRNA and plasma oxytocin levels are extremely low at the time of luteal regression (Ivell et al., 1982; Schams et al., 1985). Kotwica and Skarzynski (1993) demonstrated that, though noradrenaline infusion reduced the total amount of oxytocin in day 15 and 16 bovine CL, there was no effect on either spontaneous luteolysis or interestrous interval. These results have led the authors to propose that, in the cow, luteal oxytocin may have a permissive rather than a direct role in the control of luteolysis (Kotwica and Skarzynski, 1993). Clearly, more research is required to clarify the role of luteal oxytocin in the control of luteolysis in the cow.

Role of Progesterone

Progesterone has been suggested to have a major role in the control of luteolysis by affecting the timing of endometrial $PGF_{2\alpha}$ and/or oxytocin receptors during the estrous cycle (Vallet et al., 1990a; Silvia et al., 1991). Secretion of $PGF_{2\alpha}$ during the late estrous cycle occurs only after continuous exposure to progesterone for 10-14 days (see Silvia et al., 1991). Active immunization of ewes against progesterone results in erratic cyclicity and frequently prolonged maintenance of luteal function (French and Spennetta, 1981; Thomas et al., 1985). *In vivo* and *in vitro* endometrial secretion of $PGF_{2\alpha}$ is low in ovariectomized ewes, but can be restored by exogenous progesterone administration for 7-14 days (Louis et al., 1977; Scaramuzzi et al., 1977). Uterine secretion of $PGF_{2\alpha}$ can only be stimulated by administration of oxytocin in ovariectomized ewes or cows after pretreatment with progesterone for 7-10 days (Nukker et al., 1977, Homanics and Silvia, 1988; Lafrance and Goff, 1988). This occurs in the absence of supplementation of either E2 or OT; and there may be differences in OT-stimulated $PGF_{2\alpha}$ release between the intact cow and ewe (Howard et al., 1990).

Administration of progesterone on or before day 4 of the estrous cycle will advance pulsatile secretion of endometrial $PGF_{2\alpha}$ and cause premature luteolysis in the ewe and cow (Zimbelman et al., 1959; Loy et al., 1960; Ginther, 1968;

Harms and Malven, 1969; Ginther, 1970a; Ottobre et al., 1980; Garrett et al., 1988). Treatment of ewes with mifepristone (RU486), a progesterone receptor antagonist, during the first half of the estrous cycle delays the onset of pulsatile $PGF_{2\alpha}$ secretion and delays luteal regression (Morgan et al., 1993).

Prostaglandin (PG) synthetase is the first rate limiting enzyme in the conversion of arachidonic acid to $PGF_{2\alpha}$ (Silvia et al., 1991). Progesterone may play an essential role in stimulating the synthesis of endometrial PG synthetase (Silvia et al., 1991). The amount of immunoreactive PG synthease in uterine epithelium cells is increased when ovariectomized ewes are treated with progesterone (Raw et al., 1988). Treatment of intact ewes with progesterone during the first four days of the estrous cycle advances the increase in concentrations of mRNA encoding for PG synthetase in uterine tissue as well as causing premature luteolysis (Eggleston et al., 1990). In intact ewes, the activity of PG synthetase in ovine endometrial tissues increases between day 11-14 of the estrous cycle (Huslig et al., 1979). The change in PG synthetase activity appears to be due to greater cellular content of the enzyme rather than an increase in specific activity. Concentration of PG synthetase increases between day 4-10 postestrus, with maximum levels attained between day 10-16 of the estrous cycle (Salamonsen and Findlay, 1990).

Progesterone also affects oxytocin receptor content within the uterine endometrium. Vallet et al. (1990a) demonstrated that administration of

progesterone to ovariectomized ewes for at least 7 days resulted in decreased endometrial oxytocin receptor levels. However, progesterone administration for 12 days caused an increase in endometrial oxytocin receptors and increased oxytocin-induced release of $PGF_{2\alpha}$ from the endometrium. It is possible that 12 days of progesterone exposure causes a down-regulation of progesterone receptor and therefore removes the inhibitory effects of progesterone on endometrial synthesis of oxytocin receptors (McCracken et al., 1984; Vallet et al., 1990a). Loss of progesterone inhibition of epithelial oxytocin receptors may be related to the results which indicate that endometrial progesterone receptors in the ewe decline in epithelium after day 15 of the cycle (Spencer and Bazer, 1995).

Uterine secretion of $PGF_{2\alpha}$ can be stimulated by removal of progesterone during the late estrous cycle (Challis et al., 1976, Kindahl et al., 1981; Ottobre et al., 1984; Vincent and Inskeep, 1986). An increase in endometrial concentration of oxytocin receptors was detected within 6 h of progesterone withdrawal in the ewe (Leavitt et al., 1985). Silvia et al. (1991) suggest that the increase in $PGF_{2\alpha}$ concentration observed after progesterone withdrawal results from the increase in the uterine responsiveness to oxytocin due to increased levels of endometrial oxytocin receptors. In a subsequent study, Silvia and Raw (1993) demonstrated that pulsatile secretion of $PGF_{2\alpha}$ is greatly diminished in ovariectomized ewes, but could be partially restored by injection of progesterone and there was a tendency

for combined treatment of progesterone and estradiol to increase the number of pulses. Thus indicating that, while progesterone is a major factor in determining when luteolysis occurs (see Silvia, 1992), estrogens appear to have an important modulatory role in control of luteolysis.

Role of Estrogen

Studies have indicated that estrogen from developing follicles play an important role in the initiation of luteolysis in ruminants. Though progesterone is one of the major regulators for the timing of luteolysis, estrogen can modify oxytocin-induced $PGF_{2\alpha}$ secretion from the endometrium and may continue the final stages of luteolysis after progesterone levels decline in response to $PGF_{2\alpha}$ (McCracken et al., 1984; Vallet et al., 1990a; Silvia et al., 1991).

Studies have indicated that ablation of ovarian follicles, thus removing follicular estrogen, extend the interestrous interval in both ewes (Karsch et al., 1970) and cows (Villa-Godoy et al., 1985). The effects of estrogens on luteolysis are most pronounced in domestic ruminants that have previously been exposed to progesterone for greater than 7 days. Estradiol administration, during the mid-luteal phase of the estrous cycle, will induce premature luteolysis in intact ewes (Stormshak et al., 1969; Ginther, 1970b; Hawk and Bolt, 1970; Cook et al., 1974) or cows (Loy et al., 1960; Greenstein et al., 1958; Wiltbank et al., 1961). An increase in the pulsatile release of endometrial $PGF_{2\alpha}$ is associated with the

premature luteolysis (Ford et al., 1975; Lacroix and Kann, 1986; Hixon and Flint, 1987). Estradiol has been shown to enhance oxytocin-stimulated $PGF_{2\alpha}$ secretion from the endometrium of ovariectomized ewes supplemented with progesterone both *in vitro* and *in vivo* (McCracken, 1980; Lafrance and Goff, 1988; Vallet et al., 1990a). Administration of estradiol increases the rate of turnover of arachidonic acid in lipid storage pools (Toth and Hertelendy 1986) and *in vitro* conversion of arachidonic acid to $PGF_{2\alpha}$ is enhanced by estradiol administration (Ham et al., 1975; Naylor and Poyser, 1975; Wlodawer et al., 1976; Schatz et al., 1987). These data indicate that estrogens may stimulate PG synthetase activity.

Vallet et al. (1990a) demonstrated, in the ewe, that estrogen has a biphasic effect on endometrial oxytocin receptor content during the estrous cycle. During the first two days of estrogen exposure (i.e. the mid-luteal phase ovarian follicular wave) there was a stimulatory effect on endometrial oxytocin receptor levels. After 5 days of estrogen exposure, an inhibitory effect on endometrial oxytocin levels was observed that corresponds to luteolysis. These researchers have also indicated that, in the ewe, estrogens may modify $PGF_{2\alpha}$ release from the endometrium. A combination of estradiol and progesterone may generate the high-amplitude, short-duration peaks of oxytocin-induced $PGF_{2\alpha}$ secretion on days 15-16 of the estrous cycle (Vallet et al., 1990a). High amplitude, short duration pulses of $PGF_{2\alpha}$ secretion have been shown to be the most effective in

causing luteolysis in ewes (Schramm et al., 1983). Collectively, these data suggest that estrogen modifies the mode of $PGF_{2\alpha}$ release from the endometrium.

Endometrial estradiol receptor (ER) gene expression appears to be key for control of luteolysis. During the estrous cycle, steady state ER mRNA levels are highest on day 1; decline between days 1 and 6; and increase between days 11 and 15 of the estrous cycle (Spencer and Bazer, 1995). By using *in situ* hybridization, Spencer and Bazer (1995) demonstrated that ER mRNA abundance decreased in the endometrial epithelium, stroma and myometrium between days 1 and 6 of the estrous cycle. Endometrial ER mRNA levels were low or undetectable in all uterine cell types, but increased during days 13 to 15 of the estrous cycle; first in the endometrial luminal and shallow glandular epithelium and then in the deep glands and stroma (Spencer and Bazer, 1995). Immunocytochemical localization of the ER protein, for the most part, mirrors the mRNA data, showing the same profiles during the estrous cycle and early pregnancy (Spencer and Bazer, 1995). It has been demonstrated, in ovariectomized ewes injected with progesterone and estrogen, that ER mRNA/protein levels are abundant in the endometrial luminal and glandular epithelium (Spencer et al., 1995a). In cyclic ewes treated with a luteolytic dose of E_2 benzoate, endometrial ER were increased by 12 hours in the epithelium and 48 hours in the stroma post-injection. Endometrial OTR increased by 12 hours

and were maximal by 36-48 hours post-injection (Spencer et al., 1995b). In recombinant ovine interferon-τ (roIFN-τ) infused ewes, endometrial ER and OTR did not increase after E_2 injection (Spencer et al., 1995b). This indicates that the induction of luteolysis in E_2-treated ewes involves sequential increase in ER mRNA and protein in the endometrial epithelium that precedes the maximal increase in endometrial OTR (Spencer et al., 1995b).

Role of $PGF_{2\alpha}$ and Oxytocin

During the late luteal phase of the estrous cycle of domestic ruminants, luteal regression is caused by pulsatile (5-8 pulses/24h) secretion of $PGF_{2\alpha}$ from the uterine endometrium (McCracken et al., 1970; McCracken et al., 1972; Nancarrow et al., 1973 Thorburn et al., 1973; Peterson et al., 1975; Kindahl et al., 1976, Inskeep and Murdoch, 1980; Fredriksson et al., 1984). Pulsatile release of $PGF_{2\alpha}$ is usually evident just prior to the onset of luteal regression, before any significant decline in serum progesterone concentrations (Zarco et al., 1988). Silvia et al. (1991) proposed two mechanisms which may explain the pulsatile release of $PGF_{2\alpha}$ from the uterine endometrium. First, it is possible that the endometrium possesses an endogenous pacemaker that triggers pulsatile release of endometrial $PGF_{2\alpha}$ at 4-8 hour intervals. The second mechanism proposed is that the uterus receives an external signal delivered in a pulsatile

manner from another endocrine tissue, possibly oxytocin secreted from the neurohypophysis.

Endometrial secretion of $PGF_{2\alpha}$ is stimulated by oxytocin administration during the estrous cycle in many species (Roberts and McCracken, 1976; Leaver and Seawright, 1982; Lafrance and Goff, 1985; Goff et al., 1987; Kieborz et al., 1990; Jenkin, 1992). Several studies have indicated that pulses of oxytocin or oxytocin-associated neurophysin occur concurrently with pulses of $PGF_{2\alpha}$ during luteolysis in both sheep (Fairclough et al., 1980; Webb et al., 1981; Flint and Sheldrick, 1983; Jenkin, 1992) and cattle (Vighio and Liptrap, 1986). The oxytocin involved with endometrial $PGF_{2\alpha}$ secretion is synthesized and released from the corpus luteum (Walters et al., 1984; Hooper et al., 1986; Moore et al., 1986); and in the ewe the majority of luteal oxytocin is contained within the large luteal cells (Rodgers et al., 1983). Recently it has been demonstrated in cows, on day 8 of the estrous cycle, that secretion and replenishment of luteal OT is not dependent upon *de novo* protein synthesis but is probably due to post-translational processing of a pro-hormone (Abdelgadir et al., 1994); whether or not this is the case at the time of luteolysis remains to be determined.

McCracken et al. (1984) suggested, in the ewe, that endometrial $PGF_{2\alpha}$ and luteal oxytocin form a positive feedback loop that ultimately culminates in luteolysis. During spontaneous episodes of $PGF_{2\alpha}$ secretion, concentrations of

$PGF_{2\alpha}$ in the utero-ovarian vein increase before any change in serum oxytocin is detected (Moore et al., 1986), implying that activation of the feedback loop begins at the uterus. The mechanism which activates the positive feedback loop is not presently known. As previously indicated, Silvia et al. (1991) suggested that neurohypophyseal oxytocin serves as an the external signal to initiate $PGF_{2\alpha}$ release from the endometrium. In the ewe, pulses of $PGF_{2\alpha}$ are frequently associated with increases of oxytocin from both the CL and posterior pituitary (Hooper et al., 1986). Likewise, McCracken et al. (1991) theorize that it is neurohypopheseal oxytocin secreted in a pulsatile manner, that serves as the initial pulse generator signal of $PGF_{2\alpha}$ secretion from the endometrium.

The 4-8 hour interval between successive pulses of $PGF_{2\alpha}$ may be regulated by transient changes in uterine responsiveness to oxytocin and luteal responsiveness to $PGF_{2\alpha}$ (see Silvia et al., 1991). Acute exposure of the endometrium to oxytocin induces refractoriness to further stimulation for 6 h in ewes (Sheldrick and Flint, 1986). Uterine refractoriness to oxytocin can be maintained for several days during chronic exposure to oxytocin causing an extension of estrous cycle length in ewes (Flint and Sheldrick, 1985) and cows (Kotwica et al., 1988; Gilbert et al., 1989; Howard et al., 1990; Lutz et al., 1990). The ability of $PGF_{2\alpha}$ to stimulate luteal oxytocin secretion is suppressed after acute exposure to $PGF_{2\alpha}$ (Lamsa and McCracken, 1990) and the suppression

can only occur late in the estrous cycle (Roberts and McCracken, 1976; Leaver and Seawright, 1982; Lafrance and Goff, 1985; Goff et al., 1987; Kieborz et al., 1990). Several studies have indicated that concentrations of endometrial oxytocin receptors increase at the time of luteal regression (Sheldrick and Flint, 1985; Meyer et al., 1988; Soloff and Fields, 1989; Fuchs et al., 1990). In the ewe, OTR mRNA first appears, specifically, in the endometrial surface epithelium on days 14-15 of the estrous cycle (Stevenson et al., 1994) and the uterus is unable to secrete $PGF_{2\alpha}$, in response to OT, until at least day 14 of the estrous cycle (Silvia et al., 1992). Therefore all of the components necessary for the positive feedback loop between luteal oxytocin and endometrial $PGF_{2\alpha}$ that culminates in luteolysis are present only during the late luteal phase of the estrous cycle. While the positive feedback loop of oxytocin and $PGF_{2\alpha}$ result in luteolysis, progesterone is the major hormone regulating when luteolysis can occur in the estrous cycle of domestic ruminants (Vallet et al., 1990b).

It appears that there are cell-specific profiles of $PGF_{2\alpha}$ secretion from bovine endometrial cells. It has been demonstrated in separated bovine endometrial cells, that the endometrial epithelial cells primarily secrete $PGF_{2\alpha}$ whereas the endometrial stromal cells secrete primarily PGE_2 (Fortier et al., 1988; Asselin et al. 1996). Also, only cultured endometrial epithelial cells and not stromal cells respond to OT treatment with increased $PGF_{2\alpha}$ secretion (Asselin et al., 1996). This indicates that the endometrial epithelial cells are the main target

for OT to stimulate endometrial $PGF_{2\alpha}$ secretion. However, it should be noted that this *in vitro* study was performed with endometrial cells from cows slaughtered during the early days of the estrous cycle (Asselin et al., 1996). It remains to be seen if OT-induced $PGF_{2\alpha}$ secretion is present in cultured bovine endometrial epithelial cells from cows slaughtered near the time of luteolysis.

The Mechanism of Functional and Structural Luteolysis

During the last several years much of the research concerning the mechanism of luteolysis has focused on the actual functional and physical destruction of the CL. As stated before, $PGF_{2\alpha}$ is the luteolytic signal in domestic ruminants (McCracken et al., 1972; see Pate, 1994). It has been suggested that while $PGF_{2\alpha}$ initiates functional luteolysis (decreased progesterone production), while additional biological mechanisms are necessary for structural luteolysis (luteal tissue degradation) (see Pate, 1994). A major effect of $PGF_{2\alpha}$ on luteal cells is to inhibit lipoprotein utilization which limits the precursor for steroidogenesis (see Pate and Townson, 1994) resulting in functional luteolysis. The inhibition of luteal steroid synthesis, mediated by $PGF_{2\alpha}$, which proceeds structural luteolysis, is reversible until the actual structural destruction of the CL (see Pate, 1994).

There is evidence that a complex interactions of immune cells and associated cytokines, induced and modulated by $PGF_{2\alpha}$ during the late estrous

cycle, is involved in the mechanism of both structural and functional luteolysis (see Pate, 1994; Pate and Townson, 1994).

Recently it has become evident that apoptosis, or programmed cell death is the biological mechanism involved in structural luteolysis (see Pate and Townson, 1994). Apoptosis is morphologically characterized by nuclear and cytoplasmic condensation of individual cells (shrinkage) and detachment from the extracellular matrix (see Kerr et al., 1972; Buja et al., 1993). This is followed by loss of the nuclear membrane, fragmentation of nuclear chromatin and subsequent formation of apoptotic bodies (condensed nuclear material and cytoplasm). These apoptotic bodies are phagocytized by adjacent cells and do not usually induce an inflammation response (see Buja et al., 1993). Apoptosis is initiated by an intracellular Ca^{2+} influx and subsequent activation of endogenous endoribonucleases which fragments cellular internucleosomal DNA into multiples of 185-bp fragments termed oligonucleosomes (Martin et al. 1994; Juengel et al., 1993). This is contrasted to necrosis, which is cell death due to injury, and involves morphological and metabolic changes that are exaggerated by the degradative reactions following cell death and usually induces a inflammation response (see Buja et al., 1993).

As stated before, apoptosis appears to be involved in the structural destruction of the CL. Almost two decades ago, O'Shea et al. (1977) indicated, in the ewe, that the endothelial cells that make up the capillaries of the CL undergo

apoptosis during luteolysis. Subsequent studies have shown that, in addition to the endothelial cells, the parenchymal (large and small luteal cells) and fibroblast also undergo apoptotic cell death during luteolysis (Sawyer et al., 1990; Juengel et al., 1993). Furthermore, Sawyer et al. (1990) indicated that, while evidence of apoptosis in all three luteal cell types is evident by 36 h post-$PGF_{2\alpha}$ treatment; the luteal endothelial cells were the first to undergo apoptosis; in that nuclear changes indicative of apoptosis was first evident in the endothelial cells 12 h post-$PGF_{2\alpha}$ treatment.

Recently, it has been demonstrated that oligonucleosome formation, a positive indication of apoptosis, occurs in the CL during both spontaneous and $PGF_{2\alpha}$-induced luteolysis in the cow (Juengel et al. 1993) and ewe (Rueda et al., 1995a; Murdoch, 1995). These researchers also state that oligonucleosome formation does not appear until after the fall of luteal and serum progesterone, confirming that there is a distinct separation between structural and functional luteolysis. In a subsequent study in cattle, Juengel et al. (1994) determined that luteolysis is associated with an increase in luteal tissue inhibitor of metalloproteinase-1 and -2(TIMP-1, TIMP-2) which precedes oligonucleosome formation indicative of luteolysis (Juengel et al., 1993). Metalloproteinases (i.e. collagenase, gelatinase, stromelysin) degrade the proteinaceous components of the extracellular matrix and have an important role in tissue remodeling (Juengel et al., 1994). TIMPs regulate metalloproteinase activity by inhibiting the function

of metalloproteinases. During luteolysis, metalloproteinases and TIMPs could be involved in dissociation of apoptotic cells from the extracellular matrix (Juengel et al., 1994) as well as other mechanisms involved in structural demise of the CL.

Recent studies have begun to elucidate the mechanism of activation of luteolysis and associated apoptosis in luteolysis. Several studies have demonstrated that oxidative stress, caused by reactive oxygen intermediates (i.e. O_2^-; H_2O_2) are involved in the triggering of functional luteolysis (Sawada and Carlson, 1994; Musicki et al., 1994) as well as apoptosis (see Buttke and Sandstrom, 1994). Rueda et al. (1995b) suggest that oxidative stress in luteal cells at the time of luteolysis may result from reduced expression of enzymes which function to protect cells from damage from reactive oxygen intermediates. These authors have implicated several enzymes in the protection of luteal cells from the destructive effects of reactive oxygen species; including superoxide dismutase (SOD) which functions to metabolize reactive oxygen intermediates to H_2O_2; as well glutathione peroxidase (GSHPx) and catalase which function to rapidly convert H_2O_2 to H_2O (Rueda et al., 1995b). Isolated bovine CL from day 21 of the estrous cycle had high levels of internucleosomal DNA cleavage characteristic of apoptosis; as well as nondetectable levels of mRNA of SOD and catalase, which indicate a greatly reduced capacity to metabolize reactive oxygen intermediates, thus leaving the luteal cells susceptible to damage (Rueda et al., 1995b). This is contrasted to isolated CL from day 21 of gestation which had no

internucleosomal DNA cleavage and very high levels of the message for SOD, GSHPx and catalase; indicating that the CL of pregnancy is protected from the damaging effects of reactive oxygen intermediates (Rueda et al., 1995b). These results have led these authors to theorize that oxidative stress in luteal cells at the time of luteolysis, which results from reduced expression of enzymes which function to protect cells from damage from reactive oxygen intermediates, cause the luteal cells to undergo apoptosis and subsequent structural luteolysis (Rueda et al., 1995b). Further studies are needed to fully determine the cause and effect relationship between oxidative stress and luteolysis.

A recent study, in the ewe, has demonstrated a temporal relationship between decreased progesterone levels during $PGF_{2\alpha}$-induced luteolysis, apoptosis and induction of heat shock protein-70 (HSP-70) in luteal tissue (Murdoch, 1995). The rapid increase in HSP-70 occurred only in the prostaglandin-responsive large luteal cells, and preceded functional and structural luteolysis. Heat shock proteins are involved in intracellular protein folding and trafficking and are induced during times of cellular stress (Morimoto, 1993). Further research is needed to determine if and how induction of heat shock proteins is involved in the initiation of luteolysis

In order for the conceptus to survive, it must inhibit luteolysis to maintain progesterone secretion for at least a portion of gestation. Ruminant conceptuses

accomplish CL maintenance through secretion of Type I trophoblast interferons during early gestation.

Maternal Recognition of Pregnancy

In order to successfully establish pregnancy, the mechanism leading to luteolysis during the estrous cycle must be inhibited and a suitable uterine environment provided for the developing conceptus. It is clear that specific biochemical signals secreted by the conceptus induce the maternal uterus to maintain CL function and create a uterine environment favorable to conceptus attachment and growth. This phenomenon has been termed "maternal recognition of pregnancy" (Short et al., 1969).

In domestic ruminants, the conceptus prevents luteolysis by inhibiting the synthesis and/or secretion of the uterine luteolysin, $PGF_{2\alpha}$ (McCracken et al., 1972; McCracken et al., 1984; Thatcher et al., 1986). The ruminant conceptus synthesizes and secretes a number of polypeptides during the period of trophoblast elongation which occurs between day 12-15 in the ewe and day 16-18 in the cow. These specific conceptus polypeptides have been demonstrated to be the signal for maternal recognition of pregnancy in the ewe and cow (see Bazer et al., 1994) and have been termed ovine trophoblast interferon-τ (oIFN-τ) and bovine trophoblast interferon-τ (bIFN-τ) respectively. The biological actions of trophoblast IFNs have been extensively reviewed (see Bazer et al., 1986;

Bazer et al., 1989; Bazer et al., 1994; Leaman et al., 1992; Roberts et al., 1989a,b; Roberts et al., 1996; Stewart et al., 1989a,b; Thatcher et al., 1986; Thatcher et al., 1989a). The following will review the current theory of maternal recognition of pregnancy via the action of trophoblast IFNs in the ewe and cow.

Ovine Trophoblast Interferon-τ

Moor and Rowson (1966) first demonstrated that removal of sheep conceptuses (embryoblast and extraplacental membranes) from the uterus on day 13 or later of gestation resulted in extended CL lifespan, while removal of the conceptus prior to day 13 resulted in normal estrous cycle length. Intrauterine infusion of day 14-15 ovine conceptus homogenates into cyclic ewes significantly extended the interestrous interval (Rowson and Moor, 1967; Ellinwood et al., 1979). However, intrauterine infusion of day 21-23 (Martal et al., 1979) or day 25 (Rowson and Moor, 1967) sheep placental homogenates were not effective in extending the interestrous interval. These results indicated that the day 13-15 sheep conceptus produces a substance that extends estrous cycle length. The authors concluded that the substances were proteineous in nature since treatment of conceptus homogenates with heat or proteases effectively removed the ability to extend cycle length (Rowson and Moor, 1967; Martal et al., 1979).

Godkin et al. (1982) identified and purified the major secretory product released by the early ovine conceptuses following *in vitro* culture. The product was subsequently termed ovine trophoblast protein-1 (oTP-1) (Godkin et al.,

1984b). Biosynthetic studies (Godkin et al., 1982; Hansen et al., 1985) determined that oTP-1 is produced in large quantities between day 13-23 of gestation, specifically by the trophoblast of the ovine conceptus.

The secretion of oTP-1 coincided with elongation of the conceptus from a spherical morphology on days 12-13 (3-5 mm in diameter) to an elongated filamentous form (15-100 mm in diameter) by day 15 of gestation (Ashworth and Bazer, 1989). oTP-1 consists of 3-4 isoforms with a molecular weight of approximately 19 kDa (Godkin et al., 1984a; Anthony et al, 1988). A single day 16 conceptus is estimated to synthesize 100-500 μg of oTP-1 within a 24 h culture period *in vitro* (Ashworth and Bazer, 1989). oTP-1 appears to act locally within the uterus, as it is detectable in uterine flushings but not in maternal serum during pregnancy (Godkin et al., 1984b; Kazemi et al., 1988).

oTP-1 is the only protein secreted by the conceptus which is involved in preventing luteolysis. Intrauterine infusion of oTP-1 into the lumen of nonpregnant ewes between days 12-20 of the estrous cycle prolongs the lifespan of the CL (Godkin et al., 1984a; Vallet et al., 1988). Conceptus secretory products, from which oTP-1 had been removed by immunoabsorption, failed to extend the interestrous interval of the cyclic ewe (Vallet et al., 1988).

Conceptus synthesis of oTP-1 has been confirmed by Northern blot analysis of oTP-1 mRNA from total cellular RNA of day 12-22 ovine conceptuses (Hansen et al., 1988; Stewart et al., 1989a). Studies have indicated that oTP-1

gene expression is significant between days 13-21 of gestation, with maximal synthesis on day 14 of gestation (Hansen et al., 1988; Stewart et al., 1989a). From the peak on day 14, oTP-1 mRNA decreases 5 fold by day 16, 15 fold by day 18 and 170 fold by day 20 of gestation (Hansen et al., 1988). These results have been confirmed by the more sensitive procedure of in situ hybridization (Farin et al., 1989). On day 13-21 of gestation, all of the oTP-1 hybridization signal was associated with the trophectoderm, with essentially no hybridization in the inner-cell mass, yolk sac or extra-embryonic endoderm (Farin et al., 1989).

Imakawa et al. (1987) cloned and sequenced cDNA to oTP-1. These researchers discovered that oTP-1 has 40-55% sequence homology to the 166 amino acid (AA) bovine interferon-αI (IFN-αI) and 65-70% homology to the 172 AA bovine IFN-ω. Since trophoblast IFNs are produced in large quantity only by the trophoblast and have type-specific functions, they have been given their own classification of interferon-τ (IFN-τ) (Roberts et al., 1992; Bazer et al., 1994). oIFN-τ is encoded by multiple, distinct genes that appear to be expressed in a tissue-specific manner, and are differentially and developmentally regulated during pregnancy (Leaman et al., 1992; Nephew et al., 1993). However, it is not yet known if the different oIFN-τ gene products have specialized biological effects during maternal recognition of pregnancy.

Bovine Trophoblast Interferon-τ

The bovine conceptus undergoes trophoblastic elongation on approximately day 15-16 of pregnancy (Betteridge et al., 1978; Betteridge et al., 1980). Northey and French (1980) demonstrated that removal of conceptuses from day 17 pregnant cows resulted in extended interestrous interval compared to cows that had conceptuses removed on day 13. *In vitro* culture media from day 16 conceptus homogenates extend CL function when infused into the uterine lumen of cyclic cows on days 10-16 (Betteridge et al., 1978; Betteridge et al., 1980). Intrauterine infusion of day 17 and 18 conceptus homogenates extended luteal function in nonpregnant cows compared to those infused with day 12 homogenates (Northey and French, 1980). Bartol et al. (1985) characterized the proteins secreted by elongating bovine conceptuses, and the major protein involved in CL maintenance was termed bovine trophoblast protein-1 (bTP-1) (Helmer et al., 1987), later changed to bovine IFN-τ (bIFN-τ) (Roberts et al., 1992). Unlike oIFN-τ, bIFN-τ is a group of glycoproteins that consists of at least two groups of three-four isoforms (Mr 22 kDa and 24 kDa) with pI = 6.5-7.0 (Helmer et al., 1987; Imakawa et al., 1989; Stojkovic et al., 1995). bIFN-τ is produced by the mononucleated cells of the trophectoderm (Lifsey et al, 1989), and specifically packaged within the Golgi complex and associated clear vesicles on days 18-23 of gestation (Morgan et al., 1993). Antiserum to oIFN-τ immunoprecipitated all isoforms of bIFN-τ which indicates that bIFN-τ and oIFN-τ

are structurally related. As with oIFN-τ, bIFN-τ has been shown to be a member of the 172 amino acid IFN-ω family (Imakawa et al., 1989; Stewart et al., 1990; Hansen et al., 1991), having between 65-70% homology to bIFN-ω and 40-55% to bIFN-α. The ruminant trophoblast IFNs are highly homologous, in that oIFN-τ and bIFN-τ transcripts are approximately 90% homologous to each other and their inferred amino acid sequence are approximately 80% identical (Roberts et al., 1992).

Biological Actions of Interferons

It was surprising to discover that the signal for maternal recognition of pregnancy in ruminants was an interferon. Since the trophoblast IFNs share many of the biological effects indicative of general Type I IFNs, as well as effects unique to oIFN-τ and bIFN-τ, it is necessary to review of the general biological functions of Type I IFNs.

Interferons are cytokines produced by cells of the immune system which have a variety of antiviral and antiproliferative effects (Pestka et al., 1987). There are three major types of interferon: Leukocyte or alpha interferon (IFN-α), fibroblast or beta interferon (IFN-β) and immune or gamma interferon (IFN-γ) (Pestka et al., 1987). Both IFN-α and IFN-β are referred to as Type I IFNs, while IFN-γ is referred to as Type II IFN.

Many different cell types produce IFN-α. Namalwa cells, peripheral blood leukocytes (PBMC) and human natural killer cells (Ortaldo et al., 1983; Ortaldo et al., 1984; Pestka et al., 1987) are just a few of the cell types that are capable of secreting IFN-α. As a family, IFN-α are 166 amino acid polypeptides, with molecular weight of 16-27 kDa and pIs ranging from 5.5-6.5. The amino acid sequence of IFN-α are well conserved, with approximately 70% amino acid homology between IFN-α from different cell types and species (Pestka et al., 1987).

Human IFN-β was first derived from diploid fibroblasts (Knight, 1976). Interferon-β consists of one glycoprotein species (Pestka et al., 1987) with molecular weight of approximately 20 kDa and a specific antiviral activity of 2-5 X 10^8 units/mg protein. The amino acid sequence of IFN-β is similar between humans, mice and cattle (Pestka et al., 1987).

Human IFN-γ have been purified from peripheral blood lymphocytes. It consists of three forms of glycoprotein with molecular weights of 16, 20 and 25 kDa respectively and specific antiviral activity of 1-25 X 10^7 units/mg protein (Yip et al., 1982; Braude, 1984). The difference in molecular weight between the three forms of IFN-γ appears to be due to differences in the extent of glycosylation (Friedlander et al., 1984; Rinderknecht et al., 1984; Braude, 1984).

Interferons function biologically to protect the organism from viral infection and tumor growth (Pestka et al., 1987). Type I IFNs are characterized by their antiviral, antiproliferative and cytotoxic effects (Eife et al., 1981; Evinger et al., 1981; Evinger and Pestka, 1981; Ortaldo et al., 1983; Ortaldo et al., 1984; Fulton et al., 1986; Cross et al., 1990). Type I IFNs inhibit a wide variety of viruses in a range of cell types. Recombinant bovine and human IFN-α and IFN-β reduce the virus yield of bovine herpes virus-1, parainfluenza-3, respiratory syncytial virus and vesicular stomatitis virus (VSV) in MDBK, human fibroblast and Chinese hamster ovary cells (Evinger et al., 1981; Evinger and Pestka, 1981; Fulton et al. 1986; Cross et al., 1990). Type I IFNs have very powerful antiproliferative effects. A low dose (3-5 U/ml) of IFN-α inhibits cell multiplication by approximately 50% in human Daudi cells (Evinger and Pestka, 1981). To inhibit tumor growth, IFNs destroy cells by rapidly augmenting the cell-mediated cytotoxicity of natural killer cells and monocytes (Evinger and Pestka, 1981; Ortaldo et al. 1983; Ortaldo et al. 1984; Pestka et al., 1987). There is a positive correlation between antiviral activity of IFN-α and its ability to augment natural killer cell activity (Ortaldo et al. 1983; Ortaldo et al. 1984).

Another major effect of Type I IFNs is to modulate the expression and shedding of tumor-associated (Giacomini et al., 1984) and major histocompatibility complex (MHC)-associated (Pestka et al., 1987; Morris, 1990) cell surface antigens. Alterations in surface antigens may be an important

mechanism by which IFNs modulate intercellular interactions to inhibit tumor growth. There is no apparent correlation between antiviral activity and the shedding of tumor-associated (Giacomini et al., 1984) or MHC-associated antigens (Morris, 1990).

Interferon-α/β Receptors

Interferons exert their actions through their specific cell surface receptors (Friedman, 1967). IFN-α and IFN-β bind to the same specific receptor, while IFN-γ binds to a separate receptor (Pestka et al., 1987). The IFN-α/β receptor is a cell surface receptor with molecular weight of approximately 150 kDa (Joshi et al., 1982; Faltynek et al., 1983). A second receptor complex with molecular weight of approximately 300 kDa has been identified in similar studies (Pestka et al., 1987). The binding of IFN-α or IFN-β to their receptor is saturable. In general, there are approximately 2×10^2 to 6×10^3 IFN-α/β receptors/cell with a dissociation constant of 1×10^9 (Peska et al., 1987). When IFN-α binds its receptor at 37°C it is immediately internalized by receptor-mediated endocytosis as has been demonstrated both biochemically (Branca and Baglioni, 1982; Branca et al., 1982; Zoon et al, 1983; Sarkar and Gupta, 1984) and with electron microscopy (Kushnaryov et al., 1982; Zoon et al., 1983; Kushnaryov et al., 1985). The binding of Type I IFNs to the IFN-α/β receptor stimulates down-regulation of the IFN receptor. Several studies have indicated a decrease in cell-surface IFN receptors when cells were incubated with specific IFN ligands (Branca and

Baglioni, 1982; Branca et al., 1982; Zoon et al., 1983; Sarkar and Gupta, 1984). Down-regulation of IFN-α/β receptors can be stimulated following incubation with low concentrations of IFN-α or IFN-β but not with IFN-γ (Branca and Baglioni, 1982; Sarkar and Gupta, 1984). Several studies have demonstrated recovery of normal IFN receptor levels after down-regulation. Recovery of original IFN receptor levels can be inhibited by cycloheximide, indicating that protein synthesis is needed for IFN receptor synthesis (Aguet and Blanchard, 1981; Branca and Baglioni, 1982; Sarkar and Gupta, 1984). In MDBK cells, there appears to be a lag of approximately 4 hours between the removal of IFN and the recovery of normal levels of IFN receptors after down-regulation (Branca and Baglioni, 1982). These researchers demonstrated that this is not true for all cell types. The IFN-α/β receptors appear to have a half-life of 2-4 hours in Daudi, MDBK, or murine L1210 cells (Aguet and Blanchard, 1981; Branca and Baglioni, 1982).

One of the most important discoveries concerning IFNs is the recent elucidation of the intracellular mechanisms associated with IFN-α binding to its cell surface receptor. Schindler et al. (1992) proposed the following mechanism for intracellular responses to IFN stimulation (see Figure 1). In this biological mechanism, IFN-α binds to its cell surface receptor and stimulates an intracellular protein tyrosine kinase (PTK). The PTK then phosphorylates the 3 separated subunits of the interferon-stimulated gene factor 3-α (ISGF-3). The 3 phosphorylated subunits (113, 91, 84 kDa respectively) interact, in the cytoplasm,

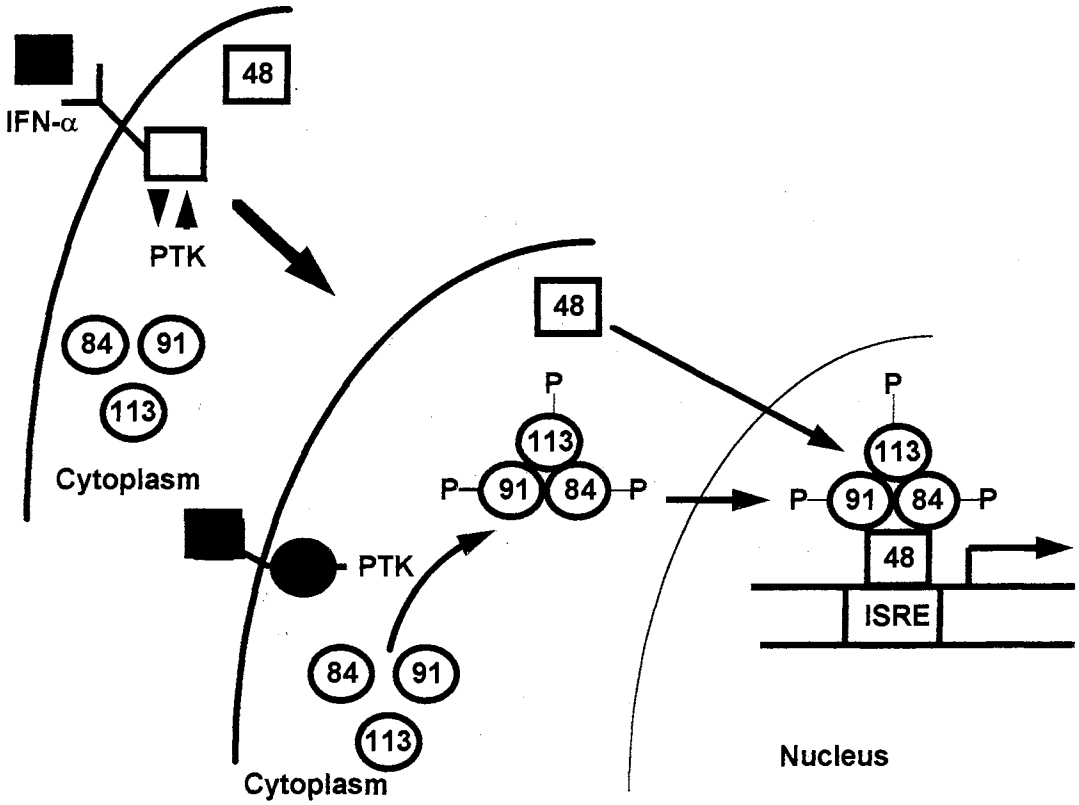

Figure 1. Diagram of proposed mechanism of intracellular events following the binding of IFN-α to the Type 1 IFN cell surface receptor and the subsequent association of the 113, 91, 84, 48 kDa subunits to form interferon-stimulated gene factor-3 (ISGF-3). PTK=protein tyrosine kinase; ISRE=interferon-stimulated response elements (From Schindler et al., 1992).

and form a complex. This complex combines with a 48 kDa gene-binding subunit and then migrates to the nucleus. This completed ISGF-3 complex then binds to the interferon-stimulated response elements (ISRE) associated with the promoter of IFN-responsive genes and this activates the transcription of the IFN-responsive genes. Since IFN-τ also binds to the Type 1 IFN receptor (Stewart et al., 1987; Roberts et al., 1992; Bazer et al., 1994), it is presumed that trophoblast IFN activate the IFN-responsive genes associated with maternal recognition of pregnancy by a similar mechanism.

The binding of IFN-α or IFN-β to cell surface receptors is necessary but not sufficient for cellular activation. Studies have shown that anti-IFN antibodies inhibit the antiviral response in cells exposed to IFNs. Also, IFN-α or IFN-β microinjected directly into cells fail to inhibit VSV replication (Higashi and Sokawa, 1982; Huez et al., 1983). These results indicate that the IFN-receptor complex must form for IFNs to have biological effects within the cell. There is a functional discrimination on the biological effects stimulated by both natural (Evinger et al., 1981; Evinger and Pestka, 1981) and recombinant (Rehberg et al., 1982) IFN-αs; even though the IFN-α/β receptor binds to all of the IFN species. An IFN-α with high antiviral activity does not necessarily have high antiproliferative (Evinger et al., 1981) or natural killer activity (Ortaldo et al., 1983; Ortaldo et al., 1984). The basis for this functional discrimination is not known.

One of the earliest cellular responses to IFN exposure is the stimulation of specific genes termed IFN-stimulated response elements (ISRE) (Levy et al., 1988; Levy et al., 1989). Hannigan and Williams (1991) demonstrated that arachidonic acid metabolism is involved in the IFN stimulation of ISRE. Exposure of cells to inhibitors of prostaglandin synthetase, result in amplification of ISRE-binding and gene transcription (Hannigan and Williams, 1991). As stated earlier in this review, the trophoblast IFN, that result in maternal recognition of pregnancy in ruminants, are Type I IFNs, however it remains to be determined if this mechanism is involved in the establishment of pregnancy in ruminants.

Biological Actions of Trophoblast Interferons

The trophoblast IFNs have biological effects similar to other IFNs. Roberts et al. (1989a) demonstrated that oIFN-τ possesses antiviral activity indicative of IFNs. The antiviral activity of oIFN-τ secreted by ovine conceptuses was determined to be greater than $1X10^7$ antiviral units/mg protein (Roberts et al. 1989a) which is as potent as any known IFN-α (Pestka et al., 1987). Interferons have also been shown to modulate cell differentiation and proliferation (Peska et al., 1987). oIFN-τ possesses antiproliferative activity that is indicative of IFNs (Roberts et al., 1989a), as oIFN-τ inhibited growth and suppressed mitogen-stimulated incorporation of [^{3}H]-thymidine into ovine lymphocytes at concentrations similar to that of IFN-α (Roberts et al., 1989a).

Trophoblast IFNs exhibit antiviral activity indicative of Type I IFNs. However, trophoblast IFNs are poorly inducible by virus and double stranded RNA in day 11 ovine conceptuses (Farin et al., 1991). Also, bIFN-τ contributes <0.1% of the total IFN produced by bovine leukocytes exposed to Sendai virus (Cross and Roberts, 1991). This may result from the virus-inducible gene sequence motifs for trophoblast IFNs being arranged differently than the other Type I IFNs (Hansen et al., 1991). These researchers have demonstrated that the genes for the trophoblast IFNs possess distinct 5'-promoter and 3'-flanking sequences from that of other IFN-αII (Hansen et al., 1991; Roberts et al., 1992; Bazer et al., 1994). The 5'-promoter regions differs from the virus inducing motifs of other types of IFNs (Hansen et al., 1991; Leaman and Roberts, 1992; Roberts et al., 1992; Bazer et al., 1994). This is supported by the poor virus-inducibility of IFN-τ from ovine conceptuses (Leaman et al., 1992; Roberts et al., 1992). Upstream promoters of general Type I IFNs typically diverge around -150 bp (Leaman and Roberts, 1992; Leaman et al., 1992; Roberts et al, 1992; Bazer et al., 1994). In ruminant species, there is cross-species sequence identity of IFN-τ promoters to approximately -400 bp upstream of the transcription start site (Leaman and Roberts, 1992; Leaman et al., 1992; Bazer et al., 1994), suggesting that regions further upstream from the IFN-τ gene are important regulatory regions. Because IFN-τ promoter constructs are expressed in uninduced transformed cells of trophoblast origin (i.e. JAR and BeWo cells) but not in nontrophoblast cells, it is postulated that trophoblast-cell-specific factors activate

transcription of the IFN-τ genes via as yet undefined response elements (Roberts et al., 1992; Bazer et al., 1994).

Trophoblast IFNs do not exhibit the cytotoxic effects that are indicative of other Type I IFNs (Pontzer et al., 1991). This explains why the large amount of IFNs secreted by the conceptus, during maternal recognition of pregnancy, does not harm the cells of the uterine endometrium. Trophoblast IFNs do share the antiproliferative effects of general Type 1 IFNs (Pestka et al., 1987) on most cell types including: lymphocytes (Newton et al., 1989; Niwano et al., 1989; Fillion et al., 1991; Skopets et al., 1992), oviduct epithelial cells (Kamwanja and Hansen, 1993) WISH and MDBK cells (Pontzer et al., 1991). However a recent study (Davidson et al., 1994) demonstrated that neither rbIFN-τ nor rbIFN-α have antiproliferative effects on bovine endometrial epithelial or stromal cells. This indicates that the bovine endometrium is resistant to the antiproliferative effects of Type 1 IFNs; and just as IFN-τ has evolved to be the pregnancy recognition signal in ruminants, the bovine endometrium has evolved to be resistant to the antiproliferative effects of IFNs. Therefore the growth of the endometrium is not compromised by the large amounts of IFN-τ secreted by the conceptus during early pregnancy.

It is not known what regulates the expression of trophoblast IFN genes in domestic ruminants. Roberts et al. (1989a) hypothesized that expression may be under the control of maternal hormones, or some factor in the uterine

environment. Expression could possibly result from the activation of master genes in the conceptus which lead to a coordinated biochemical response and culminate in maternal recognition of pregnancy.

Trophoblast Interferon Endometrial Receptors

Trophoblast IFNs and IFN-α compete for the same endometrial receptors (Stewart et al., 1987; Stewart et al., 1989a; Li and Roberts, 1994). The receptors for oIFN-τ are located in the surface and upper glandular epithelium of the endometrium (Godkin et al., 1984b). Knickerbocker and Niswender (1989) demonstrated that though receptors for oIFN-τ are located in CL, liver and kidney tissue, receptor concentration is highest in the endometrium. Hansen et al. (1989) suggest that there are two types of endometrial trophoblast IFNs receptor: a common receptor that competitively binds both trophoblast IFN and IFN-α, and a specific, low affinity endometrial receptor that binds only trophoblast IFN.

Several studies have evaluated the ability of rbIFN-αI to mimic the antiluteolytic effect of trophoblast IFNs. Intrauterine infusion of large amounts of rbIFN-αI into cyclic ewes (Stewart et al., 1989b) or cows (Plante et al., 1988; Plante, et al., 1989; Thatcher et al., 1989a) during the period of maternal recognition of pregnancy, extended the estrous cycle. However, it took considerably more rbIFN-αI to extend the interestrous intervals than trophoblast IFN. Recently, Salfen et al. (1995) demonstrated that intrauterine infusion of

rbIFN-α_I1 lengthened the luteal lifespan in cows anticipated to have short cycles by decreasing uterine secretion of $PGF_{2\alpha}$.

Intramuscular injection of rbIFN-α1 increased pregnancy rate in treated ewes (Nephew et al., 1990; Schalue-Francis et al., 1991). The studies did not demonstrate an increased incidence of stillbirths, abnormal offspring or post-partum death, indicating that the embryos that were rescued were viable and would have been lost had IFNs not been administered (Nephew et al., 1990; Schalue-Francis et al., 1991). However, a recent study (Imig et al., 1995) demonstrates that intramuscular injection of ewes with roIFN-τ resulted in hyperthermia and decreased pregnancy/lambing rates. Likewise, intermuscular injection of rbIFN-α_I1 tended to reduce pregnancy rates in heifers (Barros et al., 1992a). Intramuscular administration of IFN-α causes fever and a lowering of serum progesterone levels in cattle (Barros et al., 1992a,b; Newton et al., 1990; Plante et al., 1991). The hyperthermia is dependent on prostaglandin production, because a prostaglandin inhibitor given concurrently with rbIFN-α blocks the hyperthermic response (Barros et al., 1992b). These biological effects were mimicked by intramuscular injection of recombinant bIFN-τ, in that injection of 5 mg of rbIFN-τ extended interestrous interval and also resulted in hyperthermia and lower progesterone levels (Meyer et al., 1995b). However, intramuscular

injection of 1 mg of rbIFN-τ extended interestrous interval but did not cause hyperthermia or lower progesterone levels (Meyer et al., 1995b).

Trophoblast Interferons and Maternal Recognition of Pregnancy

The trophoblast IFNs have many biological effects which may be involved with the mechanism to inhibit luteolysis. It has been hypothesized that the trophoblast IFNs affect prostaglandin synthesis from arachidonic acid, possibly by inhibiting prostaglandin synthetase (Helmer et al., 1989b). It is possible that during evolution, the general relationship of IFN-α and arachidonic acid metabolism has been specialized to cause inhibition of endometrial PG synthetase in ruminants during early gestation. In addition, intrauterine infusion of recombinant oIFN-τ extends interestrous interval and abolishes oxytocin- (OT) induced release of PGF (Meyer et al., 1995a). *In vitro* studies, using monolayers of cultured endometrial epithelial cells, demonstrate that natural and recombinant bIFN-τ attenuate both the basal and OT-induced release of $PGF_{2\alpha}$ and PGE_2 into culture media (Danet-Desnoyers et al., 1994). However bIFN-τ has no effect on stromal $PGF_{2\alpha}$ or PGE_2 release (Danet-Desnoyers et al., 1994). Natural bIFN-τ inhibits endometrial prostaglandin synthesis through induction an intracellular inhibitor of prostaglandin synthesis (Helmer et al., 1989b). The prostaglandin synthetase inhibitor has been localized in the cytosolic fraction of endometrium from pregnant cows (Basu and Kindahl, 1987a,b; Gross et al., 1988) and may directly inhibit prostaglandin synthetase and/or the subsequent conversion of

arachidonic acid to prostaglandins (Gross et al., 1988). The prostaglandin inhibitor inhibits both PGF and PGE synthesis (Helmer et al., 1989b). It may be that the prostaglandin synthesis inhibitor is compartmentalized in the surface epithelium, so that PGF secretion is inhibited in the surface epithelium while PGE secretion is not inhibited in the stroma (Bazer, 1992).

Vallet and Lamming (1990b) demonstrated that ovine conceptus secretory proteins, but not rbIFN-α, decreases the concentration of oxytocin receptors in the endometrium. Trophoblast IFNs also alter the ovarian follicular population by suppressing the follicular waves on the ipsilateral but not the contralateral ovary (Guilbault et al., 1986; Ginther et al., 1989). It has previously been hypothesized that the trophoblast IFNs may "stabilize" endometrial progesterone receptors and either directly or indirectly prevent upregulation of endometrial estrogen and oxytocin receptors (Bazer 1992; Bazer et al., 1994). The combination of these effects would inhibit the mechanism of luteolysis. Trophoblast IFN, secreted by the conceptus, function to inhibit a prostaglandin synthetase, and to decrease the amount of endometrial oxytocin receptors. Therefore, the endometrium is not competent to secrete pulsatile $PGF_{2\alpha}$ in response to any pulses of oxytocin that may come from the posterior pituitary or the CL. The suppression of follicular waves on the ipsilateral ovary also supports the model of preventing luteolysis. Reducing the secretion of estrogen from the ovary, prevents any enhancement of the pulsatile release of $PGF_{2\alpha}$ from the endometrium (Thatcher et al., 1989b).

The possibility that the trophoblast IFNs prevent downregulation of progesterone receptors and upregulation of estrogen receptors also fit with the model of preventing luteolysis and maintaining CL function. However, data from our laboratory (Geisert, unpublished results) indicate that progesterone receptors in the endometrial epithelium of pregnant cows decline prior to the time of maternal recognition of pregnancy and remain absent throughout the rest of early gestation. This decline in progesterone receptors is similar to that observed during comparable days of the estrous cycle (Geisert, Ott and Bazer, unpublished data). These results have been recently confirmed in the cyclic ewe (Spencer and Bazer, 1995).

In domestic ruminants, trophoblast IFNs stimulate a selective group of secretory proteins from both endometrial explants and primary endometrial epithelial cells in culture. In the ewe, Vallet et al. (1987), demonstrated that oIFN-τ stimulates secretion of specific proteins from endometrial explants *in vitro*. A subsequent study determined that one of the proteins, induced by trophoblast IFN, is an acidic protein with a molecular weight of 14 kDa which has tentatively been identified as β-microglobulin, a component of the Type I major histocompatibility complex (Vallet et al., 1991).

Recent *in vitro* studies, in the cow, have demonstrated that treatment of bovine endometrial explants with rbIFN-τ stimulated the production of three endometrial secretory proteins (Naivar et al. 1995). The 8 kDa and 28 kDa

endometrial secretory proteins were stimulated specifically by rbIFN-τ but not rbIFN-α (Naivar et al. 1995). A subsequent study has identified the 8 kDa protein as ubiquitin and also identified a related 16 kDa secretory protein termed bovine ubiquitin cross-reactive protein (bUCRP) (Austin et al., 1996). bUCRP was released from endometrial explants of day 15 pregnant cows; and treatment of pregnant bovine endometrial explants with rbIFN-τ, roIFN-τ or rbIFN-α stimulated the secretion of bUCRP (Austin et al., 1996). Ubiquitin appears to be constitually produced by endometrial explants (Austin et al., 1996). These researchers hypothesize that ubiquitin and bUCRP may be involved in modulation and elimination of endometrial proteins during maternal recognition of pregnancy (Austin et al., 1996).

Induction or enhancement of specific uterine proteins may serve as histotroph, growth factors or for immunoprotection of the conceptus. All of these factors contribute to creating an uterine environment conducive to conceptus growth. Collectively, these data indicate that oIFN-τ is the major substance produced by the sheep conceptus which prevents luteolysis and possibly helps establish a uterine environment permissive to conceptus growth during early pregnancy.

Control of the endometrial estrogen receptors (ER) appear to be key for the prevention of luteolysis in ruminants. As stated before, recent studies have demonstrated that upregulation of ER in the endometrial luminal epithelium and

shallow glands, during day 11-15 of the estrous cycle, is necessary to begin the process of luteolysis in the ewe (Spencer and Bazer, 1995; Spencer et al., 1995a).

Recently, it has become evident that time- and cell type-specific regulation of the endometrial ER expression is the means by which the trophoblast IFNs inhibit the mechanism of luteolysis (Spencer and Bazer, 1995; Spencer et al., 1995a,b). During early pregnancy (days 11 to 25) ER mRNA abundance was low or absent in the endometrial luminal epithelium, shallow glands and stroma. Immunocytochemical localization of the ER protein, for the most part, mirrors the mRNA data, showing the same profiles during the estrous cycle and early pregnancy (Spencer and Bazer, 1995). It has been demonstrated that treatment of ovariectomized ewes, with progesterone and estrogen, increases ER mRNA/protein levels in the endometrial luminal and glandular epithelium (Spencer et al., 1995a). However, ovariectomized ewes injected with progesterone and estrogen and then infused with roIFN-τ, have low levels of ER mRNA/protein which are not different from background levels (Spencer et al., 1995a). These researchers also demonstrated that endometrial oxytocin receptor (OTR) levels were higher in control ewes than in ewes infused with roIFN-τ (Spencer et al., 1995a). Likewise, Stevenson et al. (1994) demonstrated that OTR mRNA is first present in the endometrial surface epithelium on day 14-15 of the ovine estrous cycle, corresponding with the initiation of luteolysis; but

endometrial OTR mRNA is absent on the corresponding days of pregnancy. In addition, the ability of OT to induce endometrial $PGF_{2\alpha}$ secretion from pregnant ewes is reduced compared to that of cyclic ewes (Silvia et al., 1992).

A related study demonstrated that infusion of roIFN-τ to cyclic ewes resulted in lower endometrial ER mRNA levels compared to ewes receiving an infusion of control proteins, and ER mRNA was more abundant in the luminal and glandular epithelium of control ewes compared to roIFN-τ ewes (Spencer et al., 1995c). However, endometrial progesterone receptor (PR) levels did not differ between control and roIFN-τ ewes. Endometrial PR were present in the endometrial stroma and epithelium in control ewes, but were found only in the endometrial stroma of roIFN-τ ewes (Spencer et al., 1995c). Endometrial OTR density was lower in the endometrium of ewes injected with roIFN-τ compared to control ewes (Spencer et al., 1995c). On day 14 of the estrous cycle, concentrations of 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ (PGFM) were increased by exogenous OT administration in control ewes, whereas PGFM levels were not effected by OT injection in roIFN-τ ewes. These results indicate that antiluteolytic effect of IFN-τ is to prevent the upregulation of the endometrial ER which prevents the upregulation of endometrial OTR and abrogates the uterine release of $PGF_{2\alpha}$ (Spencer et al., 1995c).

The combinations of the results of these studies have led Bazer's group at Texas A&M University to propose the following model for the physiological changes in the ovine endometrium during maternal recognition of pregnancy (see Figure 2). During early pregnancy, trophoblast secretion of IFN-τ extends the "progesterone block" that results in the suppression endometrial ER gene expression in the luminal epithelium and subsequently prevents OTR formation and pulsatile release of $PGF_{2\alpha}$ (Spencer et al., 1995a). Trophoblast IFNs do not prolong the progesterone block by stabilization or upregulation of endometrial PR, because endometrial epithelial PR levels are downregulated in: cyclic (Spencer and Bazer, 1995; Spencer et al., 1995a,b,c) and pregnant (Spencer and Bazer, 1995) or IFN-τ treated (Spencer et al., 1995a,b,c) ewes. The intracellular mechanism by which IFN-t inhibits the gene expression of endometrial epithelium ER is currently unknown.

One of the intracellular mechanisms by which Type I IFNs have their characteristic biological effects is to stimulate the 2',5'-oligoadenylate [2-5(A)] system to inhibit protein synthesis by degradation of cellular mRNA (Johnson and Torrence., 1984; Peska et al., 1987). Trophoblast IFNs have been shown to stimulate 2-5(A) synthetase in bovine endometrial explants (Short et al., 1991). To determine if the 2-5(A) system is involved in maternal recognition of pregnancy in ruminants, it is necessary to review the properties of the 2-5(A) system.

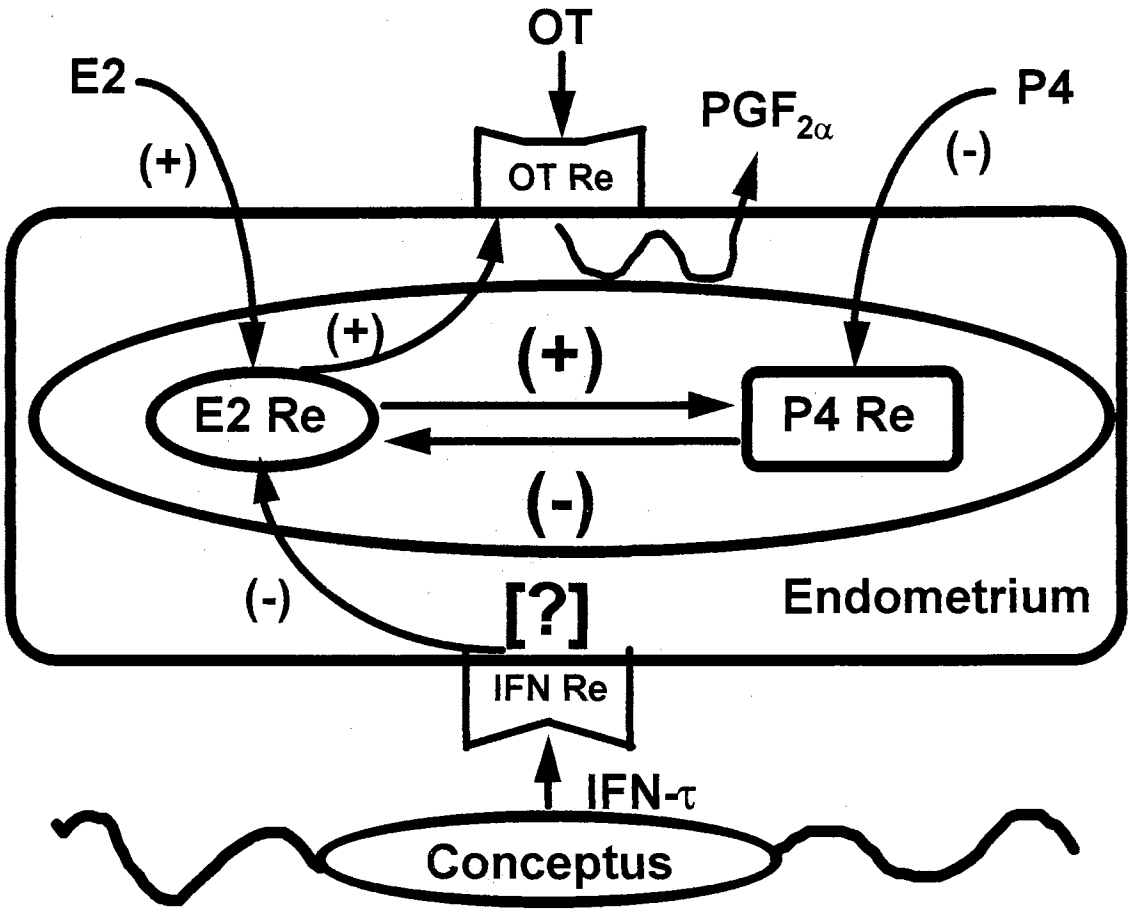

Figure 2. Diagram of the proposed mechanism of inhibition of luteolysis by conceptus secretion of trophoblast IFN in domestic ruminants during maternal recognition of pregnancy; E2=estradiol; P4=progesterone $PGF_{2\alpha}$=prostaglandin $F_{2\alpha}$; IFN-τ =interferon-τ; E2 Re= estradiol receptor; P4 Re=progesterone receptor; OT Re=oxytocin receptor; IFN Re=Type 1 interferon receptor. (From F.W. Bazer, personal communication.).

2',5'-oligoadenylate System

Interferons possess antiviral activity which is triggered through activation of several intracellular mechanisms (Pestka et al, 1987). The 2',5'-oligoadenylate [2-5(A)] system is the most well-characterized biochemical mechanism involved in interferon action (Johnston and Torrence, 1984; Pestka et al., 1987). The 2-5(A) system consists of three major enzymes: the 2-5(A)-dependent synthetase, endoribonuclease, and phosphodiesterase (see Figure 3). These enzymes function to catalyze the hydrolysis of oligoadenylate nucleotides that possess a 2',5'-phosphodiester bond instead of the usual 3',5'-phosphodiester bond (Johnston and Torrence, 1984; Pestka et al., 1987).

The 2-5(A) system induces its biological effect through inhibition of intracellular protein synthesis by selective degradation of mRNA. Johnston and Torrence (1984), proposed the current model of IFN action which suggests that IFN, binding to target cell surface receptor, stimulates double-stranded RNA catalyzed activation of inactive 2-5(A) synthetase (see Figure 3). Active 2-5(A) synthetase catalyzes formation of 2'-5'-oligonucleotides from intracellular stores of adenosine triphosphate (ATP). Formation of 2',5'-oligoadenylate then activates a 2-5(A)-dependent endoribonuclease which degrades mRNA and inhibits protein synthesis. The 2-5(A) oligonucleotide is relatively unstable and undergoes rapid degradation to ATP and AMP by the action of 2'-phosphodiesterase (Pestka et al., 1987).

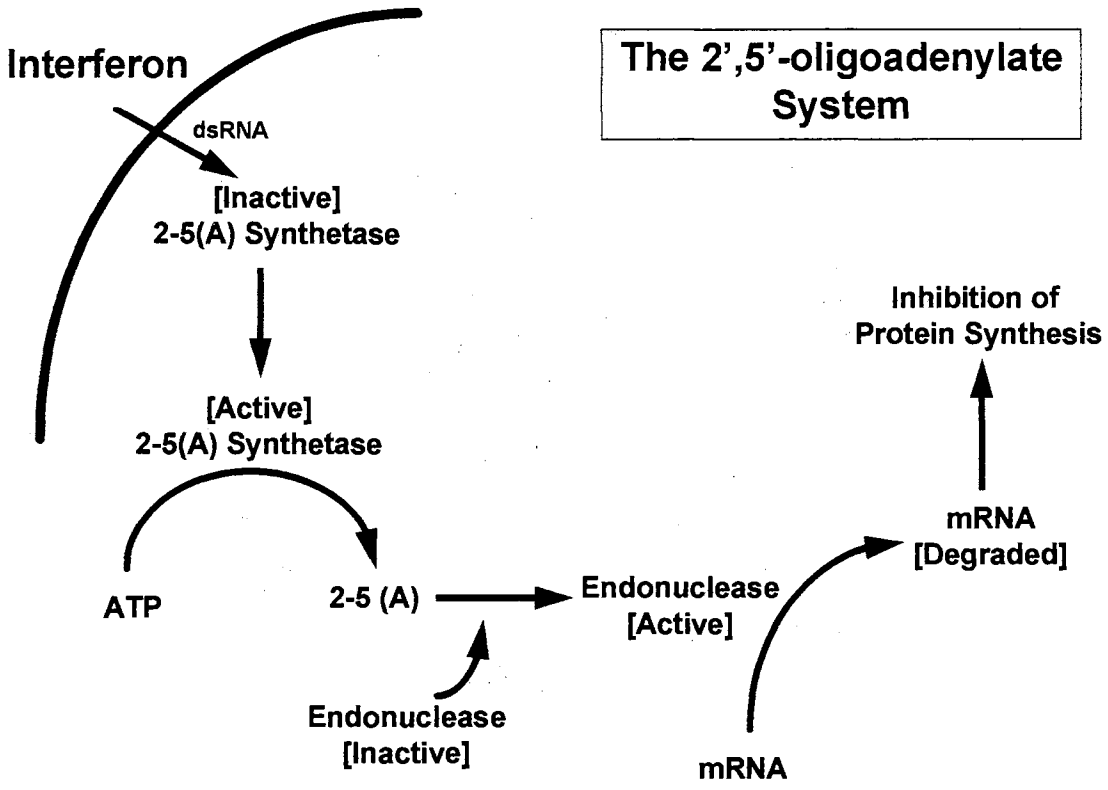

Figure 3. The mechanism of dsRNA-catalyzed, interferon-induced 2',5'-oligoadenylate [2-5(A)] system in the inhibition of protein synthesis in response to interferon. (From Johnston and Torrence, 1984).

The 2-5(A) system can be stimulated by both Type I and II IFNs. The magnitude of 2-5(A) synthetase induction by IFNs is dependent upon type of IFN and growth state of the cell (Lengyel, 1982). Induction of 2-5(A) synthetase ranges from approximately 10X basal levels in human HeLa cells (Baglioni et al., 1979) to approximately 10,000X basal levels in chick embryo cells (Ball, 1979). Although all three types of IFN (α, β, γ) are able to induce 2-5(A) synthetase in human and mouse cells (Baglioni and Maroney, 1980; Verhaegen-Lewalle et al., 1982; Benech et al., 1985), the degree of induction varies with the type of IFN (Baglioni and Maroney, 1980). Also, 2-5(A) synthetase concentrations are affected by hormonal status (Stark et al., 1979), glucocorticoid treatment (Krishnan and Baglioni, 1980), cellular differentiation status (Yarden et al., 1984) and stage of the cell cycle (Wells and Mallucci, 1985).

The 2-5(A) synthetase can be inhibited by NAD^+ and tRNA (Ferbus et al., 1981). This inhibitory action is due to these compounds occupying the nucleotide acceptor site of the enzyme preventing ATP binding to the synthetase. The 2-5(A)-dependent endoribonuclease catalyzes the cleavage of both viral and cellular RNA on the 3' side of UA, UG or UU sequences (Floyd-Smith et al., 1981; Wreschner et al., 1981).

The 2-5(A)-dependent endoribonuclease degrades many types of single-stranded RNA which can be either synthetic or natural (Pestka et al., 1987). Viral infection without IFN treatment (Silverman et al., 1982) or degradation of the 2-

5(A) oligonucleotide, by the 2-5(A)-dependent phosphodiesterase (Eppstein et al., 1979; Minks et al., 1979), functionally inactivates the 2-5(A)-dependent endoribonuclease.

In mammals, 2-5(A) synthetase is one of the first responses of the body to viral infection (Schattner et al., 1982; Johnson and Torrence, 1984). In cattle, 2-5(A) synthetase content in cultured Madin-Darby bovine kidney (MDBK) cells is stimulated by injection of modified live virus and bIFN-α (Short and Fulton, 1987). Intramuscular injection of bIFN-αI increases 2-5(A) synthetase concentration in bovine peripheral blood mononuclear cells (Short and Fulton, 1987; Perino et al. 1990).

In domestic ruminants, there is circumstantial evidence to indicate that 2-5(A) synthetase is involved in the mechanism of maternal recognition of pregnancy. Mirando et al. (1991) indicated that endometrial 2-5(A) synthetase increased during early pregnancy of the ewe; and was increased, in cyclic ewes, following intrauterine infusion of oIFN-τ compared to saline infusion. It is assumed that IFN-τ, secreted by the conceptus *in vivo* (Bazer et al, 1994) stimulates 2-5(A) synthetase in the endometrium. Porcine conceptuses secrete two different types of trophoblast IFNs during the period of CL maintenance that have been identified to be an IFN-α and IFN-γ (Harney and Bazer, 1989; Short et al., 1992). These porcine trophoblast IFNs are structurally and functionally different from IFN-τ produced by the ruminant conceptus (Bazer et al., 1994).

Also, while porcine conceptus secretory proteins increased 2-5(A) synthetase activity in monolayers of Madin-Darby bovine kidney (MDBK) cells and in bovine endometrial explants, they had no measurable effect on 2-5(A) synthetase activity of porcine endometrial explants (Short et al., 1992). This is not unexpected, since conceptus secretion of estrogens and not trophoblast IFN, is the signal for maternal recognition of pregnancy in the pig (Bazer and Thatcher 1977). However, a recent study demonstrated that porcine trophoblast IFN-α and IFN-γ stimulate 2-5(A) synthetase activity in separated porcine endometrial cells, in a cell-specific manner (D'Andrea et al., 1994). In porcine endometrial epithelial cells, 2-5(A) synthetase activity was moderately induced by porcine trophoblast IFN-γ and induced to a lesser extent by porcine trophoblast IFN-α. Conversely, stroma cells were highly sensitive to the trophoblast IFN-α but weakly sensitive to the trophoblast IFN-γ (D'Andrea et al., 1994). These researchers suggest that the porcine trophoblast IFNs may have an immunoprotective role to protect the conceptus from viral infection during early gestation and this effect is confined locally to the endometrium (D'Andrea et al., 1994).

In the cow, Short et al. (1991) indicated that the content of endometrial 2-5(A) synthetase significantly increased on day 18 of gestation, corresponding with the production of large amounts of bIFN-τ by the conceptus. These researchers also demonstrated that increased 2-5(A) synthetase in MDBK cells, as measured by antiviral activity, can be stimulated by conceptus secretory proteins enriched in

bIFN-τ. Likewise, Barros et al. (1991) demonstrated that bIFN-α stimulated 2-5(A) synthetase activity in bovine endometrial explants; and this was correlated with decreased secretion of PGF.

To date, most studies of the role of 2-5(A) synthetase activity in domestic ruminants have utilized endometrial explants. Therefore, changes of 2-5(A) synthetase could only be observed at the whole tissue level. Recently, a technique has been developed to separate the bovine endometrium into separate populations of surface epithelium, glandular epithelium and stroma cells (Schmitt et al., 1993). This technique of cell separation allows for observation of 2-5(A) synthetase levels of the three endometrial cell types. Since it appears that the antiluteolytic effects of trophoblast IFNs are localized to the uterine endometrium (see Bazer et al., 1992; 1994), cell-specific effects of IFN-τ may be important in the establishment of pregnancy in ruminants.

Schmitt et al. (1993) demonstrated that, during the bovine estrous cycle, 2-5(A) synthetase activity in surface epithelium, glandular epithelium and stroma is greatest on day 5 and 10 and declines almost 10 fold for surface epithelium and stroma and approximately 3 fold in glandular epithelium by day 15 of the estrous cycle. The decrease in endometrial 2-5(A) synthetase, observed on day 15, is coincident with the initial ability of the endometrium to secrete $PGF_{2\alpha}$ (Silvia et al., 1991). Barros et al. (1991) indicated a correlation between decreased $PGF_{2\alpha}$ secretion and increased cellular 2-5(A) synthetase in IFN-treated bovine uterine

explants. It is possible that the 2-5(A) system is involved with the control of the synthesis of proteins involved in prostaglandin synthesis and/or metabolism, so that increased 2-5(A) synthetase activity results in decreased endometrial prostaglandin levels.

In pregnant cows, the presence of a conceptus significantly increased 2-5(A) synthetase in endometrial cells on days 15 and 18 of gestation compared to similar days of the estrous cycle (Schmitt et al., 1993). The effect of the conceptus was localized to the gravid uterine horn on day 15 of gestation, as 2-5(A) synthetase in the uterine horn that contained the conceptus was significantly greater than that of the contralateral horn for all three endometrial cell types. On day 18 of gestation, when the conceptus has elongated into the contralateral horn and bIFN-τ is increased in both uterine horns (Short et al., 1991), 2-5(A) synthetase activity of the contralateral horn was similar to that of the ipsilateral horn (Schmitt et al., 1993). These data indicate that conceptus secretion of trophoblast IFNs stimulate 2-5(A) synthetase in endometrial cells as the conceptus elongates throughout the uterine horns. Conceptus stimulation of 2-5(A) synthetase is localized mainly to the endometrial epithelium. On day 15 and 18 of pregnancy, epithelial (surface and glandular) 2-5(A) synthetase was greater than that of the stroma (Schmitt et al., 1993). It is possible that the tight junctional complexes and desmosomes, characteristic of endometrial epithelium (Findlay et al., 1990) prevent trophoblast IFN from maximally stimulating 2-5(A)

synthetase in the endometrial stroma; thus restricting the signal mainly to the epithelium in contact with the conceptus. Stromal cells did have an increase in 2-5(A) synthetase on day 18 of gestation, however the increase was not as large as that observed in the surface and glandular epithelial cells (Schmitt et al., 1993). Salamonsen and Findlay (1990) previously suggested that the effects of conceptus trophoblast IFNs on uterine prostaglandin synthetase are mediated through the endometrial epithelium. It is interesting that the decline in epithelial 2-5(A) synthetase during the estrous cycle is closely coupled to the period of luteolysis in the cow; which is restored by conceptus secretion of bIFN-τ on day 15 of pregnancy. During the ovine estrous cycle, decline in epithelial 2-5(A) synthetase is related to the loss of endometrial progesterone receptor as demonstrated through immunocytochemistry (Geisert and Bazer, unpublished data). In the ewe, there is an absence of specific nuclear staining for progesterone receptor in the uterine epithelium after day 10 of gestation (Geisert, Ott and Bazer, unpublished data). Down-regulation of the progesterone receptor in the endometrial epithelium would remove the inhibitory effect of progesterone on either cellular prostaglandin or oxytocin receptor synthesis (Silvia et al., 1991). Several studies. (Helmer et al., 1989b; Basu and Kindhal, 1987a,b) have indicated that bIFN-τ induces an intracellular inhibitor to prostaglandin synthesis. Gross et al. (1988), determined that this prostaglandin synthesis inhibitor is proteinacous, present in the cytosol and can be precipitated by ammonium sulfate. Geisert et al. (1992) suggest that, during pregnancy, trophoblast IFNs

may stimulate actions similar to progesterone receptors (inhibition of prostaglandin and oxytocin receptor synthesis) either through 2-5(A) synthetase or other IFN-stimulated mechanisms.

Changes in endometrial cellular 2-5(A) synthetase correspond to known changes in prostaglandin release, epithelial progesterone receptor content and maintenance of pregnancy, the objective of the following studies are to determine the role of the 2-5(A) system in maternal recognition of pregnancy in cattle. In these studies, we have utilized an *in vitro* system to directly infuse the 2-5(A) oligotrimer into cultured bovine endometrial cells and whole endometrial explants. In using this technique we have bypassed IFN-τ, the IFN receptor and 2-5(A) synthetase. By observing endometrial secretory protein synthesis, PGF synthesis levels in response to OT treatment and endometrial E2 receptor levels it may be possible to elucidate specific roles for the 2-5(A) system in maternal recognition of pregnancy in domestic cattle.

CHAPTER III

SECRETORY PROTEIN PROFILES OF PRIMARY AND SUBCULTURED BOVINE ENDOMETRIAL STROMAL CELLS GROWN OF TYPE I COLLAGEN GELS AND PLASTIC

Introduction

The uterus is one of the most fascinating and dynamic organs in biology. It is an organ that has been modified, by evolution, for the purpose of providing an environment conducive for prenatal growth and development in eutherian mammals. The uterus consists of two regions: the endometrium, which is lined with a serous-type of mucosa capable of differentiation, under steroid stimulation, during the estrous cycle and pregnancy; and the myometrium, which consists of smooth muscle and is involved in sperm/conceptus transport, and expulsion of the fetus at parturition (see Mossman, 1977).

The endometrium, the uterine tissue layer in direct contact with the conceptus, is responsible for establishing and maintaining a hospitable environment necessary for a successful pregnancy. In cross-section, from the myometrium to the uterine lumen, the endometrium consists of the stratum spongiosum, a loosely organized basal stromal layer and a more compressed

layer of connective tissue, the stratum compactum. The stratum compactum is covered by a basement membrane and the luminal surface epithelium. The endometrial surface epithelium is composed of a pseudostratified tall columnar layer of cells that are continuous with branching uterine glands that extend almost to the myometrium (Marion and Gier, 1971).

In vitro cell culture systems have the potential to be powerful tools to study the communication between endometrial stromal and epithelial cells. There is evidence that cultured endometrial cell types have different characteristics that are expressed in a cell type-specific manner. For example, cultured endometrial stromal cells have a faster growth rate and reach confluence earlier compared to endometrial epithelial cells (see Findlay et al., 1990). Also, in both ewes (Cherny and Findlay, 1990) and cows (Danet-Desnoyers et al., 1994), cultured endometrial epithelial cells produce primarily prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) whereas cultured endometrial stromal cells produce primarily PGE_2; and endometrial epithelial cells respond to oxytocin treatment by increasing $PGF_{2\alpha}$ and PGE_2 secretion, whereas stromal cells are nonresponsive (Danet-Desnoyers et al., 1994). These cell-type specific profiles have been useful in elucidating the roles that the different endometrial cell types play during the period of luteolysis and establishment of pregnancy.

In addition to specific characteristics of the individual endometrial cell types, stromal-epithelial cellular interactions have been implicated in many biological events during the estrous cycle and early pregnancy (Glasser and McCormack, 1981; Cunha et al., 1983; Sharpe and Ferguson, 1988). Stromal cells may dictate the rate of proliferation and differentiation of the overlying epithelial cells through secretion of paracrine growth factors and extracellular matrix proteins (Gospodarowicz et al., 1979); but more research is needed to determine the importance of endometrial epithelial-stromal interactions in reproductive processes.

Future co-culture studies to determine the interaction between epithelial and stromal cells would consist of primary cultured epithelial cells and passage (subcultured) stromal cells. It is not known if subcultured endometrial stromal cells are competent to secrete proteins similar to that of primary stromal cells. The objective of the present study is to characterize the secretory protein profile of primary, 1st, 2nd, 3rd passage endometrial stromal cells cultured on plastic or Type 1 collagen.

Materials and Methods

In vitro Culture of Bovine Endometrial Cells on Different Extracellular Matrices.

Animals. Mature cows were observed for estrous behavior twice daily (0700 and 1600). On the day 15 of the estrous cycle, cows were slaughtered and uteri were obtained within 10 minutes after exsanguation and placed in a chilled (4^{o}C) 700 ppm Roccal solution and transported on ice to the laboratory (within 20 minutes) for processing in a sterile, horizontal flow hood. Uteri were trimmed free of the broad ligament and ovaries. Each uterine horn was flushed separately with sterile Hanks balanced salt solution (HBSS) (Gibco, Grand Island. NY) and the flushes were checked for signs of infection.

Preparation of Type 1 Collagen-Coated Culture Plates. Type 1 collagen-coated culture plates were prepared as follows: Type 1 collagen (Vitrogen 100 [3.0 mg/ml] Collagen Corporation, Palo Alto, CA) was diluted to .0825 mg/ml with sterile HBSS (4^{o}C) and aliquoted (1 ml/well) to sterile 24 well culture plate in a sterile positive-flow horizontal flow hood. Plates were left to dry, uncovered, overnight under ultraviolet light with the hood fan on. After liquid had evaporated and a crystallization pattern observed, each well was washed with DMEM (1 ml/well) and stored, wrapped in plastic, at 4^{o}C.

Uterine Cell Isolation. Separation of endometrial epithelial from stromal cells populations was conducted as previously described by Schmitt et al., (1993). Briefly, uterine horns were filled with pancreatin-dispase and incubated for 1.5 h in a water bath in which the temperature was increased from 4°C to 37°C. Surface epithelial rafts were recovered from the initial enzyme wash. After removal of the enzyme wash, uterine horns were filled with Ca- and Mg-free (Incomplete) HBSS (Gibco, Grand Island. NY). After repeated washes and incubation with Incomplete HBSS, both the surface and glandular epithelial cells were effectively separated from the stromal cells.

After the last incubation to recover uterine glands, the uterine horns were infused with PBS-dispase and incubated at 37°C for 15 min. The enzyme wash was removed and stromal cells were recovered by passing through a 20-µm screen. Stromal cells were also obtained after 2 washings with Ca-and Mg-free HBSS at 20°C for 15 minutes. The respective cell populations were estimated on a hemocytometer and diluted to $1X10^6$ cells/ml. The stromal cells, recovered from cyclic cows, were suspended in DMEM (Gibco, Grand Island. NY) containing 10% sterile cow serum and 1% antibiotic-antimycotic (ABAM) + anti-PPLO agent (Gibco, Grand Island. NY), plated on 24 well plates at $1X10^6$ cells/well and cultured in a controlled atmosphere of 95% air:5% CO_2 at 37° C until approximately 70-80% confluent on either plastic of Type 1 collagen.

Stromal cells were subcultured on day 6 (1st passage), day 9 (2nd passage) and day 12 (3rd passage). At each time period, 1.0 μl (10 μCi/well) ^{35}S-methionine (specific activity = 1077.5 Ci/mmol; Dupont NEN, Wilmington, DE) was added to one culture and incubated for 24 hours; whereas a companion culture was used for the passage and subculture (see Figure 4; Table I).

^{35}S-labeled culture media were microconcentrated (Centricon 100; Amicon, Danvers, MA) and approximately 250,0000 dpm of microconcentrated sample were loaded and subjected to 1D-SDS-polyacrylamide gel electrophoresis (1D-PAGE). After 1D-PAGE, the gels were stained with Coomassie Blue and subjected to fluorography (ENHANCE; Dupont-NEN, Wilmington, DE). Fluorographs were developed after 21 days of exposure at -80^{o}C.

Results

^{35}S-labeled secretory protein profiles of stromal cells grown on Type 1 collagen.

Fluorography of microconcentrated labeled secretory products revealed a total of 23 secretory protein bands were produced by cultured endometrial stromal cells, plated on Type 1 (Figure 5). Of the secretory proteins produced by stromal cells in primary culture, a majority (96%) of the protein bands were also produced by 1st, 2nd, or 3rd passage cells (Table II).

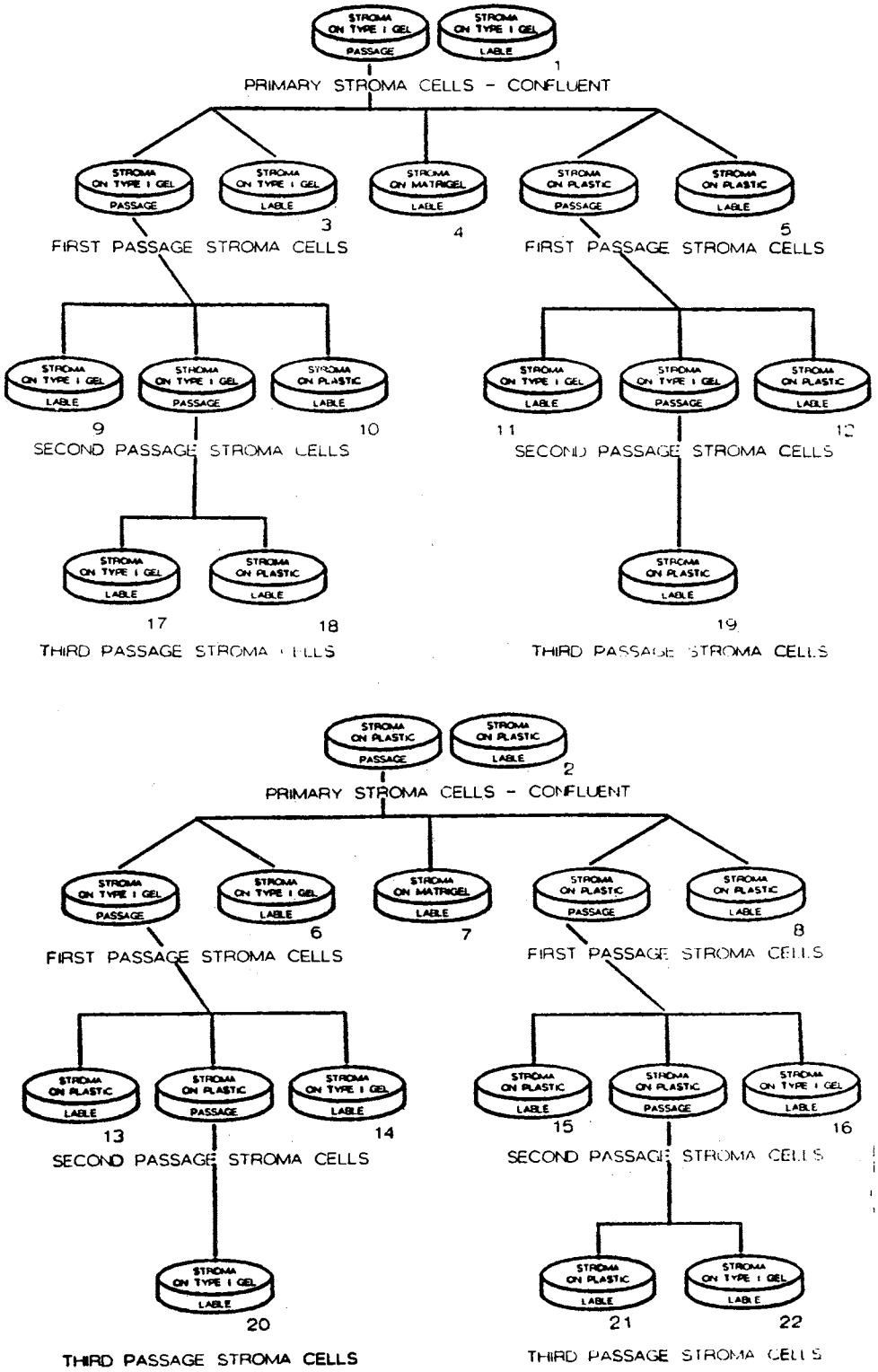

Figure 4. Diagram depicting endometrial stromal cells originally plated on Type 1 collagen or plastic (primary culture) and subsequent subcultivation scheme (numbers correspond to Dish # in Table I).

TABLE I

ENDOMETRIAL STROMAL CELLS: MATRIX TYPE AND TIME IN CULTURE

Dish #	ECM Type	Passage	Time to Confluence (days)	Total Time in Culture (days)
1	1C	1°	2	2
2	PL	1°	6	6
3	1C⇒1C	1st	4	6
4	1C⇒MG	1st	6	8
5	1C⇒PL	1st	4	6
6	PL⇒1C	1st	4	10
7	PL⇒MG	1st	6	12
8	PL⇒PL	1st	4	10
9	1C⇒1C⇒1C	2nd	3	9
10	1C⇒1C⇒PL	2nd	7	13
11	1C⇒PL⇒1C	2nd	7	13
12	1C⇒PL⇒PL	2nd	8	14
13	PL⇒1C⇒PL	2nd	3	13
14	PL⇒1C⇒1C	2nd	7	17
15	PL⇒PL⇒PL	2nd	8	18
16	PL⇒PL⇒1C	2nd	8	18
17	1C⇒1C⇒1C⇒1C	3rd	7	16
18	1C⇒1C⇒1C⇒PL	3rd	7	16
19	1C⇒PL⇒1C⇒PL	3rd	24	37
20	PL⇒1C⇒PL⇒1C	3rd	5	18
21	PL⇒PL⇒PL⇒PL	3rd	8	26
22	PL⇒PL⇒PL⇒1C	3rd	8	26

PL= Stromal cells plated on plastic.

1C= Stromal cells plated on Type 1 collagen.

MG= Stromal cells plated on Matrigel.

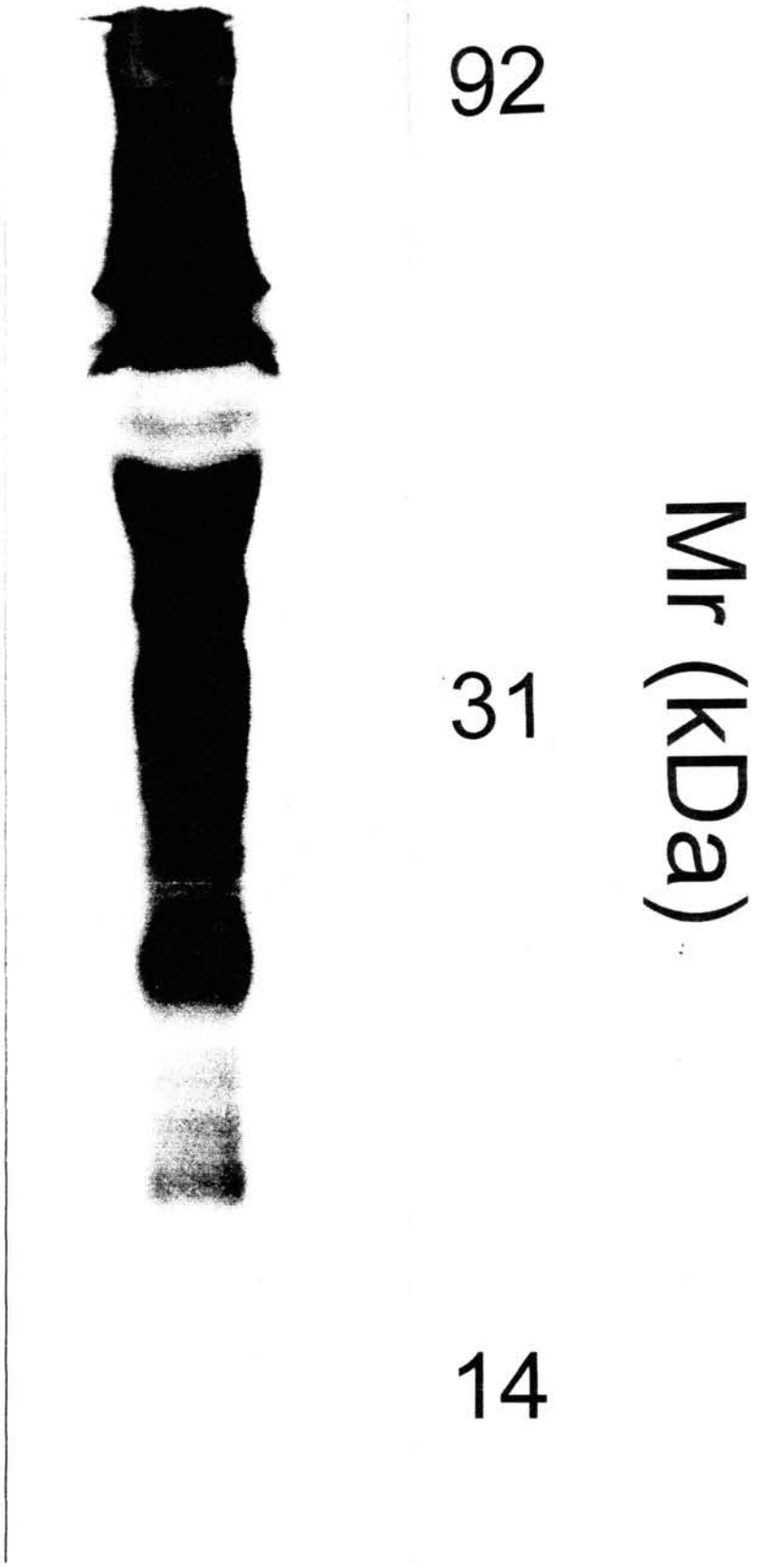

Figure 5. Representative 1-D PAGE depicting maximal ^{35}S-labeled secretory protein profile of endometrial stromal cells. Note the 23 secretory proteins indicative of cultured stromal cells

TABLE II

ENDOMETRIAL STROMAL CELLS CULTURED THROUGH 3rd SUBCULTURE: CELLS GROWN ON TYPE 1 COLLAGEN

Protein band	Apparent Mr (kDa)	1° (Day 1)	1st (Day 6)	2nd (Day 9)	3rd (Day 16)
1	> 92	X	X	X	X
2	> 92	X	X	X	X
3	92	X	X	X	X
4	72	X	X	X	X
5	59	2	2	N	N
6	52.5	X	X	F	N
7	43-46.5	2	X	2	2
8	39.5	X	X	X	X
9	34.5	2	2	2	2
10	31	X	X	X	X
11	28	X	X	X	X
12	25	X	X	X	X
13	22		X	X	X
14	21	X	X	X	
15	19.75	X	X	X	X
16	17	F	F	X	F
17	16	F	F	X	F
18	15		F	X	F
19	14.25	F			
20	14	F	X	X	X
21	13.5	F	X	F	
22	13		X	X	X
23	12	X	X	X	X
	Total bands	20	22	22	20

X= Well distinguished band.

2= Two bands very close together.

F= Faint band.

N= not resolved due to migration with albumin.

A faint 14.25 kDa protein band, observed in primary cultures, was not present in any of the passage cell cultures. However, three protein bands (13, 15, 22 kDa) were present in passage stromal cell cultures that were not observed in primary cultures (Table II). The combination of these results indicate that passage endometrial cells, plated on Type 1 collagen, are as competent or even more competent in synthesizing ^{35}S-labeled secretory proteins compared to primary cultures. This competency is maintained at least through the 2nd passage (day 9 of culture).

^{35}S-labeled secretory protein profiles of stromal cells grown on plastic.

Endometrial stromal cells in primary culture, plated on plastic, produced fewer protein bands compared to stromal cells plated on Type 1 collagen; 14 vs. 20 protein bands respectively (Table II; Table III). After 1st passage, stromal cells plated on plastic produced 23 proteins bands, which diminished to 18 protein bands by the 2nd passage. Five proteins (13, 15, 16, 21, 25 kDa) secreted by 1st passage stromal cells plated on plastic were not secreted by either primary or 2nd passage stromal cells (Table III).

Discussion

The endometrium of the uterus is a dynamic tissue which is functionally different during the estrous cycle and early pregnancy. It is becoming

TABLE III

ENDOMETRIAL STROMAL CELLS CULTURED THROUGH 3rd SUBCULTURE: CELLS GROWN ON PLASTIC

Protein band	Apparent Mr (kDa)	1° (Day 1)	1st (Day 10)	2nd (Day 18)
1	> 92	X	X	X
2	> 92	X	X	X
3	92	X	X	X
4	72	X	X	X
5	59	F	X	X
6	52.5	F	X	X
7	43-46.5	2	2	2
8	39.5	X	X	X
9	34.5	2	2	2
10	31	X	X	X
11	28	X	X	X
12	25	X	X	X
13	22		X	
14	21		X	
15	19.75	X	X	X
16	17		F	F
17	16		F	
18	15		F	
19	14.25		F	F
20	14		X	F
21	13.5		X	F
22	13		X	
23	12	X	2	X
	Total bands	14	23	18

X= Well distinguished band.

2= Two bands very close together.

F= Faint band.

N= not resolved due to migration with albumin.

increasingly evident that complex interactions between the endometrial epithelial stromal cells and associated extracellular matrix are important for normal functioning of the endometrium. However little is known of the details of this stromal-epithelial interactions.

Through recombination studies between separated epithelial and stromal tissue layers, it has been determined that the stromal tissue directs both the structure and function of the overlying epithelial cells. This control is presumably through the stromal production and secretion of locally-active "inducer molecules" (i.e. growth factors, peptide hormones, growth inhibitors) (see Cunha et al., 1983). However, little is known about the secretory protein profiles of stromal cells.

In this study, regardless of type of matrix or time in culture, the majority of the proteins secreted by stromal cells were of a low Mr (< 40 kDa). Fluorographs of separated stromal secretory proteins, plated on Type 1 collagen demonstrated that the vast majority of proteins produced during primary culture, were also produced in subsequent subculture. This indicates that passage stromal cells are capable of producing the same secretory proteins as primary cultured cells, and that passage endometrial stromal cells, cultured up to 9 days, are as competent or more competent than primary stromal cells. One secretory protein (14.25 kDa) was produced by stromal cells, plated on Type 1 collagen, in primary culture but

not produced in subsequent subcultures. Also, three secretory proteins (13, 15, 22 kDa) were produced in the subcultures and not the primary cultures. The identity of the respective secretory proteins and the significance of these different secretory protein profiles remains to be determined.

In the present study, endometrial stromal cells produced different secretory proteins, depending on the extracellular substrate utilized. Cultured stromal cells, plated on plastic, produced fewer secretory proteins compared to stromal cells plated on Type 1 collagen (14 vs. 23 proteins respectively). However, cultured stromal cells, originally plated on plastic and subsequently subcultured on plastic appear to gain competency, as indicated by secretory protein profiles. First passage stromal cells, plated on plastic, produced the full complement of 23 secretory proteins; however this is reduced to 18 secretory proteins after the second passage. These results indicate that subcultured stromal cells, plated on plastic, are more competent than stromal cells in primary culture and this competency is diminished after second passage. The biological cause and significance of these observation is currently unknown.

The three highest molecular weight ($\geq$ 92 kDa) secretory proteins were produced by stromal cells regardless of matrix type or passage number. Recently, a transmembrane proteoglycan, syndecan, has been identified as a molecule that functions as a matrix receptor by binding to cells to interstitial

collagens, fibronectin and thrombospondin (see Bernfield and Sanderson, 1990). Syndecans has been shown to be produced by both uterine epithelium and stroma cells, and have molecular weights between 92-170 kDa, the difference in molecular weights being solely attributed to the amount of glycosaminoglycans chains attached to the core protein (Boutin et al., 1991). A recent study (Boutin et al., 1991) demonstrated that rat uterine stromal cells produce syndecan; which is more prevalent in the stromal tissue immediately beneath the uterine epithelium. In the present study, it is possible that the 92 kDa and two >92 kDa secretory proteins are syndecans. *In vivo*, stromal syndecan could be involved in intracellular interactions between the uterine epithelium and stroma by localizing growth factors and facilitating interactions between growth factor and their cell surface receptors (Boutin et al., 1991). In an *in vitro* culture system, syndecans may be involved in the attachment of stromal cells to the culture plate surface. The fact that these three $\geq$ 92 kDa proteins are constitutively expressed in stromal cells, cultured on either plastic or matrix and during all observed subcultures, indicates that they may be involved in the mechanism of attachment during culture. More research is needed to determine if these three proteins are indeed syndecans; to determine if syndecans can be secreted by stromal cells; and to better determine the function of these secretory proteins *in vivo* and *in vitro*.

Components within the extracellular matrix affect cell behavior. For example, growth of differentiated cells can be induced or inhibited by various components of the extracellular matrix (see McKeehan et al., 1990). Specific components of the extracellular matrix also directly interact and determine the activity and stability of various polypeptide growth factors and regulators, such as fibroblast growth factor (see McKeehan et al., 1990). It is possible, in this study, that Type 1 collagen, either directly or indirectly, stimulates cultured endometrial stromal cells to become fully competent and produce the complete secretory protein profile indicative of cultured stromal cells; and stromal cells plated on plastic are not as competent as those plated on collagen. More research is needed understand the interaction between stromal cells and extracellular matrix and to identity proteins secreted by cultured stromal cells in the respective culture schemes. This information may help determine how the extracellular matrix and associated epithelial and stromal cells interact and function both *in vitro* and *in vivo*.

CHAPTER IV

THE ROLE OF THE 2;,5'-OLIGOADENYLATE SYSTEM DURING BOVINE MATERNAL RECOGNITION OF PREGNANCY

Introduction

To successfully establish pregnancy in domestic farm species, the mechanism leading to luteolysis during the latter part of the estrous cycle must be inhibited and a suitable uterine environment provided for the continued development of the conceptus. It is clear that specific biochemical signals, synthesized and secreted by the conceptus, induce the maternal uterus to maintain CL function and create a uterine environment favorable to conceptus attachment, growth and survival.

In domestic ruminants, the conceptus prevents luteolysis by inhibiting the synthesis and/or secretion of the uterine luteolysin, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (McCracken et al., 1972; McCracken et al., 1984; Thatcher et al., 1986). The ruminant conceptus synthesizes and secretes a number of polypeptides during the period of trophoblast elongation, which occurs between day 12-15 in the ewe and day 16-18 in the cow. Specific conceptus secretory polypeptides, termed

ovine trophoblast interferon-τ (oIFN-τ) and bovine trophoblast interferon-τ (bIFN-τ), have been demonstrated to be the signal for maternal recognition of pregnancy in the ewe and cow (see Bazer et al., 1994). The biological actions of trophoblast IFNs have been extensively reviewed (see Bazer et al., 1986; Bazer et al., 1989; Bazer et al., 1994; Leaman et al., 1992; Roberts et al., 1989a,b; Roberts et al., 1996; Stewart et al., 1989a,b; Thatcher et al., 1986; Thatcher et al., 1989a).

Recently, the following model has been proposed for the physiological changes in the ovine endometrium during maternal recognition of pregnancy (Spencer et al., 1995c). During early pregnancy, trophoblast secretion of IFN-τ extends the "progesterone block" that results in the continued suppression of endometrial estradiol receptor (ER) gene expression in the luminal epithelium and subsequently prevents oxytocin receptor (OTR) formation and pulsatile release of $PGF_{2\alpha}$ (Spencer et al., 1995a). Trophoblast IFNs do not prolong the progesterone block by stabilization or upregulation of endometrial PR, because endometrial epithelial PR levels are downregulated in cyclic (Spencer and Bazer, 1995; Spencer et al., 1995a,b,c) and pregnant (Spencer and Bazer, 1995) or IFN-τ treated (Spencer et al., 1995a,b,c) ewes. The intracellular mechanism by which IFN-τ inhibits the gene expression of endometrial epithelium ER is currently unknown.

The biological mechanism by which the trophoblast IFNs prevent luteolysis

in domestic ruminants is not completely understood. It appears that the actions of interferons are local, since the receptors for oIFN-τ are present in ovine endometrium (Godkin et al., 1984a; Knickerbocker and Niswender, 1989) and are competitively blocked by recombinant bovine IFN-α (Hansen et al., 1989). One major characteristic that the trophoblast IFNs share with other IFNs is the ability to stimulate the 2',5'-oligoadenylate [2-5(A)] system; specifically 2-5(A)-dependent synthetase and endoribonuclease. These enzymes are involved in the antiviral and antiproliferative effects of IFNs through degradation of specific mRNA to selectively inhibit protein synthesis (Johnson and Torrence., 1984). Short et al. (1991) indicated that endometrial 2-5(A) synthetase content increases significantly on day 18 of gestation compared to day 15 and 18 of the estrous cycle. In pregnant cows, conceptus stimulation of 2-5(A) synthetase is localized mainly in the endometrial epithelium (surface and glandular) of only the gravid horn, indicating that conceptus secretion of bIFN-τ stimulated 2-5(A) synthetase in the uterine horn containing the conceptus and only in the cell types in direct contact with the conceptus (Schmitt et al., 1993).

Changes in endometrial cellular 2-5(A) synthetase correspond to known changes in prostaglandin release (Barros et al., 1991), endometrial oxytocin receptor content (Stephenson et al., 1994) and maintenance of pregnancy (see Bazer et al., 1994). The objective of the present studies is to determine the role

of the 2-5(A) system in maternal recognition of pregnancy in cattle. In these studies, we have utilized an *in vitro* system to directly introduce the 2-5(A) oligotrimer into cultured bovine endometrial cells and whole endometrial explants. In using this technique we have attempted to bypass IFN-τ, the IFN receptor and 2-5(A) synthetase. By observing endometrial secretory protein synthesis and $PGF_{2\alpha}$ synthesis levels in response to oxytocin (OT) treatment, it may be possible to elucidate specific roles for the 2-5(A) system in maternal recognition of pregnancy in domestic cattle.

Materials and Methods

Animals. In all experiments, cyclic Angus and Angus X Hereford cows of approximately the same age (4-7 years) and weight (600 kg) were observed for estrous behavior twice daily (0700 and 1600). On the appropriate day of slaughter, uteri were obtained from cows within 10 minutes after exsanguation and placed in a chilled (4°C) 700 ppm Roccal solution and transported on ice to the laboratory (within 20 minutes) for processing in a sterile, horizontal positive-flow hood. Uteri were trimmed free of the broad ligament and ovaries. Each uterine horn was flushed separately with sterile Hanks balanced salt solution (HBSS) (Gibco, Grand Island. NY) and the flushes were checked for signs of infection.

Uterine Cell Separation. Isolation of epithelial and stromal cells populations was conducted as previously described by Schmitt et al. (1993) (see Appendix A). Briefly, each uterine horn was separated, filled with a 4.8 mg/ml dispase-pancreatin (Boehringer Mannheim, GmbH, FRG; Gibco, Grand Island. NY) solution and incubated in 3 water baths (4°C, 20°C and 37°C) for 60, 20 and 10 minutes, respectively. Surface epithelial rafts were recovered from the initial enzyme wash. Following removal of the enzyme wash, uterine horns were filled with Ca- and Mg-free HBSS (Gibco, Grand Island. NY). After a 30 minute incubation in room temperature HBSS, additional surface epithelial rafts were recovered from the wash. Incubation with Ca- and Mg-free HBSS was repeated 2-3 times and the surface epithelial rafts recovered were subsequently pooled with those recovered from the first 2 washes. Uterine glands were also obtained from the 2 to 3 additional incubations with Ca- and Mg-free HBSS. Surface epithelial rafts and glands were identified microscopically and then isolated on 20-μm screens. The surface and glandular epithelial cells were further dissociated by being suspended in Ca-and Mg-free HBSS and allowed to incubate at 37°C for approximately 3 hours.. After the last incubation to recover uterine glands (Wash 5), the uterine horns were infused with 1X PBS-dispase (Gibco, Grand Island. NY; Boehringer Mannheim, GmbH, FRG) and incubated at 37°C for 30 minutes to obtain stromal cells. Stromal cells were obtained following 2-3 additional incubations with Ca-and Mg-free HBSS at 20°C for 30 minutes.

Stromal cells were recovered by passing through a 20-μm screen and suspended in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island. NY) + 10% sterile cow serum + 2% antibiotic-antimycotic (ABAM)+anti-PPLO agent (Gibco, Grand Island. NY). The surface and glandular epithelial cells were also suspended in DMEM + 10% cow serum + 2% ABAM+anti-PPLO and differential plated for 60 minutes to remove any contaminating stromal cells. After differential plating, the three respective cell populations were estimated on a hemocytometer and diluted to $1X10^6$ cells/ml and plated on sterile 24 well plates at approximately $1X10^6$ cells/well and cultured in a controlled atmosphere of 95% air:5% CO_2 at 37°C. Monolayers were cultured to approximately 50% confluence (5-7 days).

Experiment 1. Lipofection of Cultured Bovine Endometrial Cells.

Experiment 1a: Preliminary Study (Lipofectin optimization). A preliminary study was performed to optimize the amount of lipofection agent (Lipofectin; Gibco, Grand Island NY) to be used in the culture system. Madin-Darby bovine kidney cells (MDBK; American Type Culture Collection, Rockville, MD) cells were cultured to approximately 50% confluence and the following treatments were made. **(LO3 treatment)** = Lipofectin (1 mg/ml; Gibco, Grand Island NY) was diluted (1:4; 1:16; 1:20 1:32; 1:40) with sterile DMEM and added (1:1) to $1X10^{-4}$ M 2-5(A) oligonucleotide trimer (kindly supplied by Dr. P.F. Torrence; National Institute of Health, Bethesda, MD); **(L treatment)** = Lipofectin (1:4; 1:16; 1:20

1:32; 1:40 dilution) was added (1:1) to sterile DMEM; **(C treatment)** = sterile DMEM.

After addition of diluted treatments (200 μl/well), MDBK monolayers were incubated for 3 hours at 38.5°C; 5%CO_2 and then washed with HBSS. Monolayers were then covered with DMEM + 1% sterile cow serum and labeled with 2 μl ^{14}C-Leucine/well (444,000 cpm/well; Amersham, Arlington Heights, IL) at 38.5°C; 5% CO_2, for 6 hours. After incubation, endometrial cell were washed twice with HBSS and covered with 5% TCA (1 ml/well) and incubated at 4°C for 30 minutes. The endometrial cells were lysed by addition of .1M NaOH (1 ml/well) and incubated at 38.5°C for 60 minutes and neutralized by addition of 1M HCl (100 μl/well). The cell lysates were collected, transferred to scintillation vials, and incorporation of ^{14}C-Leucine into intracellular protein synthesis levels was analyzed, in the cell lysates, on a scintillation counter.

To optimize the lipofection technique for uterine cells, endometrial stromal cells were separated and cultured as described above. Upon reaching 50% confluence, stromal monolayers were washed twice with sterile HBSS and treated with the following: **(LO3 treatment)** = Lipofectin (1 mg/ml) was diluted (1:8; 1:16; 1:20) with sterile DMEM and added (1:1) to $1X10^{-4}$ M 2-5(A) oligonucleotide trimer; **(L treatment)** = Lipofectin (1:8; 1:16; 1:20 dilution) was added (1:1) to sterile DMEM; **(C treatment)** = sterile DMEM. The stromal monolayers were

cultured, lysed and processed for measurement of intracellular protein synthesis as described previously.

In this preliminary study, ^{14}C-labeled intracellular protein synthesis levels (as % control) of MDBK monolayers treated with liposomes which contained 2-5(A) oligonucleotide trimer (LO3) were compared to those treated with Lipofectin (L) alone. In MDBK cells, 1:16 dilution of Lipofectin was effective in transfecting 2-5(A) trimer into cell monolayers (as evidenced be decreased ^{14}C-labeled protein synthesis) without apparent detrimental effect (Table IV). However, treatment of bovine endometrial stromal monolayers with 1:16 dilution of Lipofectin resulted in an unacceptable decrease in intracellular protein synthesis (Table IV); indicating that 1:16 dilution of Lipofectin was cytotoxic to cultured stromal cells.

Subsequent analysis of intracellular protein production for cultured endometrial stromal cells (Table IV) determined that a 1:20 dilution of Lipofectin:DMEM (200 μl/well) optimized 2-5(A) trimer transfection while minimizing the cytotoxic effects of Lipofection. Therefore, in all subsequent studies, 1:20 dilution of Lipofectin:DMEM was used to transfect 2-5(A) oligonucleotide trimer.

Experiment 1b. Intracellular Protein Profiles-Separated Endometrial Cells

Cyclic animals (n=7) were slaughtered on day 10 postestrus (estrus = day 0).

TABLE IV

PRELIMINARY STUDY: LIPOFECTIN OPTIMIZATION

Cell Type	Treatment	Lipofectin dilution	Intracellular Protein Inhibition (% Control)
MDBK	LO3	1:4	88.76
		1:16	90.64
		1:20	98.95
		1:32	81.8
		1:40	32.35
	L	1:4	41.73
		1:16	9.7
		1:20	30.28
		1:32	12.77
		1:40	29.55
ST	LO3	1:8	18.99
		1:16	100
		1:20	84.75
	L	1:8	66.09
		1:16	76.27
		1:20	39.22

MDBK = Madin-Darby bovine kidney cells

ST = Bovine endometrial stromal cells

LO3 = Lipofectin (1:20) + 2-5(A) oligonucleotide liposomes

L = Lipofectin (1:20) + DMEM

Endometrial cells were separated and cultured as described above. Upon reaching 50% confluence, the endometrial cell monolayers in each well were washed twice with HBSS and treated, in duplicate, with one of the following treatments (200 μl/well): **(LO3 treatment)** = Lipofectin (1 mg/ml) was diluted (1:20) with sterile DMEM and added (1:1) to $1X10^{-4}$ M 2-5(A) oligonucleotide trimer; **(L treatment)** = Lipofectin (1:20 dilution) was added (1:1) to sterile DMEM; **(C treatment)** = sterile DMEM. After addition of diluted treatments, endometrial cell monolayers were incubated for 3 hours at 38.5°C; 5% CO_2 and then washed with HBSS.. Monolayers were then covered with DMEM + 1% sterile cow serum and labeled with 2 μl ^{14}C-Leucine/well (444,000 cpm/well; Amersham, Arlington Heights, IL) and incubated at 38.5°C; CO_2, for 6 or 24 hours. After the respective incubation times, cells were lysed and processed for scintillation counting as described above.

Experiment 2. Lipofection of Cultured Endometrial Explants (Secretory Protein Profile).

Endometrial ^{35}S-labeled Secretory Protein Profiles. Cyclic animals (n=4) were slaughtered on day 15 postestrus (estrus = day 0). Uteri were collected and the ipsilateral horn to the CL was isolated and processed as described above. The ipsilateral uterine horn to the CL was opened along the anti-mesometrial

border and strips of endometrium (intercaruncular tissue only) were separated from myometrium and cut into explants approximately 2-3 mm in diameter. Approximately 200 mg of endometrial tissue was placed into single wells of a sterile 6-well culture plate containing 2 mls sterile DMEM. The explants were pre-incubated for 3 hours at 37°C, with gentle rocking, to equilibrate the tissue. The explants were then washed twice with HBSS and subsequently treated with one of the following treatments: 1:1 dilution (1 mg/ml) Lipofectin (1:20) + $1X10^{-4}$ M 2-5(A) oligoadenylate trimer **(LO3)**; 1:1 dilution Lipofectin (1:20) + $3X10^{-4}$ M adenosine monophosphate (AMP; kindly supplied by Dr. P.F. Torrence; National Institute of Health, Bethesda, MD) **(LOAMP)**; $1.6X10^{6}$ antiviral units (AVU)/ml roIFN-τ **(IFN)** (kindly provided by Dr. F.W. Bazer; Texas A&M University, College Station, TX); or DMEM **(C)** and incubated, with gentle rocking, for 3 hours at 37°C.

In this study AMP is used as a positive control for adenosine treatment. As stated before, the intracellular activator of the 2-5(A) dependent endoribonuclease is a trimer of ATP linked by 2'-5' phosphodiester bonds, instead of the usual 3'-5' (see Johnston and Torrence, 1984). It has been demonstrated, in all cell types studied, that AMP does not activate the 2-5(A)-dependent endoribonuclease (see Johnston and Torrence, 1984). Therefore, in the present study, liposomes containing AMP were used as a control for 2-5(A) trimer

treatment. The LOAMP treatment contains $3X10^{-4}$ M AMP, which is the same amount of adenosine contained in the $1X10^{-4}$ M 2-5(A) trimer (LO3) treatment.

Following treatment incubation, endometrial explants were washed twice with HBSS, cultured in methionine-deficient DMEM (Gibco, Grand Island. NY), supplemented with 2.5 μl ^{35}S-methionine (25 μCi/well; Dupont NEN, Wilmington, DE) and cultured for 24 hours at 37°C, with gentle rocking. The explant-conditioned media was collected and frozen at -20°C for later quantification of ^{35}S-labeled secretory proteins.

^{35}S-labeled culture media were microconcentrated (Centricon 100; Amicon, Danvers, MA) and approximately 250,0000 dpm of microconcentrated sample were loaded and subjected to 2D-SDS-polyacrylamide gel electrophoresis (2D-PAGE) as previously described (Gries et al., 1989). After 2D-PAGE, the gels were stained with Coomassie Blue and subjected to fluorography (ENHANCE; Dupont-NEN, Wilmington DE) and placed on Kodak XAR X-ray film (Sigma, St. Louis, MO). Fluorographs were developed after 21 days of exposure at -80°C.

Experiment 3. Lipofection of Cultured Endometrial Explants ($PGF_{2\alpha}$ Profile).

Endometrial Prostaglandin $F_{2\alpha}$ Levels in Response to Oxytocin Treatment.

Cyclic cows (n=3) were slaughtered on day 15 postestrus (estrus = day 0). Uteri were collected and the ipsilateral horn to the CL isolated and processed into

endometrial explants, and put into duplicate plates, as previously described in Experiment 2.

Endometrial explants (200 mg/ml) were pre-incubated in DMEM for 3 hours at 37°C to equilibrate the tissue. The explants were washed twice with HBSS and cultured for an additional 3 hours. Media was collected and stored at -80°C for later analysis of basal $PGF_{2\alpha}$ secretion, which was used as a covariant in the $PGF_{2\alpha}$ assay analysis . The explants were washed twice with HBSS and the following treatments were added: 1:1 dilution Lipofectin (1:20) + 1 X 10^{-4} M 2-5(A) oligoadenylate trimer **(LO3)**; 1:1 dilution Lipofectin (1:20) + 3 X10^{-4} M AMP **(LOAMP)**; 1.6 X 10^{6} AVU/ml roIFN-τ **(IFN)**; or DMEM **(C)** and incubated in a culture box, for 3 hours at 37°C, with gentle rocking. The explants were then treated (2 ml/well) with 2 μg/ml oxytocin (OT) (Sigma, St. Louis. MO) and incubated with gentle rocking for 60 minutes at 37°C. Medium was changed 30, 60 and 90 minutes post-OT treatment. The collected media was then frozen at -80°C for later analysis of $PGF_{2\alpha}$ production.

Prostaglandin $F_{2\alpha}$ Radioimmunoassay. Production of $PGF_{2\alpha}$ from endometrial explant, in culture, was analyzed as previously described (Mirando et al. 1993). The sensitivity of the $PGF_{2\alpha}$ assay was 100 pg/ml and the intra- and interassay CV were 5.73% and 12.11%, respectively.

Statistical Analysis.

In Experiment 1, data were subjected to least squares analysis of variance (ANOVA) using the General Linear Models (GLM) procedures of Statistical Analysis System (SAS, 1986). Inhibition of ^{14}C-labeled intracellular protein synthesis was analyzed across endometrial cell types using the model: cell type, time of incubation, cell type*time of incubation, plate (cell type*time of incubation), treatment, treatment*cell type, treatment*time of incubation, treatment*cell type*time of incubation. Difference in mean ^{14}C-labeled intracellular protein synthesis levels across cell type, time of incubation and treatment were analyzed by Bonferroni's test (SAS, 1986).

In Experiment 3, endometrial secretion of $PGF_{2\alpha}$ was analyzed using the following model: cow, plate(cow), treatment, treatment*cow, treatment*plate(cow), well(treatment*plate*cow), hour, hour*treatment, hour*cow, hr*plate(cow). Plate(cow) was used for the error term for cow; well(treatment*plate*cow) was used for the error term for plate(cow); treatment*cow was used for the error term for treatment; treatment*plate(cow) was used for treatment*cow; well(treatment*plate*cow) was used for the error term for treatment*plate(cow); hour*cow was used for the error term for hour; hour*plate(cow) was used for the error term for hour*cow. Differences in mean $PGF_{2\alpha}$ secretion between treatments and hours post-OT were analyzed by

Bonferroni's test (SAS, 1986).

Results

Experiment 1. Lipofection of Cultured Endometrial Cells.

Endometrial ^{14}C-labeled Intracellular Protein Synthesis. Treatment of bovine endometrial explants with 2-5(A) oligoadenylate trimer liposomes (LO3) and subsequent culture for 6 hours, decreased ($P<.05$) ^{14}C-labeled intracellular protein synthesis compared to DMEM-treated (C) controls (Figure 6). The three endometrial cell types responded similarly to LO3 treatment. The inhibition of intracellular protein synthesis, as percentage of control, was: SE (84.4±9.8%); GE (66.2±9.8%); ST (81.8±9.8%) respectively. This is greater inhibition ($P<.05$) than L treatment levels, as percentage of control: SE (25.8±9.8%); GE (17.2±9.8%); ST (29.5±9.8%) (Figure 6).

The initial inhibition of ^{14}C-labeled intracellular protein synthesis, following 3 hour treatment incubation, was transient. Intracellular protein synthesis levels returned to control levels following treatment of separated bovine endometrial cells with 2-5(A) oligoadenylate trimer liposomes (LO3) and subsequent culture for 24 hours (Figure 7). The percent inhibition of ^{14}C-labeled intracellular protein synthesis, after 24 hours of culture, of SE cells (9.25±12.7%) was less ($P<.05$) than either GE (37.6±9.15%) or ST (56.7±7.4%) cells respectively.

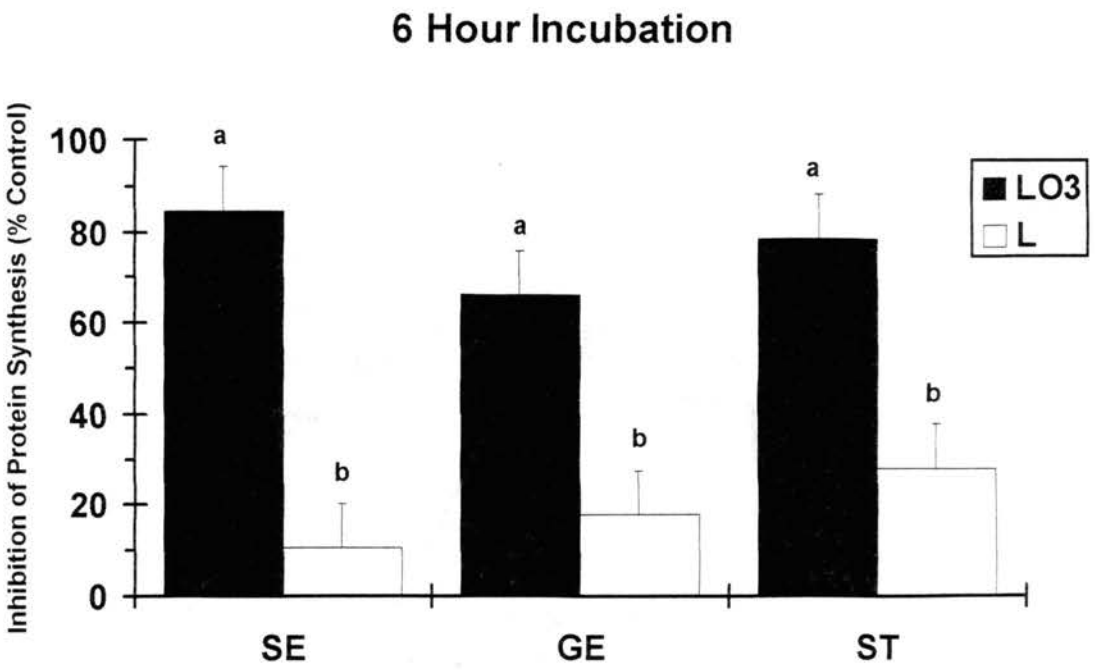

Figure 6. Inhibition of ^{14}C-labeled intracellular protein synthesis, as a percent of control, in separated bovine endometrial cells, treated with 2-5(A) trimer liposomes and cultured for 6 hours *in vitro*. SE = surface epithelium; GE = glandular epithelium; ST = stroma; LO3 = 2-5(A) oligonucleotide trimer liposomes; L = Lipofectin.

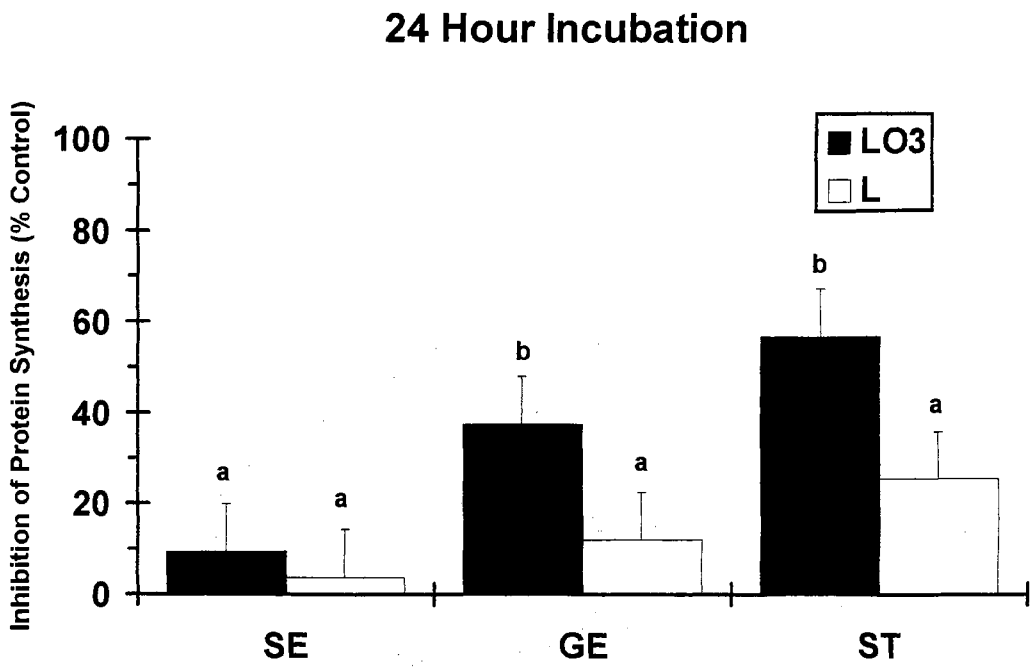

Figure 7. Inhibition of ^{14}C-labeled intracellular protein synthesis, as a percent of control, in separated bovine endometrial cells, treated with 2-5(A) trimer liposomes and cultured for 24 hours *in vitro*. SE = surface epithelium; GE = glandular epithelium; ST= stroma; LO3 = 2-5(A) oligonucleotide trimer liposomes; L = Lipofectin.

Experiment 2. Lipofection of Cultured Endometrial Explants.

Endometrial ^{35}S-labeled Secretory Protein Profiles. Bovine endometrial explants treated with roIFN-τ for 3 hours and subsequently cultured for 24 hours had basically the same secretory protein profiles as explants given the other treatments (LO3, LOAMP, C). However, IFN-τ-treated bovine endometrial explants produced and secreted two proteins (Mr = 28 kDa; pI = 4.5 and 4.8, respectively) that were not observed in the secretory protein profiles of explants given any of the other treatment (Figure 8 and 9). Also, treatment of bovine endometrial explants with roIFN-τ resulted in the enhancement of one secretory protein (Mr = 28 kDa and pI = 4.2); which was also produced, albeit at markedly lower levels, by explants given LO3, LOAMP or C treatment (Figure 8 and 9).

Treatment of bovine endometrial explants with 2-5(A) oligoadenylate trimer liposomes (LO3) for 3 hours and subsequent culture in methionine-deficient DMEM for 24 hours did not alter ^{35}S-labeled secretory protein profiles compared to fluorographs of LOAMP or C treated explant cultures. There was no discernible difference in the number or type of proteins produced between the 3 treatments (Figure 8 and 9).

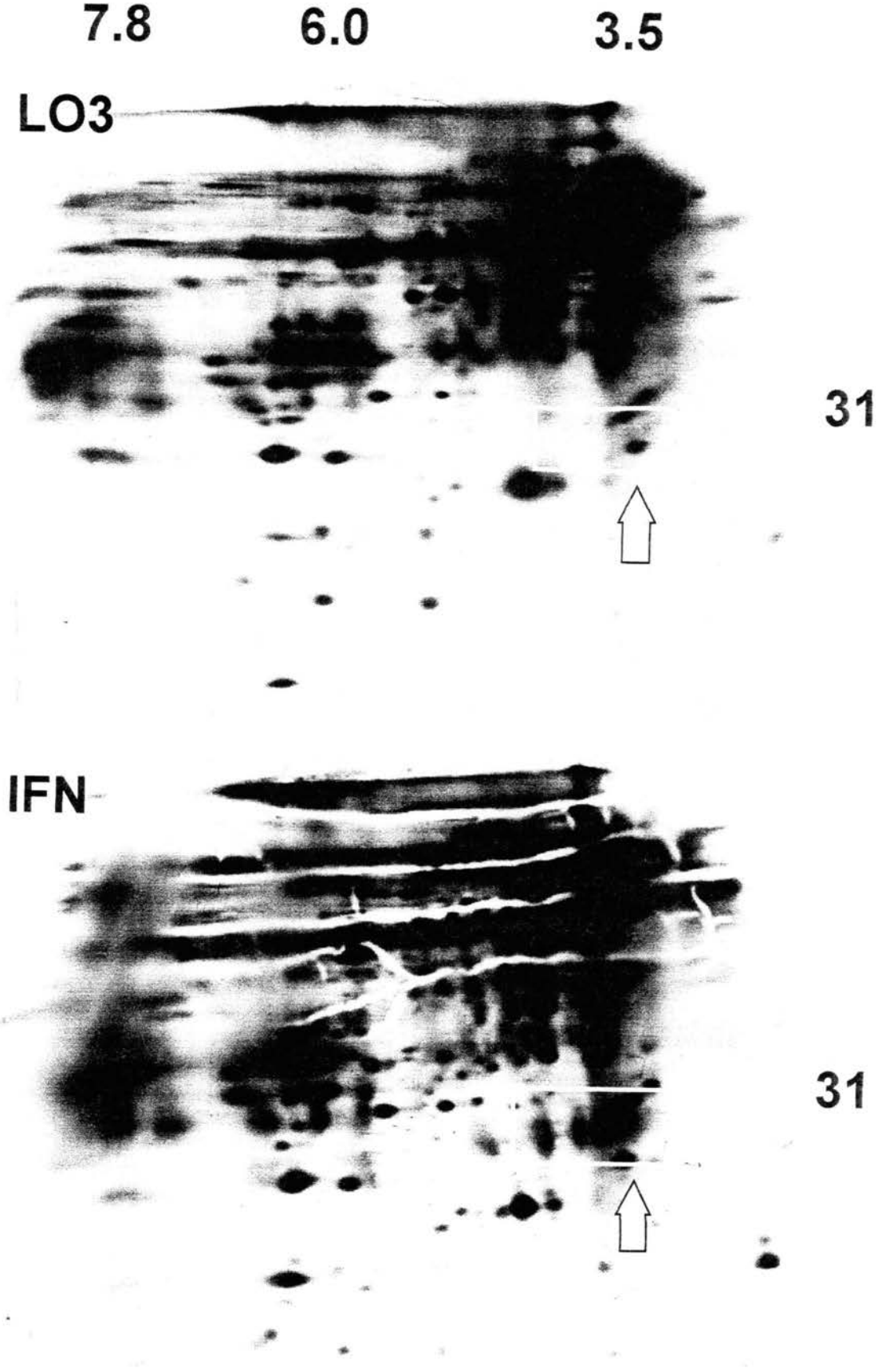

Figure 8. Representative autoradiographs of ^{35}S-labeled secretory protein profiles of bovine endometrial explants treated with: (A). 1X10^{4} M 2-5(A) oligonucleotide trimer + 1:20 lipofectin (LO3). (B). 1.6X10^{6} AVU/ml roIFN-τ (IFN). Area in box represents secretory proteins stimulated or enhanced in cultures treated with roIFN-τ.

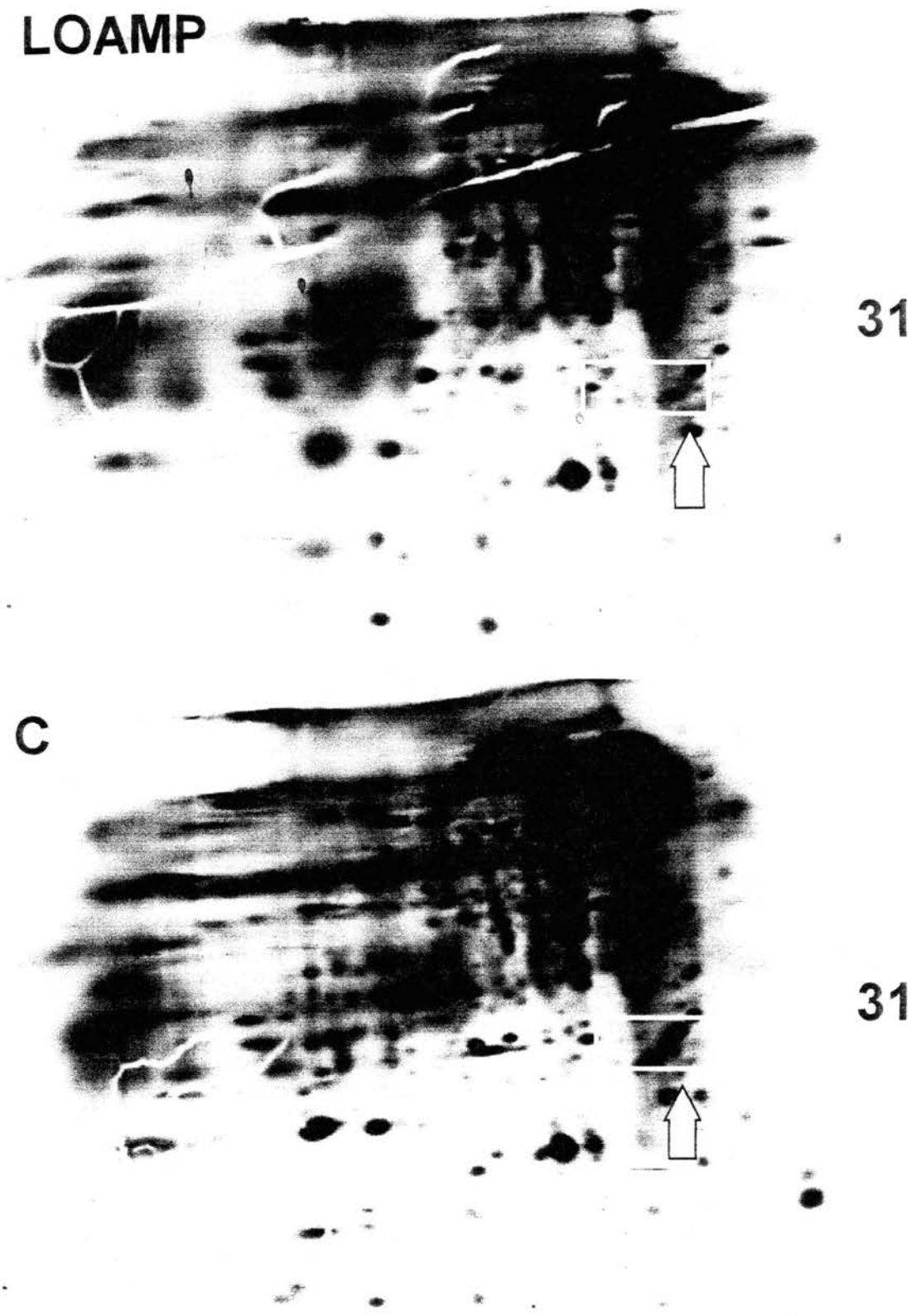

Figure 9. Representative autroradiographs of ^{35}S-labeled secretory protein profiles of bovine endometrial explants treated with: (A). $3X10^{4}$ M AMP + 1:20 lipofectin (LOAMP). (B). DMEM(C). Area in box represents secretory proteins stimulated or enhanced in cultures treated with roIFN-τ.

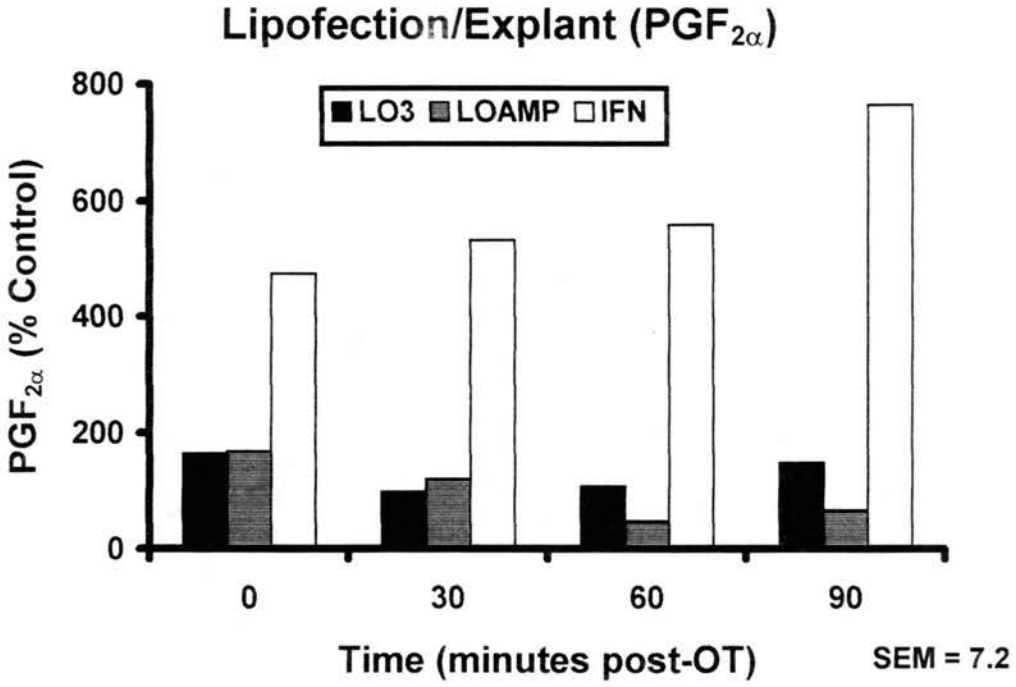

Figure 10. Average endometrial $PGF_{2\alpha}$ release (% control) from bovine endometrial explants treated with 2-5(A) liposomes and subsequently treated with oxytocin (OT) *in vitro*.

Experiment 3. Lipofection of Cultured Endometrial Explants.

Endometrial Prostaglandin $F_{2\alpha}$ Levels in Response to Oxytocin Treatment.

Analysis of endometrial $PGF_{2\alpha}$ levels in response to oxytocin treatment demonstrated significant ($P<.05$) cow to cow variability in $PGF_{2\alpha}$ (pg/ml) secretory levels, but not a significant cow*treatment interaction. To reduce the variability in $PGF_{2\alpha}$ secretion between cows, $PGF_{2\alpha}$ secretory data for each treatment is expressed as percentage of the $PGF_{2\alpha}$ level of the control well (% control) for each respective plate.

Treatment of bovine endometrial explants with 2-5(A) oligoadenylate trimer liposomes (LO3) for 3 hours and subsequent 24 hour culture stimulated a ($P<.05$) increase in $PGF_{2\alpha}$ secretion ($P<.05$) compared to control, and these levels were similar for each sampling time (164.1%$\pm$21.3; 99.2%$\pm$10.2; 107.9%$\pm$12.9; 148.7%$\pm$8.36 for 0; 30; 60; 90 minutes, respectively; Figure 10). The $PGF_{2\alpha}$ levels of LO3-treated explants were not different from that of endometrial explants treated with AMP liposomes (LOAMP) at any of the sample times (168.1%$\pm$16.5, 120.6%$\pm$15.2; 48.3%$\pm$6.9; 66.4%$\pm$6.6, for 0; 30, 60, 90 minutes, respectively; Figure 10). These results indicate that the increased $PGF_{2\alpha}$ secretion is probably due to exposure to the lipofection agent and not due to oligonucleotide treatment. Recombinant oIFN-τ stimulated $PGF_{2\alpha}$ secretion (474.5%$\pm$50.1; 533.1%$\pm$54.11;

559.9%±49.5; 764.9%±57.8) for (0, 30; 60; 90 minutes) respectively, that was different ($P<.05$) from $PGF_{2\alpha}$ levels of the other treatments for each sampling time (Figure 10). Treatment with oxytocin did not affect release of $PGF_{2\alpha}$ from bovine endometrial explants.

Discussion

It is well established that trophoblast IFNs secreted from the conceptus are involved in maternal recognition of pregnancy in domestic ruminants (see Bazer et al., 1984, 1989; Roberts et al., 1989a,b, 1990). Intrauterine infusion of purified oIFN-τ (Godkin et al., 1984a; Vallet et al., 1988) or highly enriched bIFN-τ complex (Helmer et al., 1989a) prolongs CL function in cyclic ewes and cows, respectively. However, conceptus secretory proteins from which oIFN-τ had been removed by immunoabsorption fail to lengthen the interestrous interval of cyclic ewes (Vallet et al., 1988). The trophoblast IFNs have demonstrated antiviral and antiproliferative effects characteristic of IFN-α (Peskta et al., 1987; Roberts et al., 1990).

Spencer et al. (1995c) propose that conceptus secretion of IFN-τ, during early pregnancy, extends the "progesterone block" that results in the continued suppression of endometrial estradiol receptor (ER) gene expression in the luminal epithelium and subsequently prevents upregulation of oxytocin receptor (OTR)

and pulsatile release of $PGF_{2\alpha}$ (Spencer et al., 1995a). Trophoblast IFNs do not prolong the progesterone block by stabilization or upregulation of endometrial PR, because endometrial epithelial PR levels are downregulated in cyclic (Spencer and Bazer, 1995; Spencer et al., 1995a,b,c), pregnant (Spencer and Bazer, 1995) or IFN-τ treated (Spencer et al., 1995a,b,c) ewes. The mechanism by which IFN-τ inhibits ER gene expression is currently unknown.

One of the major characteristics of IFNs, including the trophoblast IFNs is to stimulate the 2-5(A) system (Johnston and Torrence, 1984; Short et al., 1991; Schmitt et al., 1993). In ewes, endometrial 2-5(A) synthetase activity is increased during early pregnancy and after rbIFN-α injection (Mirando et al., 1991). In the cow, Short et al. (1991) observed a significant increase in total endometrial 2-5(A) synthetase on day 18 of gestation that is concurrent with conceptus production of bIFN-τ. A subsequent study determined that this increase in endometrial 2-5(A) synthetase during early gestation was specific to the uterine horn containing the conceptus and only in the endometrial surface and glandular epithelium and not the underlying stromal cells (Schmitt et al., 1993). These results were interesting because it demonstrated that endometrial 2-5(A) synthetase activity was not only stimulated in the uterine horn containing the conceptus but also only in the endometrial cell types in direct contact with the conceptus and conceptus-secreted bIFN-τ. Also, bIFN-τ has been shown to be as effective as rbIFN-α in

inducing intracellular 2-5(A) synthetase activity in cultured endometrial cell monolayers (Schmitt et al., 1993). Increased endometrial 2-5(A) synthetase activity, in bovine endometrial explants, is correlated with the blocking of endometrial PGF secretion by bIFN-α (Barros et al., 1991). The combination of these results have provided circumstantial evidence that the 2-5(A) system could play a role in the mechanism of maternal recognition of pregnancy.

Lipofection in a highly efficient technique originally used to transfect exogenous DNA directly into cultured cells (Felgner et al., 1987). In this technique, oligonucleotides are coated in cationic lipid which form liposomes. The liposomes are subsequently phagocytized by the cultured cells, and the liposome-enveloped oligonucleotides are then released into the cell and a portion of the exogenous DNA becomes localized in the nucleus (Felgner et al., 1987). In Experiment 1, we modified the lipofection technique, not to incorporate exogenous DNA into the nucleus of cells, but rather to incorporate the activator of the 2-5(A)-dependent endoribonuclease [2-5(A) oligonucleotide trimer] into cultured bovine endometrial cells and observe subsequent ^{14}C-labeled intracellular protein synthesis levels.

The results from Experiment 1 indicate that 2-5(A) oligonucleotide trimer can be successfully introduced directly into cultured bovine endometrial cells and activate the 2-5(A)-dependent endoribonuclease. A significant decrease in ^{14}C-

labeled intracellular protein synthesis levels was observed after incubation of cultured endometrial cells with 2-5(A) trimer liposomes and subsequent 6 hour culture in the presence of ^{14}C-Leucine. This effect was transient in that cultured cells, treated as above but cultured in the presence of ^{14}C-Leucine for 24 hours, had intracellular protein synthesis levels that returned to control levels. Thus, the lipofection technique was not cytotoxic to the cell monolayers and the decrease in intracellular protein synthesis levels was initiated by the activation of the 2-5(A) system.

While the functional half-life of 2-5(A) trimer is not known, studies with mouse L cells demonstrated that microinjection of 2-5(A) trimer prior to infection with vesicular stomatitis virus (VSV) inhibited virus growth and lasted for approximately 12 hours (Higashi and Sokawa, 1982). These data are consistent with our observation of the transient inhibition of ^{14}C-labeled intracellular protein synthesis in cultured bovine endometrial cells. The identity and function of the proteins that are being inhibited by lipofection of 2-5(A) trimer have not been evaluated.

The results of Experiment 2 indicate that treatment of bovine endometrial explants, *in vitro*, with roIFN-τ induces changes in secretory protein profiles compared to control cultures. These changes were not seen in endometrial explants treated with 2-5(A) trimer liposomes, indicating that the effect of IFN-τ on

the synthesis and secretion of these secretory proteins is not mediated through the 2-5(A) system; but rather through some other intracellular mechanisms.

In a recent study, Naivar et al. (1995) described a similar protein (28 kDa; pI=6.3) which was stimulated by treatment with rbIFN-τ but not rbIFN-α in endometrial explants from both cyclic and pregnant cows. In the present study, it is possible to see that the purported 28 kDa IFN-τ-stimulated protein are two secretory proteins (28 kDa; pI=4.5, 4.8, respectively), whose secretion is stimulated by IFN-τ; and one secretory protein (28 kDa; pI=4.2) whose secretion is greatly enhanced by treatment with IFN-τ. Currently the function of these IFN-τ -stimulated secretory proteins is unknown. More research is needed to identify the structure and function of these IFN-τ-stimulated secretory proteins, as well as the mechanisms by which these proteins are differentially stimulated to be secreted by IFN-τ but not IFN-α.

The results, from Experiment 3, indicate that day 15 bovine endometrial explants are not responsive to oxytocin (OT), in terms of increased $PGF_{2\alpha}$ production, and that the 2-5(A) system is not involved in the increased $PGF_{2\alpha}$ secretion following roIFN-τ treatment. In the present study, roIFN-τ stimulated a large release of $PGF_{2\alpha}$ from bovine endometrial explants that was not seen in explants treated with 2-5(A) liposomes. This indicates that the increase in $PGF_{2\alpha}$,

in response to IFN-τ, is not mediated through the 2-5(A) system.

The increase in $PGF_{2\alpha}$ in response to IFN-τ treatment could be due to an effect of IFN-τ not associated with maternal recognition of pregnancy. The trophoblast IFNs possess antiviral activity characteristic of Type 1 IFNs (Roberts et al., 1989a) and bind to the same Type 1 IFN receptor as IFN-α (Stewart et al., 1987; Stewart et al., 1989a; Li and Roberts, 1994). Pottathil et al. (1980) demonstrated that the establishment of an IFN-mediated antiviral state involves the activation of fatty acid cyclooxygenase, the enzyme that catalyzes the first step in prostaglandin biosynthesis. In the present study, it is possible that IFN-τ treatment stimulated an antiviral response in the endometrial explants characterized by a large release of $PGF_{2\alpha}$. Danet-Desnoyers et al. (1994) described slight increase in $PGF_{2\alpha}$ in response to IFN-τ treatment in separated endometrial stromal cells. Since endometrial explants are mostly stromal cells with a thin layer of overlying epithelial cells, it is not surprising that the $PGF_{2\alpha}$ profiles in response to IFN-τ treatment are similar between explants and cultured stromal cells.

Data from the present study indicate that OT was not effective in inducing $PGF_{2\alpha}$ release from bovine endometrial explants. Recently studies have identified cell specific effects of OT-induced release of PGF from separated

bovine endometrial cells (Danet-Desnoyers et al., 1994; Asselin et al. 1996). These researchers observed that bovine endometrial epithelial cells were responsive to OT treatment whereas endometrial stromal cells were not, and hypothesized that on day 15 of gestation endometrial OT receptors are present on epithelial cells and not on stromal cells (Danet-Desnoyers et al., 1994). Recently in the ewe, OT receptor mRNA has been localized in the endometrial luminal epithelium of days 14-15 of the estrous cycle (Stevenson et al., 1994). Therefore it appears that the endometrial epithelial cells are the main target for OT to stimulate endometrial $PGF_{2\alpha}$ secretion and not endometrial stromal cells (Asselin et al., 1996).

The endometrial $PGF_{2\alpha}$ profiles of endometrial explants in response to OT treatment, in the present study, are similar to those shown for separated endometrial stromal cells exposed to OT (Danet-Desnoyers et al., 1994). There was no difference in PGF levels from endometrial stromal cells cultured in the presence of OT and those of untreated controls over a 90 minute incubation period (Danet-Desnoyers et al., 1994). As stated before, endometrial explants are mostly stromal cells with a thin layer of overlying epithelial cells. Hence, it is not surprising that the $PGF_{2\alpha}$ profiles in response to OT treatment are similar to those of cultured endometrial stromal cells. The relatively large amount of stromal tissue appears to mask the OT-induced increase in PGF secretion from

the epithelial cells (Danet-Desnoyers et al., 1994; Asselin et al. 1996).

It is possible, in this study, that there are cell-cell interactions between the epithelial and stromal cells in endometrial explants that are not present in cultures of the separated endometrial cell types; and that these interactions are involved in the IFN-stimulated $PGF_{2\alpha}$ release of endometrial explants. Also, endometrial tissue was collected and treated on the day of slaughter (day 15 of the estrous cycle). Then IFN-τ was applied for three hours, treated with OT for 1 hour and then sampled at 30, 60, 90 minutes after removal of OT. Danet-Desnoyers (1994) collected and separated tissue on day 15 of the estrous cycle, cultured for approximately 7 days, treated with IFN for 24 hours, then sampled 30 and 90 minutes after OT treatment. During the culture period, the separated endometrial cells may have differentiated, therefore the stromal cells were no longer as responsive to IFN-stimulation, in regards to $PGF_{2\alpha}$ secretion.

Also, IFN-stimulated $PGF_{2\alpha}$ secretion may be an acute response to IFN-τ treatment and that incubation with IFN-τ for 24 hours and subsequent sampling was too late to detect the increased $PGF_{2\alpha}$ secretion. By using an *in vitro* perifusion chamber system, Vallet et al. (1989) demonstrated in ovine endometrium that short term oIFN-τ treatment enhanced OT-induced PGF secretion, while long term oIFN-τ treatment inhibited OT-induced PGF secretion. These researchers also demonstrated that endometrium from pregnant ewes

secreted more basal PGF than endometrium from cyclic ewes, indicating that oIFN-t may stimulate endometrial $PGF_{2\alpha}$ secretion *in vivo*. The biological significance of these observations have yet to be resolved.

It was observed, in this study that exposure of endometrial explants to the lipofection agent, either with 2-5(A) trimer or ATP, stimulated increased $PGF_{2\alpha}$ secretion compared to untreated controls. Felgner et al (1987) demonstrated that the lipofection agent is cytotoxic at high levels (>100 μg). In this study, 100 μg of lipofectin agent/well was used, so it is possible that we were damaging some of the cells and causing a release of $PGF_{2\alpha}$. However, as described in the preliminary study (Experiment 1a), 100 μg/ml of lipofection agent was shown to be effective in introducing 2-5(A) trimer into cultured bovine endometrial cells without causing permanent damage.

Currently, the biological mechanism by which the trophoblast IFNs prevent luteolysis in domestic ruminants is not completely understood. It appears that the actions of interferons are local, since the receptors for oIFN-τ are present in ovine endometrium (Godkin et al., 1984a; Knickerbocker and Niswender, 1989) and are competitively blocked by recombinant bovine IFN-α (Hansen et al., 1989).

The results from our experiments indicate that the 2-5(A) system is not involved in the major biological actions of trophoblast IFNs during maternal

recognition of pregnancy. Other IFN-stimulated systems have been identified and may be important for biological actions of IFN-τ. One of the earliest cellular responses to IFN exposure is the stimulation of specific genes termed IFN-stimulated response elements (ISRE) (Levy et al., 1988; Levy et al., 1989). Hannigan and Williams (1991) demonstrated that arachidonic acid metabolism and prostaglandin synthesis are involved in the IFN stimulation of ISRE. Exposure of cells to inhibitors of prostaglandin synthetase, result in amplification of ISRE-binding and gene transcription (Hannigan and Williams, 1991). It is possible that IFN-τ is activating ISRE in endometrial cells and affecting $PGF_{2\alpha}$ synthesis and preventing luteolysis.

It has also been hypothesized that ovine endometrial ER genes are associated with ISRE and may be inhibited from being expressed by conceptus secretion of trophoblast IFNs during maternal recognition of pregnancy (see Bazer et al., 1994). The ovine ER gene has not been cloned, however there is evidence that human ER contain putative ISREs (see Bazer et al., 1994). More research needs to be performed to clone the ER gene and to identify other ISRE-associated genes and determine if any are stimulated specifically by IFN-τ.

Another IFN-stimulated mechanism that may be involved in maternal recognition of pregnancy is IFN-induced Mx protein expression. In general, Mx proteins possess antiviral activity (see Pestka et al., 1987), are homologous to

GTPases and may be involved in cellular protein transport (Horisberger and Gunst, 1991). In ewes, Mx gene and protein expression has been demonstrated to be markedly enhanced in the endometrial epithelium, stroma and myometrium, on day 17 of gestation; and continues to be expressed at high levels in the luminal epithelium on day 25 of gestation (Ott et al, 1995). This is very similar to the activity of 2-5(A) synthetase in the endometrial surface and glandular epithelium during maternal recognition of pregnancy (Schmitt et al., 1993). Currently it is not known how Mx protein expression is involved in the mechanism of maternal recognition of pregnancy in ruminants. However Mx proteins could be involved in antiviral protection of the conceptus and the uterus during the establishment of pregnancy.

The specific effects of 2-5(A) system during early pregnancy remains to be determined. In this study, we have confirmed observations of the effects of IFN-τ on endometrial tissue and have shown that the 2-5(A) system does not appear to be involved in either endometrial protein or $PGF_{2\alpha}$ secretion. If the 2-5(A) system is involved in the establishment of pregnancy, it appears to be at a more subtle level than originally thought.

One of the ways 2-5(A) synthetase could function in the establishment of pregnancy is by protecting the conceptus from viral infection. In mammals, 2-5(A) synthetase is one of the first responses of the body to viral infection (Schattner et

al., 1982; Johnson and Torrence, 1984). In cattle, 2-5(A) synthetase content, in cultured MDBK cells, is stimulated by injection of modified live virus and bIFN-α (Short and Fulton, 1987). Intramuscular injection of bIFN-α increases 2-5(A) synthetase concentration in bovine peripheral blood mononuclear cells (Short and Fulton, 1987; Perino et al. 1990). It is possible that conceptus production of bIFN-τ activates the 2-5(A) system as a preventative measure to protect the conceptus from viral infection and help establish a uterine environment conducive for continued conceptus growth and development. It is also possible that the 2-5(A) system is being activated by IFN-τ to inhibit the synthesis of specific proteins within the endometrial epithelium, related specifically to the establishment of pregnancy. By identifying the intracellular mechanisms being affected by the 2-5(A) system, it may be possible to determine the role of the 2-5(A) system during early gestation; and lead to a better understanding of the effects of IFN-τ during establishment of pregnancy in the cow.

CHAPTER V

GENERAL DISCUSSION

It is hard to imagine the evolutionary challenge presented by the concept of eutharian mammals. The actual growth and development of one antigenically distinct individual within another; from a single cell to a complete organism, is truly one of the miracles of nature. It has been suggested that the evolution of the placenta of eutherian mammals is the result of genetic conflict between the mother and the fetus. Since both parties have different immediate goals and interests, an evolutionary compromise had to be brokered in order for eutherian pregnancies to become a reality (see Roberts et al., 1996).

While the production of viable offspring is the final goal of pregnancy, different species have developed different mechanisms of achieving this goal. Even if one looks at just one particular event during pregnancy; such as the mechanism by which the conceptus signals its presence to the mother, even closely related species can have evolved vastly different mechanisms to establish pregnancy. The domestic species are a good example to illustrate this phenomenon.

In the gilt, the mechanism by which the conceptus signals the mother to inhibit luteolysis and establish pregnancy is by conceptus secretion of estadiol (E2) during days 12-15 of gestation (see Bazer et al., 1986). The E2 is released into the uterine lumen and causes the endometrium to secrete the luteolysin prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in an exocrine manner (into the uterine lumen), rather than an endocrine manner (into the uterine/ovarian vasculature). This way $PGF_{2\alpha}$ is sequestered in the uterine lumen and cannot reach the ovaries and initiate luteolysis. The CLs are thus maintained and pregnancy is established (see Bazer et al., 1986).

Relatively little is known about maternal recognition of pregnancy in the mare. It is known is that intrauterine migration (12-14 times/day) of the spherical conceptus during days 12-18 of pregnancy is necessary to prevent luteolysis and establish pregnancy (see Bazer et al., 1994). It is thought that intrauterine migration allows the conceptus to deliver the maternal recognition signal to the entire surface area of the endometrium and this prevents the synthesis and/or secretion of $PGF_{2\alpha}$ to subsequently prevent luteolysis. However the identity of this factor is currently unknown.

The domestic ruminants have taken a different tact in establishing pregnancy, in that these species have adapted part of the immune system to

function as the signal of maternal recognition of pregnancy. Similar to the way that $PGF_{2\alpha}$ has been modified from being a general inflammatory agent to the specific luteolysin, the ruminants have modified Type I interferons (IFN) to be the signal for the establishment of pregnancy. It has been well established that conceptus secretion of trophoblast interferon (IFN-τ), between days 14-16 in the ewe (Ashworth and Bazer, 1989) and days 16-19 of gestation in the cow (see Bazer, 1992) is the signal for maternal recognition of pregnancy . It has been proposed (Spencer et al., 1995c) that conceptus secretion of IFN-τ, during early pregnancy, suppresses endometrial estradiol receptor gene expression in the uterine luminal epithelium and subsequently prevents upregulation of endometrial oxytocin receptor (OTR) and pulsatile release of $PGF_{2\alpha}$ (Spencer et al., 1995a). This, in effect, short circuits the luteolytic mechanism, so that $PGF_{2\alpha}$ is not released, the CL is maintained and pregnancy is established.

It is interesting that ruminants have adapted part of the immune system for maternal recognition of pregnancy. Perhaps during evolution, protection of the antigenically distinct conceptus from the maternal immune system caused simultaneous changes in both the mother and conceptus, the net result of which was the evolution of a specific subclass of Type 1 IFNs as the antiluteolytic mechanism in ruminants. Interferons, in general, are cytokines which have

antiviral, antiproliferative and cytotoxic effects (see Pestka et al., 1987). Trophoblast IFNs exhibit antiviral activity indicative of Type I IFNs. However, trophoblast IFNs are poorly inducible by virus and double stranded RNA in day 11 ovine conceptuses (Farin et al., 1991). Also, bIFN-τ contributes <0.1% of the total IFN produced by bovine leukocytes exposed to Sendai virus (Cross and Roberts, 1991). This may result from the virus-inducible gene sequence motifs for trophoblast IFNs being arranged differently than the other Type I IFNs (Hansen et al., 1991).

Trophoblast IFNs do not exhibit the cytotoxic effects indicative of other Type I IFNs (Pontzer et al., 1991). This explains why the large amount of IFNs secreted by the conceptus, during maternal recognition of pregnancy, does not harm the cells of the uterine endometrium or the conceptus itself. Trophoblast IFNs do share the antiproliferative effects of general Type 1 IFNs (Pestka et al., 1987) on most cell types including: lymphocytes (Newton et al., 1989; Niwano et al., 1989; Fillion et al., 1991; Skopets et al., 1992), oviduct epithelial cells (Kamwanja and Hansen, 1993) WISH and MDBK cells (Pontzer et al., 1991). However a recent study (Davidson et al., 1994) demonstrated that neither rbIFN-τ nor rbIFN-α have antiproliferative effects on bovine endometrial epithelial or stromal cells. This indicates that the bovine endometrium is resistant to the antiproliferative effects of Type 1 IFNs; and just as IFN-τ has evolved to be the

pregnancy recognition signal in ruminants, the bovine endometrium has evolved to be resistant to the antiproliferative effects of IFNs. Therefore the growth of the endometrium is not compromised by the large amounts of IFN-τ secreted by the conceptus during early pregnancy.

One of the major characteristics of IFNs is to stimulate the 2-5(A) system. The experiments, described in this dissertation, demonstrate that 2-5(A) oligonucleotide trimer can be successfully transfected into cultured bovine endometrial cells via a cationic lipid (lipofection) and cause a transient decrease in intracellular protein synthesis. These experiments have also demonstrated that the endometrial secretory protein and $PGF_{2\alpha}$ profiles changes, stimulated by exposure to IFN-τ, is not mediated through the 2-5(A) system.

Currently, there is no known role for the 2-5(A) system during early gestation in ruminants. However, the profile of intracellular 2-5(A) synthetase activity during early gestation (Schmitt et al., 1993) and the general biological actions of the 2-5(A) system would indicate that the 2-5(A) system may have a role in the establishment of pregnancy other than maternal recognition of pregnancy.

One of the ways 2-5(A) synthetase could function in the establishment of pregnancy is by protecting the conceptus from viral infection. In mammals, 2-5(A)

synthetase is one of the first responses of the body to viral infection (Schattner et al., 1982; Johnson and Torrence, 1984). In cattle, 2-5(A) synthetase content, in cultured MDBK cells, is stimulated by injection of modified live virus and bIFN-α (Short and Fulton, 1987). Intramuscular injection of bIFN-α increases 2-5(A) synthetase concentration in bovine peripheral blood mononuclear cells (Short and Fulton, 1987; Perino et al. 1990). It is possible that conceptus production of bIFN-τ activates the 2-5(A) system as a preventative measure to protect the conceptus from viral infection and help establish a uterine environment conducive for continued conceptus growth and development. It is also possible that the 2-5(A) system is being activated by IFN-τ to inhibit the synthesis of specific proteins, within the endometrial epithelium, related specifically to the establishment of pregnancy.

Probably the most efficient and effective way to determine which mRNAs and proteins are being affected by the 2-5(A) system is to use differential display polymerase chain reaction (ddPCR). The technique of ddPCR has been used to identify and analyze altered gene expression at the mRNA level in eukaryotic cells (Liang et al., 1994).

The sequential use of two types of primers are required for ddPCR (Liang et al., 1994; Mou et al., 1994). The first is an anchored primer, which is an oligo-

dT 13mer which anneals to the poly(A) tail-3' untranslated region of the mRNA. The second primer is an arbitrary primer 10mer of known sequence, and is added to the reverse transcription reaction mixture containing: Taq polymerase; radioactive nucleotides and dsDNA amplified using PCR (Mou et al., 1994). The amplified DNA fragments, representing the 3' termini of the mRNAs, are separated on a denaturing polyacrylamide gel and visualized using autoradiography. By analyzing the exposed X-ray film it is possible to compare which mRNAs are being differentially produced by different samples. The identified cDNAs can be eluted from the gel, sequenced and compared to the sequence of known cDNAs (Mou et al., 1994). The sequence and pattern of expression can be used in an attempt to identify the corresponding mRNA and protein and determine biological function.

In our culture system, ddPCR can be used to identify cell type- and pregnancy-dependent changes in mRNA profiles. The corresponding cDNA sequences of differentially expressed mRNA, in cyclic vs. pregnant endometrial cells, can then be used to identify which mRNAs are being expressed or not expressed and possibly determine the biological significance of the observed mRNA profiles. Also, by using the lipofection technique on separated bovine endometrial cells, it would be possible to identify the mRNAs being affected by

exposure to the 2-5(A) trimer; and compare the results to that of IFN-τ-treated cells. The combination of the lipofection and ddPCR techniques have the potential to demonstrated direct effects of the 2-5(A) system on mRNA expression and may determine the role of the 2-5(A) system during the establishment of pregnancy.

Other IFN-stimulated systems have implicated in maternal recognition of pregnancy in ruminants. One of the earliest cellular responses to IFN exposure is the stimulation of specific genes termed IFN-stimulated response elements (ISRE) (Levy et al., 1988; Levy et al., 1989). Hannigan and Williams (1991) demonstrated that arachidonic acid metabolism and prostaglandin synthesis are involved in the IFN stimulation of ISRE. Exposure of cells to inhibitors of prostaglandin synthetase, result in amplification of ISRE-binding and gene transcription (Hannigan and Williams, 1991). It is possible that IFN-τ is activating ISRE in endometrial cells and affecting $PGF_{2\alpha}$ synthesis and preventing luteolysis.

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evidence that human ER contain putative ISREs (see Bazer et al., 1994). More research needs to be performed to clone the ER gene and to identify other ISRE-associated genes and determine if any are stimulated specifically by IFN-τ.

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The mechanisms by which trophoblast IFNs cause maternal recognition of pregnancy are largely unknown. Previous studies have indicated that 2-5(A) synthetase increases at the time of maternal recognition of pregnancy in the endometrial cells in direct contact with the conceptus. The current data suggests

that the 2-5(A) system is not involved in secretory protein or $PGF_{2\alpha}$ profiles, and does not appear to be a major mechanism in the prevention of luteolysis. However, it is possible that the 2-5(A) system is involved in the establishment of pregnancy in a more subtle fashion. More research is needed to determine the intracellular mechanisms by which IFN-τ prevents luteolysis and the role of the 2-5(A) system in the establishment of pregnancy in the cow.

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APPENDIX A

UTERINE CELL SEPARATION

As developed by Dr. Michael Zavy and modified by Raymond Schmitt

Gross Preparation of Bovine Reproduction Tracts.

1. Upon receiving the reproductive tract, immediately trim off vagina and bladder if this has not already been done. Then place the tract into a plastic bag containing Roccal II (700 ppm) and massage. This effectively sanitizes the tract and begins washing away blood seepage. Place the bag and contents on ice for immediate transport to the laboratory (~15 minutes).

2. Once in the laboratory, place the reproductive tract onto a tray which has been sanitized with Roccal II and trim off the broad ligament. **NOTE** - be sure not to trim too closely or erosion of the uterine wall is likely to occur resulting in a poor separation of cellular populations. Separate the uterine horns by cutting the intercornual ligament and clamp each horn just anterior to the uterine

bifurcation. The horns are then cut free and placed into a bath containing Roccal II (700 ppm).

3. Place the uterine horns on a clean tray sanitized with Roccal II and blot dry with a sterile gauze pad. Wash the uterine lumen of each horn twice, by filling it with enough, room temperature Hank's Balanced Salt Solution (HBSS) minus phenol red (Gibco, Grand Island, NY) to cause moderate distention (generally 30-60 mls/horn). This is accomplished by inserting a luer adapter into the tip of the uterine horn to which a 60cc sterile syringe is attached. Then remove the luer adapter and strip the flush from the horn and then repeat the process. These washes remove red blood cells and cellular debris and can be discarded after inspecting the washes for signs of infection.

Epithelial Enzymatic Incubations.

1. After the wash, each uterine horn is filled with 40-60 mls, sterile (filtered through a .22 μm syringe filter) pancreatin (Gibco, Grand Island, NY) to which

dispase (Boehringer Mannheim, GmbH, FRG) had been added previously (see preparation of epithelial enzyme wash). Enzyme is added until the horns are moderately distended, but not taught. The tip of the uterine horn is then closed with a clamp and each uterine horn is placed into a ziplock bag which is filled with 250 mls HBSS + 2% antibiotic/antimycotic (ABAM) + anti-PPLO agent (Gibco, Grand Island. NY) at 4°C and incubated in an ice water bath for 60 minutes.

2. The 4°C incubation wash is then poured out, and the ziplock bags are filled with 250 mls HBSS + 2% ABAM/anti-PPLO at 20°C. The horns are then incubated in a 20°C water bath for 20 minutes.

3. The 20°C incubation wash is then poured out, and the ziplock bags are filled with 250 mls HBSS + 2% ABAM/anti-PPLO at 37°C. This incubation is carried out for 10 minutes in a 37°C water bath. At the end of 10 minutes, pour off the 37°C HBSS and put horns in room temperature (RT) HBSS + 1% penicillin/streptomycin (P/S; Pfizer, New York, NY) in a sterile horizontal

positive-flow hood, to slow down further digestion.

Separation of Epithelium.

1. Remove the clamp from the tip of the uterine horn and drain the enzyme wash from the uterine lumen by gently stripping. The flush is collected into a sterile beaker (250 mls) in a sterile horizontal positive-flow hood. Then, moderately distend the uterine lumen by filling it with 37°C HBSS - Ca^{2+}/Mg^{2+} (Incomplete HBSS; Gibco, Grand Island, NY). Incubate this for 30 minutes in RT HBSS+ 10% P/S in the sterile horizontal flow hood. Centrifuge the enzyme wash (500 X g) for 7 minutes at RT. In a sterile laminar flow hood, remove the cells with a pasteur pipette and resuspend in about 40 mls complete HBSS at 37°C in a sterile 50 ml conical centrifuge tube and store in 37°C incubator. This fraction contains surface epithelium and the majority of cells should rapidly settle to the bottom of the tube while the supernatant should appear relatively clear. **NOTE -** If the supernatant is cloudy, stromal contamination has probably occurred due to too rapid enzymatic digestion.

2. Gently massage the uterine horns to aid in loosening the epithelium and drain the wash form the horns by gently stripping. Moderately distend the uterine horn again with 37^{o}C Incomplete HBSS and incubate in RT HBSS+ P/S in the horizontal flow hood for 30 minutes. Centrifuge Wash 2 (500 X g) for 7 minutes at RT. In a laminar flow hood, remove the cells with a pasteur pipette and resuspend in approximately 40 mls HBSS (37^{o}C) in a sterile 50 mls conical centrifuge tube and store in 37^{o}C incubator. The purpose of this step is to remove the cells from the Incomplete HBSS so that the epithelial rafts remain relatively intact rather than have continued dissociation of the cells.

3. Gently massage the uterine horns and drain Wash 3 from the uterine lumen. ****Microscopically evaluate the flush at this time.**** Observe a sample of Wash 3 under the microscope to determine if it consists of primarily surface epithelial rafts, uterine glands or stromal cells. If stromal cell contamination has occurred proceed to the stromal enzyme digestion. If not, distend the horns with 37^{o}C Incomplete HBSS and incubate in RT HBSS + P/S for 30 minutes. Centrifuge Wash 3 (500 X g) for 7 minutes to pellet cells which should be

primarily glandular epithelium, but will also contain surface epithelial rafts. Remove the pellet with a sterile pasteur pipette and resuspend in 40 mls HBSS at 37°C and store in 37°C incubator. Large epithelial rafts which settle very rapidly if present can be removed and added to Wash 2. If the remaining cells are a mix of rafts and glands, discard Wash 3. If the cell population is nearly all rafts or glands use as deemed appropriate.

4. Gently massage the uterine horns and drain Wash 4 from the uterine lumen. ****Microscopically evaluate the flush at this time.**** Observe a sample of Wash 3 under the microscope to determine if it consists of primarily surface epithelial rafts, uterine glands or stromal cells. If stromal cell contamination has occurred proceed to the stromal enzyme digestion. If not, distend the horns with 37°C Incomplete HBSS and incubate in RT HBSS + P/S for 30 minutes. Centrifuge Wash 4 (500 X g) for 7 minutes to pellet cells which should be primarily glandular epithelium, but will also contain surface epithelial rafts. Remove the pellet with a sterile pasteur pipette and resuspend in 40 mls HBSS at 37°C and store in 37°C incubator. Large epithelial rafts which settle very

rapidly if present can be removed and added to Wash 2.

5. Gently massage the uterine horns and drain Wash 5 from the uterine lumen. ****Microscopically evaluate the flush at this time.**** Centrifuge Wash 5 (500 X g) for 7 minutes to pellet cells which should be primarily glandular epithelium, but will also contain surface epithelial rafts. Proceed to the stromal enzyme wash (see below). Remove the pellet with a sterile pasteur pipette and resuspend in 40 mls HBSS at 37°C and store in 37°C incubator.

Stromal Enzymatic Incubations.

1. To each uterine horn which was been denuded of epithelium, add enough 37°C PBS-dispase (see stromal enzyme wash) to moderately distend the horn (usually 80 mls/horn. Place the uterine horns into a new zip-lock bag and add 250 mls 37°C HBSS+ABAM/anti-PPLO. Incubate for 30 minutes in a 37°C water bath.

2. Pour off the HBSS and put uterine horns in RT HBSS + P/S in a

horizontal flow hood. Drain the enzyme wash (Wash 6) from the uterine lumen after gentle massage. This fraction should be enriched in single stromal cells but will also contain glandular epithelium. Moderately distend the uterine horns with 37°C Incomplete HBSS and incubate in HBSS for 30 minutes. Remove any mucoid or fibrous material (munge) and centrifuge Wash 6 (500 X g) for 7 minutes at RT. In a laminar flow hood remove pellet with a sterile pasteur pipette and resuspend in 40 mls HBSS at 37°C and store in 37°C incubator. Let settle for 10 minutes and remove cells which settle at the bottom of the centrifuge tube. These are to be added to the glandular epithelium in Wash 4. The individual stromal cells will remain suspended in HBSS.

3. Drain Wash 7 from the uterine lumen after gentle massage. This fraction will probably also contain stromal and glandular epithelial cells. Moderately distend the uterine lumen with 37°C Incomplete HBSS and incubate for 30 minutes in RT HBSS + P/S. Remove any munge and centrifuge Wash 7 (500 X g) for 7 minutes at RT. Remove pellet with a sterile pasteur pipette and resuspend in 40 mls of HBSS (37°C) and store in 37°C incubator. Let settle for

10 minutes and remove cells which have settled to the bottom of the conical tube. These are to be added to the glandular epithelium in Wash 4.

4. Drain Wash 8 from the uterine lumen after gentle message. If stromal cell or glandular epithelial cells yields appear too low, another Incomplete HBSS wash can be carried out. If yield appears acceptable, discard the uterine horns. Remove any munge from the wash. Centrifuge Wash 8 (500 X g) for 7 minutes at RT. Remove pellet and resuspend cells in 40 mls HBSS at 37^{o}C and store in 37^{o}C incubator.

Isolation of Surface Epithelial Rafts, Glandular Epithelium and Stroma:

Surface Epithelial Rafts.

1. Pour the surface epithelial collections (Washes 1 & 2) over a 20 μm screen to collect the epithelial rafts. Wash the cells 2-3 times with 50 ml HBSS (37^{o}C). Then wash the epithelial rafts off the screen and into a sterile 50 ml conical centrifuge tube using Incomplete HBSS (37^{o}C). Discard the filtrate

which contains single epithelial and stromal cells and red blood cells.

2. Allow the epithelial rafts to incubate in Incomplete HBSS for 2 hours, in a 37°C incubator, to allow the cell-cell attachments to be loosened by Incomplete HBSS. At the end of the 2 hour incubation, the epithelial cells should be broken down to clumps consisting of 8-20 cells/clump. Cells should be centrifuged (500 X g) for 7 minutes at RT. Remove pellet and resuspend cells in 25 mls Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY) + 10% cow serum + 2% ABAM/anti-PPLO and dispense into a sterile 100 mm petri dish and differential plate at 38.5°C (see below).

Glandular Epithelium.

1. Pour the glandular epithelial collections (Washes 3, 4 & 5) over a 20 μm screen to collect the epithelial rafts. Wash the cells 2-3 times with 50 ml HBSS (37°C). Then wash the endometrial glands off the screen and into a sterile 50 ml conical centrifuge tube using Incomplete HBSS (37°C). Discard the filtrate

which contains single epithelial and stromal cells and red blood cells.

2. Allow the endometrial glands to incubate in Incomplete HBSS for 2 hours, in a 37°C incubator, to allow the cell-cell attachments to be loosened by Incomplete HBSS. At the end of the 2 hour incubation, the glandular epithelial cells should be broken down to clumps consisting of 8-20 cells/clump. Cells should be centrifuged (500 X g) for 7 minutes at RT. Remove pellet and resuspend cells in 25 mls DMEM + 10% CS + 2% ABAM/anti-PPLO and dispense into a sterile 100 mm petri dish and differential plate at 38.5°C (see below).

Differential Plating (to remove stromal contamination).

1. Pour the separated cell populations, of the surface and glandular epithelial cells, into separate 100 mm petri dishes (see above) and incubate for 1 hr at 37°C, in a controlled atmosphere of 95% air:5% CO_2.

2. Gently rock the petri dishes back and forth twice and then recover the

supernatants, containing the respective epithelial cell types, from the dishes.

3. Remove a 10 μl aliquot from each of the populations of single epithelial cells and assess cell number on a hemocytometer to determine the respective cell yield.

4. Dilute the respective epithelial cell populations (~ $1X10^6$ cells/ml) and aliquot to sterile 24 well plate (1 ml/well) as needed.

5. Culture at 38.5°C in a controlled atmosphere of 95% air:5% CO_2 until approximately 70% confluent (7-10 days).

Stroma

1. It is possible to utilize cells from Washes 5-8 to obtain a population of stromal cells. Each wash is assessed for purity of the population of stromal cells which remain suspended in the cell supernatant.

2. The washes (5-8) are then poured over a 20 μm screen, and the retentate

is discarded and the filtrate (containing the single stromal cells) is retained.

3. The filtrate is centrifuged (500 X g) and the pellet resuspended in DMEM + 10% CS+ 2% ABAM/anti-PPLO.

4. Remove a 10 μl aliquot from each of the populations of single epithelial cells and assess cell number on a hemocytometer to determine the respective cell yield.

5. Dilute the stromal cells (250,000 cells/ml) and aliquot to sterile 24 well plate (1 ml/well) as needed.

6. Culture at 38.5°C in a controlled atmosphere of 95% air:5% CO_2 until approximately 70% confluent (5-7 days).

Preparation of Enzymes

The enzyme washes are to be prepared prior to starting the cell separation procedure. Make these solutions up on the day that the separation

will be carried out.

Epithelial enzyme wash. Dissolve 4.8 mg of dispase (Boehringer Mannheim, GmbH, FRG) per ml of pancreatin (Gibco, Grand Island, NY) solution. Make up 160 ml/uterus, which is a total of 768 mg of dispase dissolved in 160 mls pancreatin solution. Make this up in four 50 ml round-bottom centrifuge tubes. In each of the centrifuge tubes, dissolve 192 mg dispase by adding 10 ml of pancreatin/tube and swirl the tube frequently until nearly all the dispase has dissolved. Then add 30 ml of pancreatin tube to each tube to dilute the pancreatin/dispase to a concentration of 4.8 mg dispase/ml pancreatin. Use moderate mixing and/or agitation to completely dissolve in dispase. After the dispase is completely dissolved, clarify the solution by centrifugation at 10,000 rpm for 5 minutes. After centrifugation pour the supernatant into four 50 ml conical tubes and place on ice in a 4^{o}C cooler. When transferring the enzyme solution into the uterine lumen, pour the contents into a 60cc syringe to which a .22 μm syringe filter (Costar, Van Nuys, CA) has been fitted. Aliquot 25-80 mls of the filtered enzyme wash into the open end of each uterine segment by a

sterile 16 or 18 gauge luer stub adapter until moderately distended but not taught.

Stromal enzyme wash. Dissolve 4.8 mg of dispase in Dulbecco's PBS - Ca^{2+}/Mg^{2+} (Gibco, Grand Island, NY). This is made up as a 10X PBS solution. Make up 160 ml/uterus in four 50 ml round bottom centrifuge tubes. In each of the centrifuge tubes, dissolve 192 mg dispase by adding 4 ml of 10X PBS solution and swirl the tube frequently until nearly all the dispase has dissolved. Then add 36 ml of milli-Q water to each tube to dilute the DPBS to a 1X solution. Centrifuge the solution at 10,000 rpm for 5 minutes and transfer to 50 ml conical tubes until needed. Transfer to the uterine lumen as described for the epithelial enzyme wash.

VITA 2

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Doctor of Philosophy

Thesis: ROLE OF THE 2'.5'-OLIGOADENYLATE SYSTEM DURING BOVINE MATERNAL RECOGNITION OF PREGNANCY

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Personal Data: Born in St. Louis, Missouri, February 20, 1967, the son of Raymond Schmitt and Sally Martinez. Married on August 11, 1990 to Lisa McCart. Is impatiently waiting for the birth of his son Jacob Ryan (as of 6/17/96) any day now. Lives in a trailer park in Guymon Oklahoma, and is one of the few Reproductive Physiologists who actually got a job after graduation. He is gainfully employed (as of 6/17/96) by Seaboard Farms Inc. and spends his professional time getting to know boars on a more personal level than is usual for someone of his delicate sensibilities (ah well, its a living).

Education: Graduated from McCluer North Senior High School, Florissant, Missouri, in May, 1985; received Bachelor of Science degree in Agriculture, Animal Science from the University of Missouri-Columbia in May, 1989; received Master of Science degree in Animal Science, Reproductive Physiology from Oklahoma State University in May, 1992; completed requirements for the Doctor of Philosophy degree, Animal Breeding and Reproduction at Oklahoma State University in July, 1996. Oh, what a long strange trip it's been.

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