

Endocrine and Paracrine Regulation of Integrin $\alpha_v\beta_3$ in Bovine Endometrium

by

Sarah Kimmins

Submitted in partial fulfilment of the requirements for the degree
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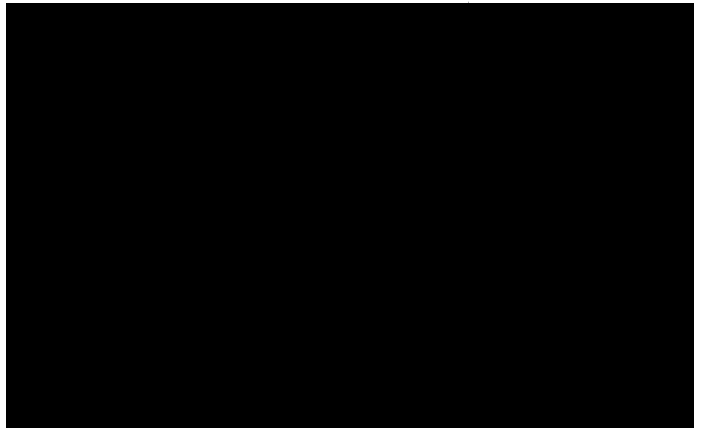
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DEDICATION

For Jean-Paul: Thank-you for your constant support and encouragement.

TABLE OF CONTENTS

TABLE OF CONTENTS	v
LIST OF FIGURES	viii
LIST OF TABLES	xi
LIST OF ABBREVIATIONS	xii
ABSTRACT	xiii
ACKNOWLEDGEMENTS	xiv
CHAPTER 1 LITERATURE REVIEW	1
1.0 Introduction	1
1.1 Development and Physiology of the Bovine Uterus	4
1.2 The Bovine Estrous Cycle	6
1.3 Day 16:Oxytocin Initiation of Prostaglandin Synthesis	10
1.4 Placentation	12
1.5 Interferon tau: The Pregnancy Recognition Protein in Ruminants	14
1.6 Estrogen Receptors	18
1.7 Integrin Structure and Function	19
1.8 Integrin Regulation	20
CHAPTER 2 ESTROUS CYCLE AND PREGNANCY EFFECTS ON THE EXPRESSION OF ESTROGEN AND PROGESTERONE RECEPTORS IN BOVINE ENDOMETRIUM	24
2.0 Introduction	24
2.1 Materials and Methods	25
2.2 Results	28

2.3 Discussion	35
CHAPTER 3 PERIIMPLANTATION EXPRESSION OF INTEGRIN $\alpha_v\beta_3$ AND OSTEOPONTIN ILLUSTRATES DIFFERENCES IN THE UTERINE CELL BIOLOGY OF SHEEP AND CATTLE	40
3.0 Introduction	40
3.1 Materials and Methods	42
3.2 Results	45
3.3 Discussion	51
CHAPTER 4 THE EFFECTS OF ESTROGEN AND PROGESTERONE ON PROSTAGLANDINS AND INTEGRIN BETA 3 (β_3) SUBUNIT EXPRESSION IN PRIMARY CULTURES OF BOVINE ENDOMETRIAL CELLS	59
4.0 Introduction	59
4.1 Materials and Methods	60
4.2 Results	67
4.3 Discussion	72
CHAPTER 5 PROSTAGLANDINS BUT NOT INTERFERON-TAU AFFECT INTEGRIN $\alpha_v\beta_3$ EXPRESSION IN BOVINE ENDOMETRIAL CELLS	78
5.0 Introduction	78
5.1 Materials and Methods	80
5.2 Results	81
5.3 Discussion	83

CHAPTER 6 THE EFFECTS OF ESTROGEN, ITS ANTAGONIST ICI 182, 780 AND INTERFERON- TAU ON THE EXPRESSION OF ESTROGEN RECEPTORS AND INTEGRIN $\alpha_v\beta_3$ ON CYCLE DAY 16 IN BOVINE ENDOMETRIUM	86
6.0 Introduction	86
6.1 Materials and Methods	88
6.2 Results	92
6.3 Discussion	97
 CHAPTER 7 IMPLICATIONS AND DISCUSSION	 102
7.0 Variation in Estrogen and Progesterone Receptors in Ruminant Endometrium:Significance and Roles in Luteolysis	102
7.1 Integrin $\alpha_v\beta_3$ and Insulin-Like Growth Factor: Potential Functions in Luteolysis and Maternal Recognition of Pregnancy	106
7.2 Cultured Endometrial Cells	109
7.3 Endocrine and Paracrine Regulation of Integrin $\alpha_v\beta_3$ in Bovine Endometrium	110
7.4 Future Directions	111
7.5 Conclusion	114
 REFERENCES	 116

LIST OF FIGURES

Figure 1.	Anatomy of the bovine uterus.	5
Figure 2.	Hormone profiles during a typical bovine estrous cycle and early pregnancy.	9
Figure 3.	An integrative model depicting oxytocin initiated prostaglandin synthesis in an endometrial cell.	11
Figure 4.	Immunofluorescent localization of the estrogen receptor in cryosections of bovine endometrium during the estrous cycle.	29
Figure 5.	Effects of cycle and pregnancy day on estrogen receptor distribution in bovine endometrium.	30
Figure 6.	Immunohistochemical localization of the progesterone receptor in cryosections of bovine endometrium.	32
Figure 7.	Estrous cycle and pregnancy effects on the distribution of progesterone receptors in bovine endometrium.	33
Figure 8.	Immunohistochemical localization of integrin $\alpha_v\beta_3$ in cryostat cross-sections of bovine endometrium during the estrous cycle and pregnancy.	46
Figure 9.	Effect of pregnancy stage on the number of subepithelial stromal cells showing positive reactivity to anti- $\alpha_v\beta_3$ antibody.	47
Figure 10.	Immunohistochemical localization of integrin $\alpha_v\beta_3$ in cryostat cross-sections of ovine endometrium during the estrous cycle and	49

pregnancy.

Figure 11.	Immunohistochemical localization of osteopontin in cryostat cross-sections of bovine endometrium during the estrous cycle and pregnancy.	50
Figure 12.	Estrous cycle and pregnancy effects on osteopontin levels in bovine endometrium.	52
Figure 13.	Immunohistochemistry for detection of osteopontin in endometrial tissue collected from cycling and pregnant sheep.	53
Figure 14.	Experiment 1 treatments.	63
Figure 15.	Reverse-transcriptase PCR of integrin β_3 subunit mRNA.	68
Figure 16.	Experiment 2 RT-PCR results for integrin β_3 subunit mRNA from cultured intercaruncular stromal cells treated with estrogen and progesterone.	70
Figure 17.	Detection of mRNA and protein for ER α and PR in bovine endometrial cell monolayers by RT-PCR and immunohistochemistry.	70
Figure 18.	Northern blot analysis of integrin β_3 subunit expression in intercaruncular stromal cells from experiment 1.	71
Figure 19.	Immunocytochemical staining of intercaruncular stromal cells for integrin $\alpha_v\beta_3$.	71
Figure 20.	Effect of steroid treatment on prostaglandin production in cultured	73

monolayers of bovine endometrial cells.

- Figure 21. Reverse-transcriptase PCR of integrin β_3 subunit mRNA from cultured intercaruncular stromal cells treated with control media, IFN- τ , PGE $_2$ or PGF $_{2\alpha}$ for 48h. 82
- Figure 22. Immunocytochemical staining of intercaruncular stromal cells for integrin $\alpha_v\beta_3$. 82
- Figure 23. Synchronization schedule. 89
- Figure 24. Immunofluorescent localization of the estrogen receptor in cryostat cross-sections of endometrium obtained on day 16 of the estrous cycle after uterine infusion with either control, IFN- τ , estrogen or ICI 182,780. 93
- Figure 25. Immunoreactivity for integrin $\alpha_v\beta_3$ in sections from heifers treated with either control, IFN- τ , estrogen or ICI 182,780. 95
- Figure 26. Effect of treatment on expression of integrin subunit β_3 as detected by Northern blot analysis of intercaruncular endometrium 96
- Figure 27. Model of integrin $\alpha_v\beta_3$ regulation in bovine endometrium 111

LIST OF TABLES

Table 1.	Primer sequences.	64
Table 2.	Uterine characteristics of heifers treated with either control, IFN- τ , estrogen or ICI 182,780.	92

ABSTRACT

Integrins are involved in reproductive processes such as uterine receptivity and embryo attachment. At the fetal-maternal interface integrins are involved cell attachment and communication. In cattle, the uterine distribution of integrin $\alpha_v\beta_3$ is different between cycling and pregnant animals. The **hypothesis** of this research was that integrin $\alpha_v\beta_3$ may be involved in the events surrounding embryo implantation. Because its expression is regulated during the estrous cycle and pregnancy it may be controlled by estrogen receptor (ER) or progesterone receptor (PR) signaling, or by local substances produced by the uterus or embryo. The **objectives** were to: 1) characterize the expression of ER and PR in the uterus during the estrous cycle and pregnancy, 2) identify factors that regulate integrin $\alpha_v\beta_3$ expression, and relate this information to the local uterine environment at luteolysis and embryo attachment and 3) compare expression patterns of integrin $\alpha_v\beta_3$ and its ligand osteopontin in the uteri of sheep and cattle. **Results and Discussion.** The pattern of ER distribution was consistent with the hypothesis that cycle termination is initiated by estrogen binding its receptor; estrogen is a known stimulant for synthesis of the luteolysin, $\text{PGF}_{2\alpha}$. Uterine infusion of the pregnancy recognition protein, interferon-tau, or an anti-estrogen blocked ER expression. Therefore in pregnancy interferon-tau may prevent luteolysis by blocking ER action. Interferon-tau did not affect integrin $\alpha_v\beta_3$. Treatment of cultured uterine cells with estrogen inhibited expression of integrin $\alpha_v\beta_3$ while prostaglandins stimulated expression. Based on these results it appears that estrogen downregulates integrin $\alpha_v\beta_3$ on day 16 of the cycle, which may promote differentiation of the epithelium into the prostaglandin secreting phenotype. If interferon-tau increases the luteoprotective substance prostaglandin E_2 , this may sustain integrin $\alpha_v\beta_3$ expression during pregnancy. Integrin $\alpha_v\beta_3$ and osteopontin were not co-expressed at the fetal maternal-interface. Therefore in ruminants integrin $\alpha_v\beta_3$ and osteopontin are not essential for attachment, but may function in the signaling pathways initiated at luteolysis and maternal recognition of pregnancy.

ABBREVIATIONS

Arginine-glycine-asparagine	RGD
Bovine endometrial cell line	BEND
Bovine serum albumin	BSA
Cyclooxygenase	COX
Estrogen Receptor	ER
Extracellular matrix	ECM
Follicle stimulating hormone	FSH
Insulin-like growth factor	IGF
Interferon-tau	IFN- τ
Interferon receptor	IFNAR
Interferon regulatory factor	IRF
Luteinizing hormone	LH
Phosphate buffered saline	PBS
Progesterone receptor	PR
Prostaglandin E ₂	PGE ₂
Prostaglandin F _{2α}	PGF _{2α}
Signaling transducer and activator of transcription	STAT

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CHAPTER 1 LITERATURE REVIEW

1.0 Introduction

During the estrous cycle and pregnancy there is communication between the hypothalamic-pituitary axis, the ovaries, the endometrium and the embryo. The ovaries produce estrogen and progesterone in response to gonadotropins, which in turn act on the uterus to regulate gene expression that either promotes or prevents pregnancy establishment. Artificial insemination is the method of breeding in dairy operations however, success rates are low; calving occurs following less than 50% of breedings (Royal et al., 2000). Some reproductive failure is the result of poor management, heat stress, or infection (Ayalon, 1978; Badinga et al., 1985). However, the majority of early embryonic loss is attributable to an unreceptive uterine environment and occurs during the critical period when the embryo must signal its presence to the dam and prevent a return to estrus (Betteridge et al., 1980; Roche et al., 1981; Humblot, 2001). This critical period is termed maternal recognition of pregnancy and coincides with the secretion of the embryonic factor interferon-tau (IFN- τ). Pregnancy establishment requires continued secretion of progesterone by the corpus luteum. In the absence of an embryo, the uterus secretes prostaglandin-F_{2 α} (PGF_{2 α}) in a pulsatile manner to cause regression of the corpus luteum and a return to estrus. While the antiluteolytic effects of IFN- τ are well documented, the effects on endometrial gene expression are just now being elucidated. The reproductive cycles of ruminants have evolved similar modes of luteolysis, maternal recognition of pregnancy and placentation. However, studies

show that there is species-specific endometrial gene expression during these processes.

Over the past ten years it has become clear that the integrin family of adhesion proteins is involved in reproductive processes such as fertilization, uterine receptivity, embryo attachment, and trophoblast invasion (reviewed in Sueoka et al. 1997; Coutifaris et al. 1998). Integrins are heterodimeric transmembrane proteins composed of an α and a β subunit and mediate cell-cell and cell-extracellular matrix (ECM) attachment. Integrins are key facilitators of cellular processes involved in tissue remodeling such as cell migration and de-adhesion (Martin, 2002). In many species endometrial expression of integrins and their ECM ligands is correlated with embryo attachment and uterine differentiation during the receptive phase and pregnancy (Bowen et al., 1997; Fazleabas et al., 1997; Kimmins and MacLaren, 1999; Apparao et al., 2001; Johnson et al., 2001a). Our work has identified estrous cycle regulation of the integrin adhesion molecule integrin $\alpha_v\beta_3$ in cattle (Kimmins and MacLaren, 1999), previously identified as a marker of endometrial receptivity in women (Lessey et al., 1992; Tabibzadeh 1992; Albers et al., 1995). However, the regulation and distribution of this integrin in cattle is unique in comparison to other species.

Expression of bovine integrin $\alpha_v\beta_3$ is estrous cycle dependent. On day 16 of the estrous cycle, expression of integrin $\alpha_v\beta_3$ is lost from stromal cells underlying the luminal epithelium (Kimmins and MacLaren, 1999). Day 16 is the central day of the bovine estrous cycle as it marks the switch from a progesterone dominated uterus (embryo receptive), to an estrogen dominated uterus (embryo non-receptive). Also

of interest is the differential expression of integrin $\alpha_v\beta_3$ in the caruncular and intercaruncular regions. Caruncles are unique to ruminants and are the privileged sites of placentation (Atkinson et al., 1984). Expression of integrin $\alpha_v\beta_3$ is restricted to intercaruncular sites, suggesting that it may be involved in the prevention of villus development (Kimmins and MacLaren, 1999). While changes in the expression of integrin $\alpha_v\beta_3$ coincide with major hormonal events, the cell-specific regulation suggests a role for local uterine products in its regulation.

In mice, humans, baboons, and ruminants, it has been postulated that co-expression of integrin $\alpha_v\beta_3$ and its ligand osteopontin on uterine epithelium is required for trophoblast attachment and/or invasion; blocking integrin $\alpha_v\beta_3$ in mice prevents embryo implantation (Coutifaris et al., 1998; Apparao et al., 2001; Johnson et al., 2001). In sheep, integrin subunits α_v and β_3 are expressed on the apical surface of uterine epithelium and on conceptus trophoblast, although no change in their expression patterns has been observed between cyclic and pregnant ewes (Johnson et al., 2001). Despite the similarity in placental anatomy of sheep and cattle, this widespread distribution pattern of integrin $\alpha_v\beta_3$ is not observed in cycling cattle. Distributions of integrin $\alpha_v\beta_3$ and its ligand osteopontin have not yet been described in pregnant bovine endometrium.

The relationship between steroid receptors, maternal recognition of pregnancy and integrin regulation, all of which are significant to embryo implantation, has not been studied in bovine endometrium. The objectives of this research were to: 1) characterize the sex steroid receptor distribution and expression in bovine endometrium throughout the estrous cycle and early pregnancy, 2) compare

uterine distribution of integrin $\alpha_v\beta_3$ and its ligand osteopontin between cattle and sheep, 3) develop an *in vitro* system to examine the effects of potential endocrine and paracrine regulators of integrin $\alpha_v\beta_3$ present in the day 16 pregnant and non-pregnant uterine environments, 4) test *in vitro* regulators of integrin $\alpha_v\beta_3$ *in utero* and 5) provide information on the paracrine dialogue between uterine epithelial and stromal cells and how it relates to the regulation of integrin $\alpha_v\beta_3$ and steroid receptors. This research will further the understanding of the signaling events that occur during maternal recognition of pregnancy and lay a foundation for further studies of the functional significance of integrins in placentation.

1.1 Development and Physiology of the Bovine Uterus

Shown in Figure 1 is the anatomy of the bovine uterus. The uterus develops as a result of estrogenic influences (arising from the ovaries) on the Müllerian ducts which give rise to the oviducts, uterus, cervix and vagina (Gilbert, 1994). Partial fusion of the ducts results in a bicornuate uterus common to cattle and sheep. The major functions of the ruminant uterus are: 1) production of the luteolytic $\text{PGF}_{2\alpha}$ and luteoprotective PGE_2 , 2) maturation of spermatozoa, 3) transport and maturation of the embryo, 4) provision of a receptive uterine environment for the conceptus, and 5) delivery of the fetus and placenta at parturition. Uterine histogenesis begins in cattle around gestation day 70 when the paramesonephric tissue becomes divided into zones that will form the endometrium and mesometrium. By gestation day 150, definitive uterine tissue layers have formed and include the subluminal densely packed stroma, the loose stroma and the inner and outer layers of smooth muscle

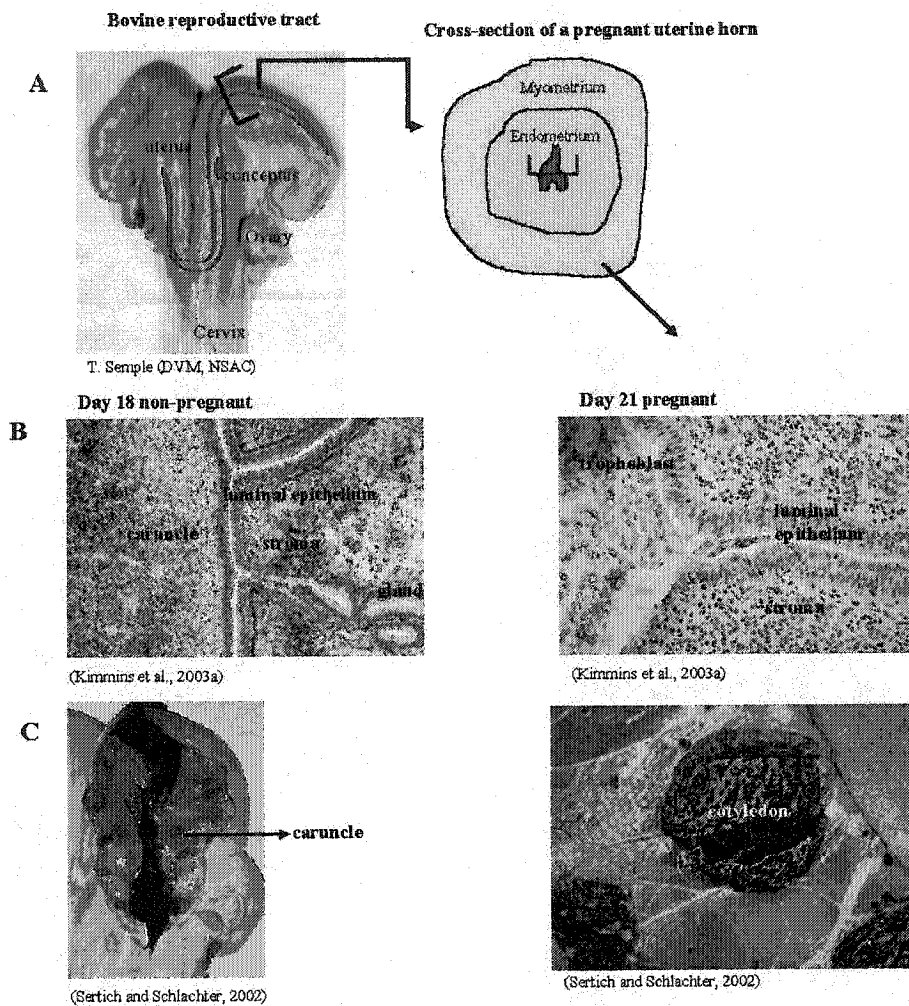


Figure 1. Anatomy of the bovine uterus. (A) The bovine uterus is bicornuate and by day 21 of pregnancy the trophoblast (forms the placenta) surrounding the conceptus fills the entire horn ipsilateral to the corpus luteum. (B) Shown are cryostat cross-sections through pregnant and non-pregnant endometrium. (C) An opened uterus 9 days post-calving shows the raised hemotrophic buttons, the caruncles, which form the maternal component of the placenta. The cotyledons fused with maternal caruncles form the functional units of the placenta, placentomes.

(Bartol et al., 1999). Also at this time the caruncular regions of the uterus form. The caruncles are dense connective tissue regions which are unique to ruminants and form the maternal component of the placenta (Atkinson et al., 1984).

The major endometrial cell types include luminal and glandular columnar epithelia, stromal cells, and endothelial cells associated with blood vessels (Van der Wielen and King, 1984). In healthy bovine endometrium, lymphocytes are present throughout the estrous cycle and are evenly distributed throughout the endometrium (Cobb and Watson, 1995). Under the influence of the ovarian steroids endometrial cells undergo cyclic patterns of growth and regression (Johnson et al., 1997; Rider et al., 1998).

1.2 The Bovine Estrous Cycle

The coordinated function of reproductive organs is under the systemic control of various hormones, neuropeptides and growth factors (Berndson et al., 1996; Monget and Monniaux, 1995; Miyamoto et al., 2000). Based on hormonal events, the bovine estrous cycle is divided into four stages: estrus (day 0), metestrus (days 1-6), diestrus (days 7-17), and proestrus (days 18-20).

In cattle, gonadotropin synthesis is regulated by estrogen and progesterone action on the hypothalamus, which influences secretion of gonadotropin-releasing hormone, which in turn controls the amount of anterior pituitary mRNA encoding luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Hamernik, 1995). During proestrus rapid follicle growth occurs under the influence of FSH and LH.

The estrogen-induced pre-ovulatory surge of LH and FSH induces ovulation (Caraty et al., 1995).

Estrus is behaviorally defined as the period when a cow is receptive to mating and is the result of high circulating levels of estradiol-17 β arising from the pre-ovulatory follicle. The synthesis of estradiol-17 β involves the two major cell types surrounding the oocyte; the outer layer of thecal cells that produce androgens, and the inner layer of granulosa cells which aromatize androgens to estrogens (Berndston et al., 1996). During proestrus and estrus, circulating levels of estradiol-17 β range from 5 pg/ml to 10 pg/ml (Wettemann et al., 1972). Estrus ends when the cow will no longer stand to be mounted by herd-mates or the bull.

Ovulation occurs during metestrus, 10-12 hours post-estrus and is characterized by declining plasma estrogen levels to 2-5 pg/ml (Wettemann et al., 1972). Following ovulation, a blood clot forms at the ovulation site, the corpus hemorrhagicum, and under the influence of LH the thecal and granulosa cells redifferentiate, becoming progesterone secreting luteal cells. This switch in steroidogenic capabilities marks the beginning of the luteal phase of the cycle, diestrus. During diestrus progesterone arising from the corpus luteum acts on the hypothalamus to suppress pulsatile release of LH and FSH (Wettemann, 1972). In addition to progesterone the corpus luteum secretes the glycoprotein oxytocin, that is involved in the initiation of luteolysis, a PGF_{2 α} mediated event (Mirando et al., 1993; Berndston et al., 1996; Ivell and Walther, 1999).

Follicular activity occurs throughout the estrous cycle and early pregnancy. Shortly after ovulation the first wave of follicle development occurs around day two

of the cycle. Heifers have two or three waves of follicle development throughout the estrous cycle that usually take place on days 2, 10 and 16 in a three wave cycle, or days 2 and 10 in a two wave cycle (Ireland et al., 2000). These waves of follicle growth are preceded by transient peaks in FSH and subsequent increases in circulating estrogen (Sunderland et al., 1994). A cohort of follicles (~24 antral follicles) grow together at the beginning of each wave, followed by atresia of all but the dominant follicle, which is defined as the largest follicle and is usually ≥ 5 mm in diameter. The ovulatory follicle is dominant at the time of luteolysis or shortly thereafter (Campbell et al., 1995). The paracrine mechanisms that underlie selection of the dominant follicle are unknown, but differential expression of growth factors such as inhibin, activin, and insulin like growth factor-I (IGF) are thought to be important (Stock and Fortune, 1993; Campbell et al., 1995; Mihm et al., 1997).

Day 16 of the bovine estrous cycle is the last day for successful embryo transfer (Betteridge and Loskutoff, 1993). Before day 16 the conceptus must signal its presence to the dam through the release of IFN- τ , which acts to block the release of PGF $_{2\alpha}$ (Godkin et al., 1997; Martal et al., 1997; Roberts et al., 1999). In the non-pregnant animal in response to increased oxytocin sensitivity, PGF $_{2\alpha}$ is released in a pulsatile manner (reviewed in McCracken et al., 1999) resulting in regression of the corpus luteum and return to estrus. In cattle on around day 10 of pregnancy, the embryonic trophoblast produces the antiluteolysin, IFN- τ , which extends the lifespan of the corpus luteum maintaining luteal levels of progesterone (Roberts et al., 1992). It is agreed that in ruminants, the primary action of IFN- τ is to prevent pulsatile release of PGF $_{2\alpha}$ and to increase the production of luteoprotective

prostaglandin E₂ (PGE₂) (Roberts et al., 1992; Asselin et al., 1998). Shown in Fig. 2 are schematic diagrams of the hormone profiles observed during a typical estrous cycle and early pregnancy.

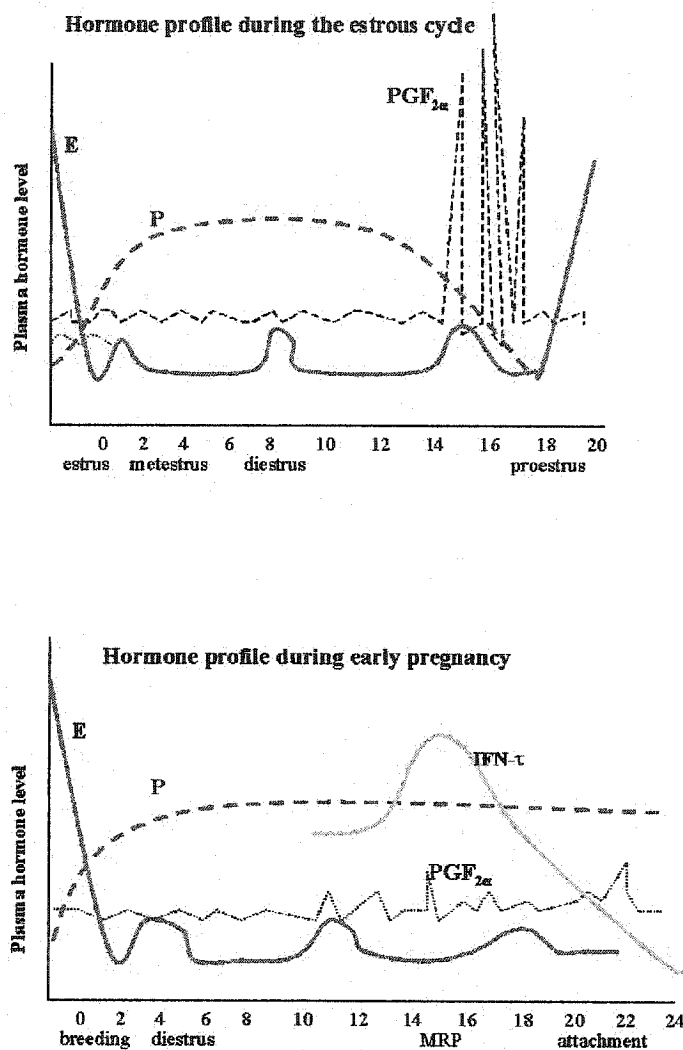


Figure 2. A schematic diagram of hormone profiles during a typical bovine estrous cycle and early pregnancy. E=estrogen, P=progesterone, PGF_{2α}=prostaglandin F_{2α}, PGE₂= prostaglandin E₂, IFN-τ=interferon tau, MRP=maternal recognition of pregnancy.

1.3 Day 16: Oxytocin Initiation of Prostaglandin Synthesis

Activation of the oxytocin-signaling pathway culminates in the pulsatile release of $\text{PGF}_{2\alpha}$, and involves a complex endocrine circuitry between the endometrium and ovaries. The development of oxytocin receptors on luminal epithelium on day 16 of the bovine estrous cycle allows the uterus to respond to oxytocin (Robinson et al., 2001). Granulosa cells of the preovulatory follicle and luteinized granulosa cells are the major sources of oxytocin. On day 16, ovarian oxytocin causes the release of $\text{PGF}_{2\alpha}$ in the uterus by the recurring activation of a positive feedback loop (Tian et al., 1995; Tsai and Wiltbank, 1998). Uterine $\text{PGF}_{2\alpha}$ is transported to the ovaries by a ventromedial pathway, where it reaches the corpus luteum causing further release of oxytocin (Tsai and Wiltbank, 1998; Salli et al., 2000).

The biochemical pathways that may be involved in prostaglandin synthesis in the uterus are described in Fig. 3. Prostaglandin production requires cyclooxygenase enzyme (COX, also known as prostaglandin H-synthase), for the conversion of arachidonic acid to the prostaglandin precursors PGG_2 and PGH_2 . There are two isoforms of COX: the usually constitutively expressed COX-1 and the induced form COX-2 (Versteeg et al., 1999). In bovine endometrium expression of COX-1 was not detected by Northern blot in tissue from cycling animals (Arosh et al., 2002), but has been detected by ribonuclease protection assay in the caruncles of third term pregnant cows (Fuchs et al., 1999). Immunohistochemical localization of COX-1 in bovine tissues is in agreement with the previous studies; weak expression was detected throughout the endometrium (Emond et al., 2002). Collectively these

results indicate that COX-1 is probably expressed at low levels in bovine endometrium and not likely to be involved in luteolysis or maternal recognition of pregnancy.

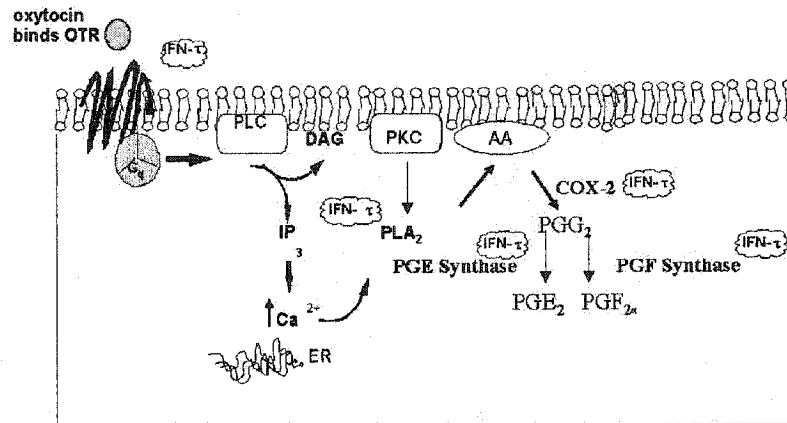


Figure 3. An integrative model depicting oxytocin initiated prostaglandin synthesis in an endometrial cell. The signaling proteins that IFN- τ may affect during maternal recognition of pregnancy are shown. Binding of oxytocin to its plasma membrane receptor (OTR) on the luminal epithelium promotes interaction of the ligand receptor complex with a G-protein that activates phospholipase C (PLC). PLC converts phosphatidylinositol bisphosphate to diacylglycerol (DAG) and inositol triphosphate (IP₃). IP₃ mobilizes calcium from the endoplasmic reticulum (ER) and DAG interacts with protein kinase C (PKC), which triggers a MAP kinase cascade and results in phospholipase A₂ (PLA₂) triggered release of membrane phospholipids such as arachidonic acid (AA). AA is then peroxidised by COX-2 to a prostaglandin intermediary followed by conversion to either PGF_{2 α} or PGE₂ by either PGF synthase or PGE synthase respectively (Versteeg et al., 1999).

On the other hand, COX-2 expression in bovine endometrium was estrous cycle dependent. Cyclooxygenase-2 expression increased in bovine endometrium just prior to luteolysis (Arosh et al., 2002). In contrast, no increase in COX-2 protein was detected by immunohistochemistry in tissues from cycling cattle collected on days 15 and 16 of the estrous cycle in comparison to other cycle days (Boos, 1998; Emond et al., 2002). These contradictory results may reflect differences in the sensitivity of detection methods or reflect the high turn-over of COX given that it is a suicide enzyme. In support of estrous cycle dependent expression of COX-2, cultured ovine and bovine endometrial cells showed increased COX-2 expression in response to oxytocin and in turn $\text{PGF}_{2\alpha}$ synthesis (Asselin et al., 1997a; Burns et al., 1997; Fuchs et al., 1999). Interestingly, for both species there was greater expression of COX-2 in caruncular than intercaruncular tissue (Charpigny et al., 1997; Fuchs et al., 1999). Physiologically it is logical that the regions of the uterus that form the maternal component of the placenta have a greater capacity for prostaglandin production. Prostaglandins are important regulators of blood flow, which would be an asset at the sites of placentation. The fetal component of the placenta, the cotyledons, are a rich source of cytokines and growth factors, which are strong stimulators of COX-2 expression and probably stimulate its expression in maternal endometrium.

1.4 Placentation

The bovine embryo enters the uterus by day 5, forms a blastocyst about day 6, and hatches from the zona pellucida by day 8 (Bettteridge and Fléchon, 1988). Early pregnancy (days 8 to 18) is characterized by a lengthy pre-attachment stage during

which the embryo elongates and the trophoblast (tissue which will form the fetal membranes) fills the uterine horn ipsilateral to the corpus luteum. Trophoblast is composed of epithelial-like cells, with apical microvilli, connected by tight junctions and arranged on a basement membrane (King et al., 1982). During the elongation period, the paracrine dialogue between the embryo and dam establishes a uterine environment that promotes growth and attachment. To prevent maternal immunological attack there is modified expression of major histocompatibility antigens on trophoblast cell surfaces. An increase in the $\text{PGE}_2:\text{PGF}_{2\alpha}$ ratio and luteal levels of progesterone (≥ 4 ng/ml) serve to locally suppress the maternal immune system by preventing lymphocyte transformation (Hansen and Liu, 1996).

Bovine embryos can be flushed up until day 18 of pregnancy, after which cell contacts between uterine epithelium and trophoblast have formed (King et al., 1982). Regions of trophoblast overlying uterine glands develop transient extensions that project into the glands, loosely attaching the conceptus to the uterus. At this time about 20% of trophoblast cells undergo division without cytokinesis, yielding binucleate cells, which are unique to ruminant placentation (Wooding, 1992). The progression of placentation from attachment to implantation occurs around day 19, as binucleate cells migrate through the trophoectodermal tight junction, fusing with uterine epithelium to form areas of syncytium, for which the bovine placenta has been classified as synepitheliochorial. The feto-maternal syncytium is essential for implantation and placentome growth, as binucleate cells produce and deliver proteins, autoids and steroid hormones to the maternal circulation such as PGE_2 , progesterone and placental lactogen (Wooding, 1992). Binucleate cells continue to

migrate throughout pregnancy, but at the caruncles the resulting trinucleate cells are quickly replaced by regrowth of epithelial cells (Wathes and Wooding, 1980).

During pregnancy the caruncles enlarge to accommodate penetration by the chorionic villi that develop in fetal areas adjacent to caruncles, the cotyledons. Once interdigitated the caruncle-cotyledon structures form the functional units of the placenta, placentomes (Amoroso, 1952; Guillomot, 1995). Formation of the ruminant placenta likely requires cell specific expression of attachment promoting molecules such as integrins, selective degradation of the cell matrix, and factors that must limit invasion such as tissue inhibitors of matrix metalloproteinases (Salamonsen, 1994). Prostanoids, growth factors, and the ovarian steroids probably regulate the local expression of these molecules.

While significant progress has been made in the characterization of human and mouse implantation, in cattle there are few studies that illustrate the relationships between attachment promoting proteins and their regulators. In cattle, invasion of fetal tissues into the maternal endometrium is restricted to the uterine epithelium. Therefore the molecular mechanisms underlying placentation in surface implanters are likely to be different from species with invasive hemochorial placentation.

1.5 Interferon tau: The Pregnancy Recognition Protein in Ruminants

Interferon tau, previously known as trophoblast protein-1, was identified as a major polypeptide produced by the conceptus in early pregnancy (Reviewed by Martal et al., 1997). When trophoblast homogenates or secreted products of the embryo were infused into sheep uteri, the corpus luteum was sustained (Rowson and Moor 1967; Ellinwood et al., 1979a), and pulsatile release of $\text{PGF}_{2\alpha}$ was attenuated

(Ellinwood et al., 1979b). The amino acid sequence of IFN- τ shares 45-70% identity with α -interferons, but it was subclassified as IFN- τ to reflect its trophoblast origin, abundance during maternal recognition of pregnancy, and the unique non-coding 3' region (Roberts et al., 1999). Interferon-tau has antiviral, antiproliferative, immunomodulatory and its expression is sexually dimorphic (Godkin et al., 1997; Martal et al., 1997; Hansen et al., 1999; Larson et al., 2001). It is exclusively produced by the trophectoderm of ruminants, from days 9 to 21 in sheep, days 11 to 25 in cattle, and days 14 to 17 in goats (Martal et al., 1997). Maximal secretion coincides with embryo elongation from days 14 to 16, and production of IFN- τ stops once trophoblast has attached to the epithelium (Mann et al., 1999). Since its identification the effect of IFN- τ on uterine function and gene expression has been a fruitful avenue for research aimed at reducing embryonic mortality.

Interferon-tau Signal Transduction

Interferon-tau binds dimeric type I interferon receptors composed of IFNAR1 and IFNAR2 subunits that are present during the estrous cycle and pregnancy (Godkin et al., 1984; Han et al., 1997). There is a high degree of sequence similarity between ovine and bovine receptors; IFNAR1 and IFNAR2 share 92% and 88% sequence identity respectively between ovine and bovine (Han et al., 1997). The signaling molecules tyrosine kinase-2, and signaling transducer and activator of transcription (STAT), associate with IFNAR 1 and 2 cytoplasmic domains, while Janus kinase-1 preferentially binds IFNAR-2 (Han et al., 1997). Genes identified to be influenced by IFN- τ either in ovine endometrium, or in cultured ovine

endometrial cells include: transcription factors STATS 1 and 2, IRF-1 and 9 (interferon regulatory factor), 2',5'-oligoadenylate synthetase (involved in antiviral response), and ISG17 (also known as ubiquitin cross-reactive protein) (Johnson et al., 2001b; Stewart et al., 2001ab). The primary pathway activated by IFN- τ is the STAT1 signaling pathway; expression of IRF-9 and STAT2 is prevented in human stromal fibroblasts with mutations in STAT1 (Stewart et al., 2001b). Moreover, IFN- τ stimulation of ovine endometrial cells causes tyrosine phosphorylation and nuclear translocation of STAT transcription factors (Stewart et al., 2001a). Once STAT homodimers have translocated to the nucleus, they recognize and bind IFN-stimulated response elements to regulate gene transcription.

Uterine Genes Affected by Interferon-tau

It was recently shown that IFN- τ stimulated gene expression in ovine endometrium was strongest in the periluminal stroma and glandular epithelium. Direct IFN- τ action on the luminal epithelium and shallow glands was prevented by expression of IRF-2 (Choi et al., 2001). Further support for the notion that IFN- τ targets the stroma is that cultured stromal cells respond to IFN- τ (Asselin et al., 1997a; Xiao and Goff., 1999; Xiao et al., 1999). Based on the aforementioned experimental evidence, it appears that IFN- τ action on the luminal epithelium may be mediated in a paracrine manner by the underlying stroma. It is not surprising the subepithelial stroma would be involved in regulating the overlying epithelium during the period of IFN- τ secretion, as this coincides with trophoblast attachment. During implantation the luminal epithelium undergoes extensive degeneration. If IFN- τ

regulated genes were to act only through the luminal epithelium, at the stage when cell-cell contacts between trophoblast and luminal epithelium become established, IFN- τ effects would be interrupted. To date uterine localization of IFN- τ gene expression has been undertaken in the ewe only.

In sheep IFN- τ acts within the uterus to prevent upregulation of oxytocin receptors and ER α in the luminal epithelium of sheep (Spencer and Bazer, 1996) and in doing so, the magnitude of PGF $_{2\alpha}$ and oxytocin pulses are reduced, and the corpus luteum is maintained (McCracken et al., 1999). In cattle the effects of IFN- τ on endometrial steroid receptor expression are unknown. In both sheep and cattle, IFN- τ is hypothesized to have luteoprotective actions. Injection of pregnant sheep with the synthetic PGF $_{2\alpha}$, Cloprostenol, induced estrus in fewer pregnant sheep than non-pregnant sheep, and the more embryos present, the less likely it was to induce luteolysis (Nancarrow et al., 1982; Silvia et al., 1984). There is a developing concept that production of PGE $_2$ is important for maternal recognition of pregnancy, and that it may be the luteoprotective substance either produced by the uterus in response to IFN- τ , and/or by the conceptus (Roberts et al., 1992; Hansen et al., 1999). Prostaglandin E $_2$ has been suggested as the luteoprotective factor because it lessened the luteolytic effects of PGF $_{2\alpha}$ (Pratt et al., 1977). An interesting possibility, that has been investigated by several research groups, is whether IFN- τ alters or stabilizes key enzymes in prostaglandin synthesis (Asselin et al., 1997ab; Xiao et al., 1998; Charpigny et al., 1999; Asselin and Fortier, 2000; Binelli et al., 2000).

During maternal recognition of pregnancy, Asselin and Fortier (2000) suggest that there is a switch in prostanoid production by endometrial cells from PGF $_{2\alpha}$ to

PGE₂. In support of this, increased levels of PGE₂ have been observed in venous blood during early pregnancy in sheep (Silvia et al., 1984b), and expression of COX-2 increased from day 16 to day 18 of pregnancy in bovine endometrium (Emond et al., 2002). Therefore, increased PGE₂ can in part be explained by the increased expression of COX-2 in pregnant endometrial stroma. In addition to the endometrium, the trophoblast could be a second source of prostanoids during maternal recognition of pregnancy. Ovine and bovine trophoblastic, but not mesodermal cells, strongly express COX-2 (Charpigny et al., 1997; Emond et al., 2002).

1.6 Estrogen Receptors

Estrogen receptors belong to the family of nuclear hormone receptors that act as transcription factors to influence gene expression. The ER protein is separated into six structural domains based on function. A highly conserved region, the DNA binding domain, is located in the amino terminus and contains two zinc finger motifs that facilitate binding of the receptor to hormone response elements in the promoters of hormone regulated genes (MacGregor and Jordan 1998). The ligand binding domain is also highly conserved and is located in the C-terminus. The hypervariable A/F region, located in the amino-terminus, has autonomous transcriptional activation function and is referred to as the AF-1 region. This AF-1 region is most often altered in receptor isoforms and interacts with growth factors such as IGF-1 to confer ligand independent activity of the ER (Aranda and Pascual 2001).

There are two ER genes, ER α and ER β , which differ significantly in their tissue distribution and ligand binding characteristics. Generally ER bind DNA as homodimers, but when both subtypes of ER are present binding as heterodimers can occur (Katzenellenbogen and Katzenellenbogen, 2000). While ER α and ER β have highly similar DNA-binding domains they share only 21% homology in the N-terminus, with ER β being truncated in the AF-1 region. Due to this difference ER ligands bind these subtypes with differing affinity. For example the selective estrogen receptor modulator, tamoxifen, acts as a partial agonist of ER α and a complete antagonist of ER β (Katzenellenbogen and Katzenellenbogen, 2000). Although the DNA binding domains of ER α and ER β are nearly identical, they do not activate estrogen responsive genes in the same manner. Furthermore how these transcription factors interact with other signaling proteins such as AP1 can be specific to the ER type (Katzenellenbogen and Katzenellenbogen, 2000).

1.7 Integrin Structure and Function

Integrins are heterodimeric transmembrane glycoproteins that serve as cell receptors for the ECM, although a few are involved in cell-cell adhesion. In mammals, 18 α and 8 β integrin subunits have been identified and these can combine to form twenty-three receptor combinations. Indeed, not all subunits are able to dimerize with each other (Hynes, 1999). Both the α and β integrin subunits contain large extracellular domains, single pass transmembrane domains and short cytoplasmic domains (integrin β_4 excluded). Integrins mediate cell-cell or cell-matrix adhesion, transduce signals, provide physical support to cells through

interaction with the cytoskeleton, regulate cell movement and can alter gene expression (Hynes, 1999). The amino acid motif, arginine-glycine-asparagine (RGD) is present in fibronectin, osteopontin, and other adhesion proteins. It is a common binding site for many integrins and contributes to the flexibility in ligand binding. Integrin $\alpha_v\beta_3$ is the most promiscuous receptor as it can bind vitronectin, fibronectin, osteopontin, von Willenbrand factor, and thrombospondin.

Osteopontin is a secreted phosphoprotein that is widely distributed in many mineralized tissues such as bone and teeth, the vascular system, the immune system and epithelial tissues such as the kidney, breast, uterus and placenta. Sex steroids, glucocorticoids, vitamin D and growth factors regulate osteopontin. Its function can be altered by extensive post-translational modification including phosphorylation, sulphation, glycosylation and cleavage (reviewed in Sodek et al., 2000), which can in part determine its interaction with cell surface receptors. For example thrombin cleavage promotes increased binding through the RGD domain (Xie et al., 2001). Integrin $\alpha_v\beta_3$ is thought to be a major receptor for osteopontin in the uterus of humans and sheep, but its distribution in the bovine uterus has not yet been studied (Coutifaris et al., 1998; Johnson et al., 2001a).

1.8 Integrin Regulation

The expression of integrins can be regulated at transcription or post-transcription through alternative splicing of mRNA, or mobilization of intracellular stores. So far ECM, growth factors, cytokines and steroids have been identified as regulators of integrin expression; however the mechanisms are complex as regulatory

factors can interact (Chen et al., 1992; Bruner et al., 1995; Grossinsky et al., 1996). Growth factor or steroid treatment of cultured cells can increase or decrease the levels of integrin mRNA, and can affect post-RNA events such as assembly of integrin dimers (reviewed in Kim and Yamada, 1997). It is now clear that regulation of integrin expression is cell-type specific and results can vary under different experimental conditions. For example, primary cultures of human endometrial cells (stromal and epithelial) maintain constant levels of integrins regardless of steroid hormone treatment but are sensitive to regulation by growth factors (Grossinsky et al., 1996; Sillem et al., 1997). In contrast, immortalized human endometrial epithelial cells downregulate integrin $\alpha_v\beta_3$ when treated with estrogen or progesterone (Castlebaum et al., 1997). Whether integrin transcription is directly controlled by the steroid receptor superfamily of transcription factors is controversial.

There is a growing body of evidence showing that integrins, steroid hormones, and growth factors have cooperative regulatory pathways. For example, bone resorption requires estrogen-stimulated transforming growth factor- β_3 (a known stimulator of integrin $\alpha_v\beta_3$ expression) and osteoclast adhesion via integrin $\alpha_v\beta_3$ to osteopontin (Igotz et al., 1989; Yang et al., 1996; Duong et al., 2000). Taken together, these results suggest that in the process of bone resorption (and probably in other cell systems) there is cross-talk between steroid, growth factor and integrin receptors. To add an even further level of complexity, there is a connection between growth factors, integrins, steroids, and ECM. Matrix metalloproteinases (MMP) are involved in ECM remodeling and their expression in endometrium is regulated by

steroids (Rodgers et al., 1994), growth factors, and cytokines such as IFN- τ (Salmonsens et al., 1994). In turn, ECM composition can regulate integrin expression and vice versa while integrins can also regulate MMP expression (Chen et al., 1992). An in vivo experiment illustrates the consequences of the interplay between steroid receptors and tissue architecture. Shymala and co-workers (1998) generated transgenic mice carrying an excess of the PR-B isoform and found that the mammary epithelium was disorganized, and lacked a distinct basement membrane. This experiment is interesting as it demonstrates a link between steroid receptors and cell-cell and cell-ECM interactions. Whether PR-B influences cell adhesion through regulation of integrin or other adhesion molecule genes remains to be elucidated.

Integrins exist in three conformational states, resting, activated and ligand bound (Ylanne, 1998). In response to an intracellular signal, integrin affinity for its ligand is altered by either tyrosine phosphorylation of the cytoplasmic domain as occurs for activation of integrin $\alpha_v\beta_3$, or by 'unlocking' of the membrane proximal hinge that is located in the C-terminal of α and β subunits (Hughes and Pfaff, 1998). Integrins require extracellular divalent cations for ligand binding and both subunits contain cation binding EF-hand-like motifs (Johannsson et al., 1997). Intracellular signaling pathways identified in integrin activation involve the Rho family of GTP-ases. Integrin avidity modulation results from Rho interaction with actin filaments and the subsequent formation of focal adhesions (Schwartz and Shattil, 2000). However, integrin activation is likely to be cell and integrin specific as alternative splicing of the β cytoplasmic domain depends on cell phenotype. Furthermore, as the cytoplasmic domain is the target for affinity modification, integrin splice variants

probably interact with a modified repertoire of intracellular signaling proteins (Vijayan et al., 2000).

CHAPTER 2 ESTROUS CYCLE AND PREGNANCY EFFECTS ON THE EXPRESSION OF ESTROGEN AND PROGESTERONE RECEPTORS IN BOVINE ENDOMETRIUM

2.0 Introduction

The bovine estrous cycle is governed by follicular estrogen and luteal progesterone. These shifts in these hormones allow for the development and ovulation of an egg, receptivity to mating, and preparation of the uterus for pregnancy. In the uterus, intercellular communication requires integration of hormonal signals from the ovaries and embryo, which in turn depends on tissue and cell specific expression of steroid receptors. The ruminant endometrium is composed of glandular and luminal epithelia overlying a well vascularized stroma (King, 1993; Guillomot, 1995; Bartol et al., 1999). The final paracrine dialogue between epithelium and the underlying stroma can be permissive or inductive, in part determining a tissue response to its hormonal environment (Hodges, 1994). The mechanisms which govern such interactions in the uterus include cell-cell contact, paracrine transmission of hormones, cytokines, signaling molecules, ECM and cell adhesion molecule interactions at tissue boundaries (Cunha and Young, 1992; Damsky and Werb, 1992; Hodges, 1994; Vićovac and Aplin, 1996; Bartol et al., 1999; Roberts et al., 1999).

In ruminants, the timing of luteolysis determines cycle length and its prevention is required for pregnancy establishment (Godkin et al., 1997; Martal et al., 1997). In sheep, altered levels and distribution of estrogen (ER) and progesterone receptors (PR) are thought to be important in the onset of $\text{PGF}_{2\alpha}$ secretion (Spencer

et al., 1995; Spencer et al., 1999). To date there has not been a comprehensive study documenting the distribution patterns and levels of steroid receptors in the bovine uterus throughout the estrous cycle and early pregnancy. This study was designed to examine the distribution and relative expression levels of uterine ER and PR during the estrous cycle and the peri-implantation period. Steroid-treated ovariectomized animals were used to separate the effects of estrogen and progesterone on receptor levels.

2.1 Materials and Methods

Animals and Tissue Collection

All procedures performed were in accordance with the guidelines of The Canadian Council on Animal Care (1993). Reproductive tracts from sexually mature mixed beef breed cattle (n=39, ≥ 3 /day) were collected at government-inspected abattoirs on days 0 (standing heat), 1, 3, 6, 10, 14, 15, 16, 17, 18, 19 and 20 of the estrous cycle, or days 16, 18, 21, 24 or 30 of pregnancy (n=3/day). To confirm cycle stage, ovarian dating was performed at collection according to the criteria of Ireland (1979). Pregnancy was confirmed by the presence of embryonic tissue in the uterus. An additional 10 animals were ovariectomized via an incision through the left paralumbar fossa after local anesthesia with lidocaine 2% (Vetoquinol Canada Inc., Joliette, PQ, Canada) in an inverted L block. Following a one week recovery period after surgery, animals received one of the following treatments for 12 days: estradiol-17 β (24 mg implant, Compudose; Eli Lilly, Quebec City, QC, Canada, n=3), progesterone (1.9 g, P₄-releasing intravaginal device [PRID]; Sanofi, Cambridge,

ON, Canada, n=2), both estrogen and progesterone (n=2), or the control, not treated (n=3). Halfway through the treatment period PRIDS were changed to maintain luteal levels (> 4 ng/ml) of circulating progesterone until slaughter. One cubic centimeter cross-sectioned blocks were dissected from the active or pregnant uterine horn, and frozen in liquid nitrogen within one hour post-mortem. Tissues were transferred to an ultra-low-temperature freezer (-80°C) for long-term storage.

Immunohistochemical Detection of ER and PR

Estrogen receptors were localized in bovine endometrium using a monoclonal antibody generated against calf uterus ER α that recognizes the epitope between amino acids 120-170 in the transactivation domain of the N-terminus (clone AER314; Neomarkers Inc., Union City, CA). Progesterone receptors were targeted using a monoclonal antibody generated against human endometrial PR-B that recognizes the hormone binding domain (clone hPRa2; Neomarkers Inc.). Optimal primary antibody concentrations were empirically determined to be 0.4 μ g/ml in 2 per cent bovine serum albumin (BSA), phosphate buffered saline, pH 7.2-7.4 (PBS), when used in conjunction with the Vectastain Elite ABC kit® (Vector Laboratories, Burlingame, CA). Controls were treated with 0.4 μ g/ml purified mouse IgG (Chemicon, Temecula, CA). Immunoperoxidase (PR) or immunofluorescent (ER) staining was performed on 5-8 μ m cryosections, collected on 3-aminopropyltriethoxysilane-coated Superfrost Plus® slides (Fisher Scientific, Whitby, ON, Canada). Cut sections were dried for 30 min, then fixed for 10 min in acetone and stored at -80 °C. Non-specific binding was minimized by an initial blocking step

using 2 per cent BSA in PBS for 30 min. Sections were incubated with primary antibody overnight at room temperature in a humid chamber, then washed 3 times for 3 min in PBS and further processed according to the ABC kit directions. Antibody-antigen interactions were visualized using 3-amino-9-ethylcarbazole (Sigma, Saint Louis, MO) and the sections were counterstained with Mayer's hematoxylin (Electron Microscopy Sciences, Fort Washington, PA). The stained sections were preserved using Aquaperm ® mounting medium (Fisher). Slides were viewed on an Olympus BH-2 microscope (Olympus Optical Company, Tokyo, Japan). Progesterone receptor images were captured with a Pixera digital camera (Pixera Corporation, Los Gatos, CA). For localization of ER, fluorochrome-conjugated streptavidin probes (Alexa-488, Molecular Probes Inc. Eugene, OR, USA) were applied for 30 min, then sections were washed in PBS and mounted in Fluoromount G (Electron Microscopy Sciences, Washington, PA, USA). Images were viewed and captured on a Leica DML microscope (Leica Microsystems, Wetzlar, Germany), equipped with an Olympus omPC camera (Olympus Optical Company, Tokyo, Japan), and photographed on Kodak Royal Gold (Eastman Kodak, Rochester NY, USA). Uterine tissue sections from each animal (3 per slide) were processed at least three times with each antibody and slides were processed as full sets which included all animals from either the pregnant or not pregnant group to minimize inter-experiment variation.

Statistical Analysis

Receptor staining intensities were scored using a five point scale, (1, not detected; 2, low; 3, moderate; 4, high; 5, very high) in the luminal epithelium, subepithelial stroma (defined as the band of compact stroma immediately adjacent to the basal lamina approximately 3-6 cells wide), compact stroma, loose stroma, superficial glands, deep glands and myometrium. Any differences between caruncular and intercaruncular tissue staining patterns were also scored. Data were analyzed using the Kruskal-Wallis test of population medians (Sprent, 1993) to identify the effects of cycle day and pregnancy stage on steroid receptor levels. When significant effects were observed the Mann-Whitney two sample rank comparison test was used to test the equality of population medians between cycle days or pregnancy stage. Scoring results were blocked by experiment to test for experimental effects, which were found to be insignificant ($P>0.05$).

2.2 Results

Estrogen Receptor Distribution

In cryosections from cycling animals, overall endometrial expression of ER was highest post-ovulation days 1-3 and lowest during the luteal phase days 7-17 (Fig. 4 and 5). Compact stroma reactivity was lower on day 17 than during proestrus (days 18-20) ($P<0.05$). On day 17 (Fig. 4a), weak reactivity was detected in the compact stroma and expression increased through proestrus (Fig. 4b and 5a), in the compact stroma and shallow glands. Through estrus, reactivity continued to spread outward from the compact stroma with staining intensity increasing in the glands and loose stroma (Fig. 5). On days 1-3, in comparison to all other cycle days examined,

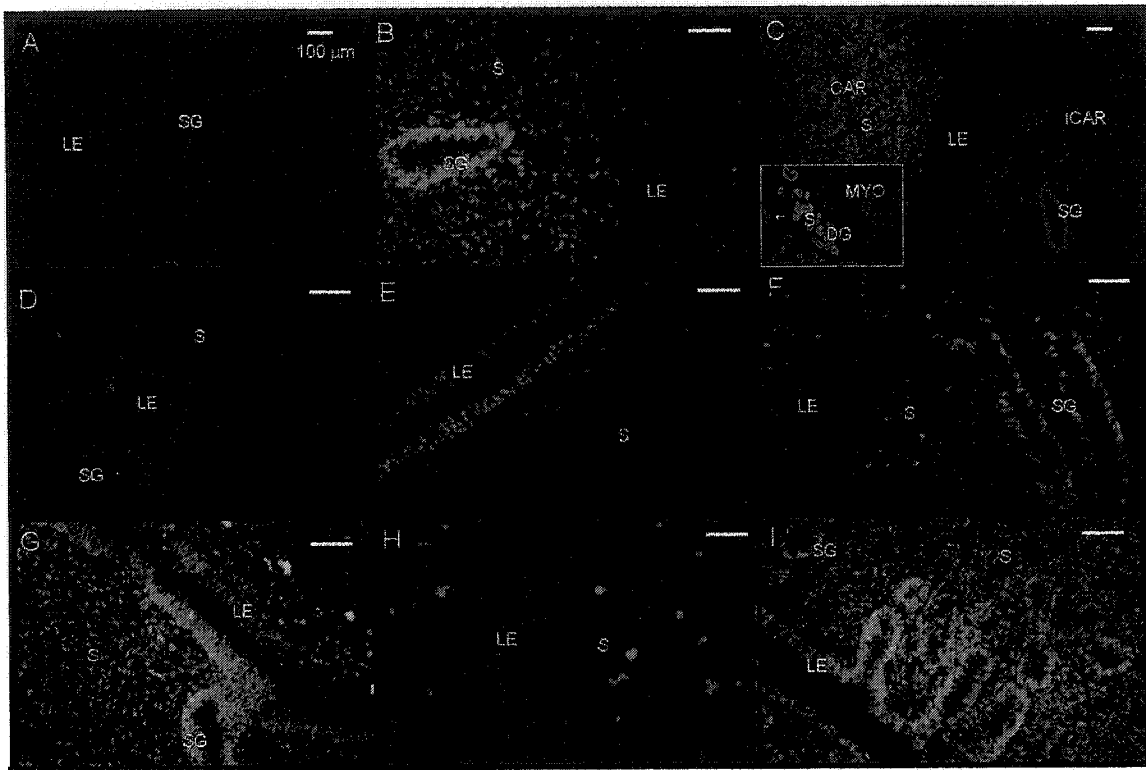


Figure 4. Immunofluorescent localization of the estrogen receptor in cryosections of bovine endometrium during the estrous cycle (A-E), day 16 of pregnancy (F), and from ovariectomized animals (G-I). Immunoreactivity is indicated by green fluorescence and was localized predominantly to nuclei of the stroma (S), shallow glands (SG), and deep glands (DG). Staining of the luminal epithelium (LE), was only detected in sections prepared from animals on days 16 and 14 of the cycle. (A) day 17, (B) day 20, (C) day 3, (D) day 15, (E) day 16, (F) day 16 pregnant, (G) OVX-E, (H) OVX-P, (I) OVX. Size of scale bar in (A) applies to all. M=myometrium, arrow indicates a blood vessel.

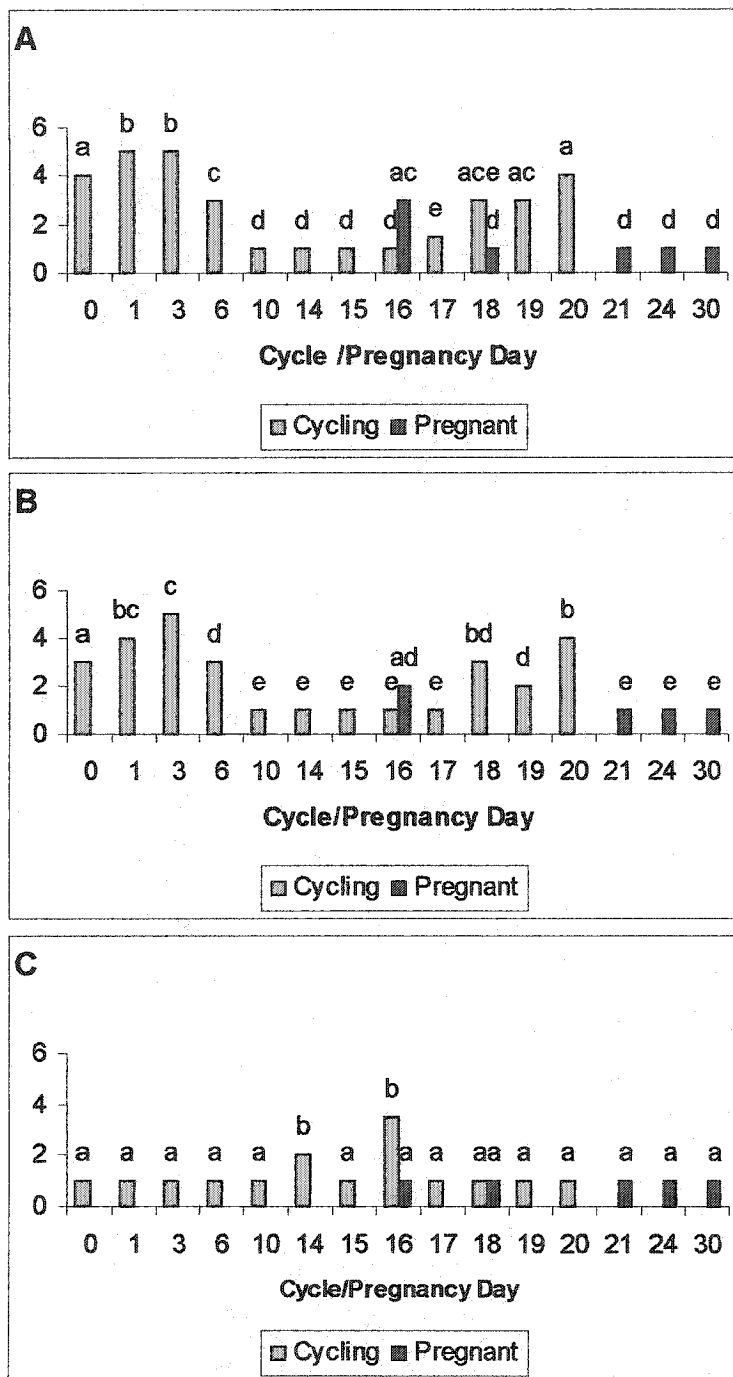


Figure 5. Effects of cycle and pregnancy day on estrogen receptor distribution in bovine endometrium. Results are expressed as median staining intensity values. Mann-Whitney's two sample rank test was used to compare equality of medians. Results were statistically compared based on cycle day and pregnancy. Bars not sharing the same letter are significantly different ($P < 0.05$). (A) shallow glands, (B) deep glands, (C) luminal epithelium.

ER expression was maximal ($P<0.05$) with strong expression occurring in the glands and moderate expression throughout the stroma (Figures 4c and 5ab). By late metestrus (day 6), ER levels had declined and disappeared from the compact stroma so that only moderate expression remained in the glands (Fig. 4d and 5ab). Staining of the luminal epithelium ($P<0.05$) was exclusive to animals collected on days 16 ($n=5$) or 14 ($n=2$) (Fig. 4e and 5c). A positive reaction was detected in the glands of day 16 pregnant animals (Fig. 4f and 5a). All other peri-implantation samples were negative ($P<0.05$).

Estrogen-treated OVX animals had the highest expression levels of ER ($P<0.05$) in the glands, luminal epithelium and compact stroma (Fig. 4g) in comparison to OVX-P and OVX-E+P treated cows. Animals receiving progesterone alone or in combination with estrogen had the lowest estrogen receptor levels in comparison to OVX-E and control animals ($P<0.05$). Little to no staining occurred in the compact stroma of OVX-P animals (Fig. 4h). Reactivity was predominantly confined to the deep glands in OVX E+P treated animals. Estrogen receptor expression in control animals was detected throughout the endometrium (Figure 4i). Estrogen receptors were not observed in the vasculature of any animals.

Progesterone Receptor Distributions

Immunoperoxidase staining for PR revealed intermediate expression in the subepithelial compact stroma and myometrium in cryosections from proestrus animals (Fig. 6a and 7a). By estrus expression had spread outward to the loose stroma (Fig. 6b and 7b), and by day three (Fig. 6c and 7b) expression was maximal

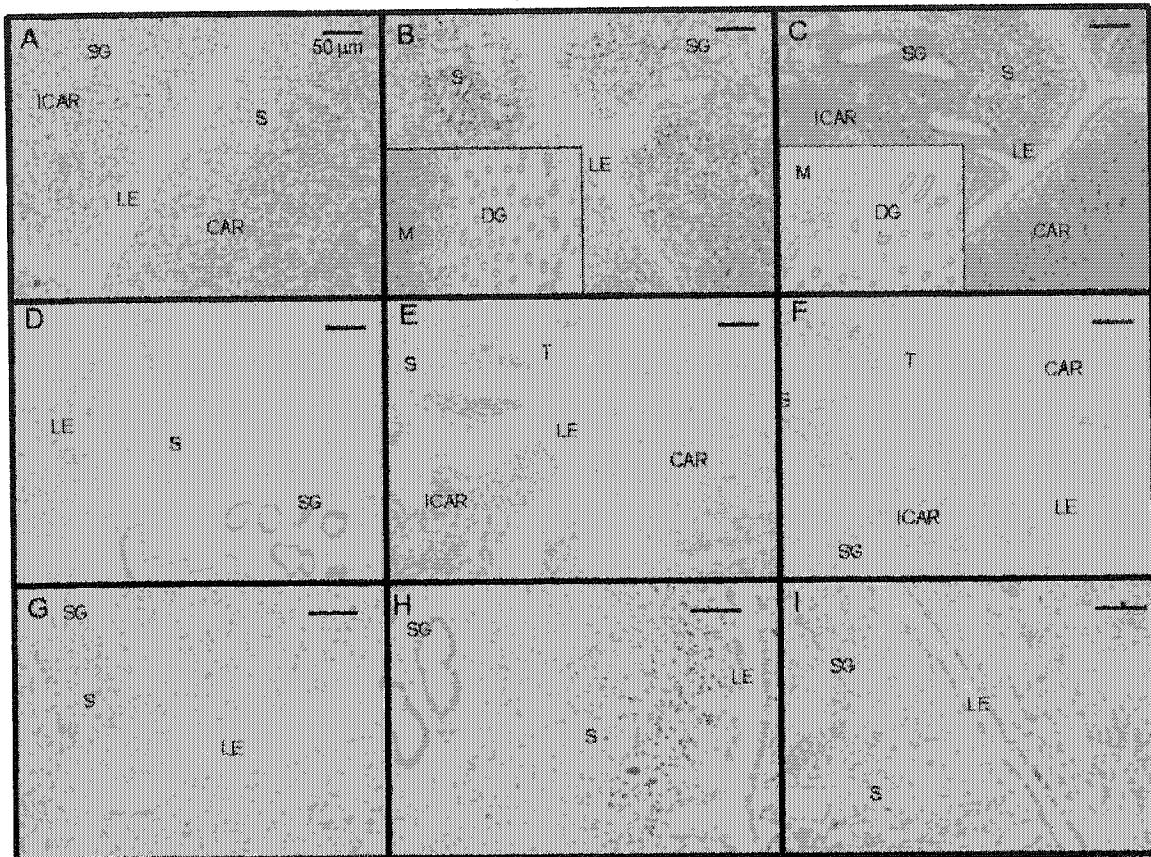


Figure 6. Immunohistochemical localization of the progesterone receptor in cryosections of bovine endometrium from cycling (A-D), pregnant (E,F) and ovariectomized animals (G-I). Red nuclei indicate the presence of immunoreactive progesterone receptor protein and were detected predominantly in the stroma (S), shallow glands (SG), deep glands (DG, shown in insets) and myometrium (M, shown in insets). Progesterone receptors were not detected in the luminal epithelium (LE). Sections were counterstained with hematoxylin. (A) day 18, (B) day 1, (C) day 3, (D) day 6, (E) day 18 pregnant, (F) day 21 pregnant, (G) OVX-P, (H) OVX-E, (I) OVX. Size of scale bar in (A) applies to all. CAR=caruncle; ICAR=intercaruncle; T=trophoblast.

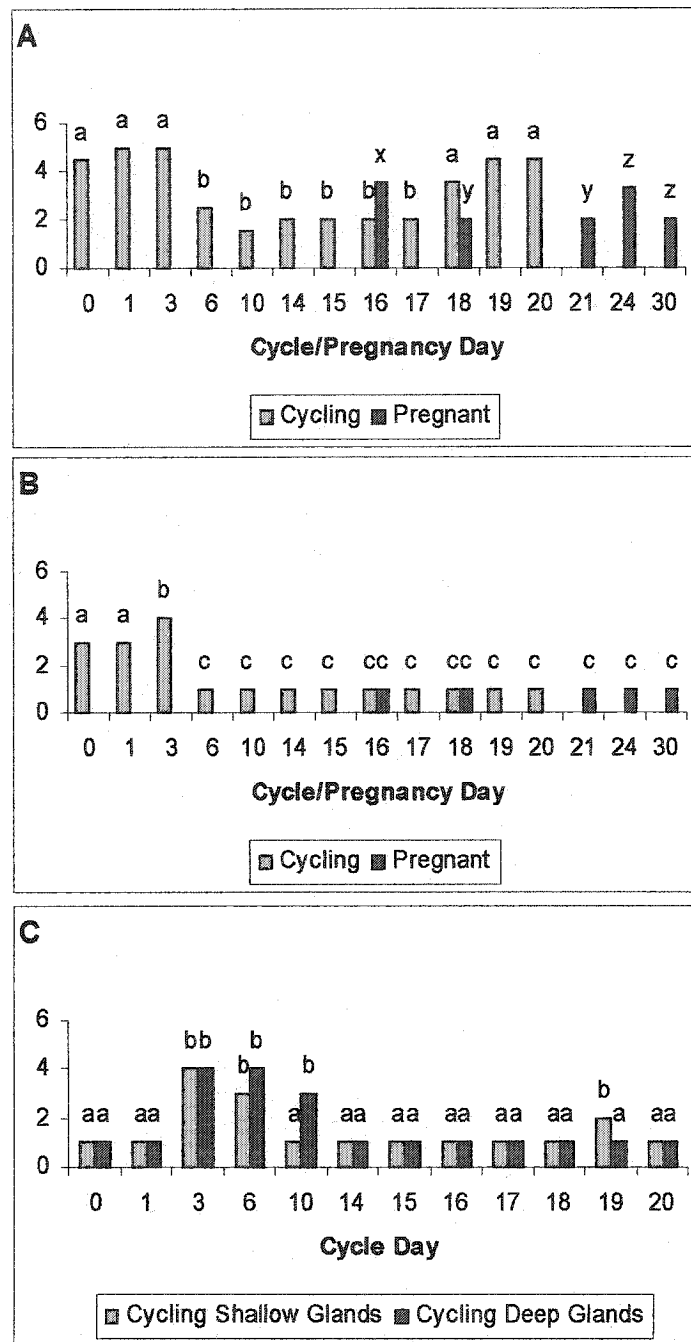


Figure 7. Estrous cycle and pregnancy effects on the distribution of progesterone receptors in bovine endometrium. Results are expressed as median staining intensity values. Mann-Whitney's two sample rank test was used to compare equality of medians. Scoring results from the compact stroma were grouped by cycle stage or pregnancy for statistical comparisons. Results from the loose stroma and glands were compared by cycle and pregnancy day. Bars not sharing the same letter are significantly different ($P < 0.05$). (A) compact stroma, (B) loose stroma, (C) glands.

in the loose stroma comparison to all other cycle days ($P<0.05$) with strong nuclear staining occurring in the stroma, glands and myometrium. By late metestrus (day 6) (Fig. 6d, and 7ac), expression was decreased in comparison to early metestrus ($P<0.05$) in the regions closest to the luminal epithelium with moderate reactivity present in the compact stroma and shallow glands (Fig. 6ac). Intermediate staining remained in the deep glands. Immunostaining was lower in diestrus samples (days 7-17) than in samples collected during other cycle stages ($P<0.05$) (Fig. 7); weak reactivity was present in the compact stroma and myometrium. No expression of PR was detected in the luminal epithelium (Fig. 6a-d) at any cycle day examined and there was no difference in reactivity between caruncular and intercaruncular regions. In contrast, in pregnant animals caruncular stroma showed reduced reactivity compared to intercaruncular stroma, particularly in the subepithelial region ($P<0.05$)(Fig. 6ef). Staining in the compact stroma was lower on days 18 and 21 in comparison to days 16, 24 and 30 of pregnancy ($P<0.05$)(Fig. 7a). For all stages of pregnancy examined the myometrium was positive and the uterine epithelium was negative.

Ovariectomized animals treated with progesterone (Fig. 6g) had reduced immunoreactivity in comparison to OVX-E (Fig. 6h), OVX-E+P, and OVX animals ($P<0.05$) (Fig. 6i). Sections from OVX-P animals showed punctate staining throughout the stroma and moderate staining of the myometrium. Endometrium from OVX, OVX- E, and OVX-E+P animals had intermediate to strong stromal and myometrial staining. Glandular staining was only detected in OVX-E animals and the luminal epithelium stained positively in OVX and OVX-E animals.

2.3 Discussion

The results from this study indicate that the tissue distribution of steroid receptors is regulated during the estrous cycle and early pregnancy in a cell specific manner that cannot be entirely attributed to regulation by the sex steroids themselves. Paracrine factors produced by local cell populations or originating from the embryo could modulate transcription of ER and PR gene expression (Spencer et al., 1995; Spencer et al., 1999; Kurita et al., 2000ab).

In sheep, mice and rats, estrogen influences expression of estrogen and progesterone receptors in uterine stromal and epithelial cells (Wathes and Hammon, 1993; Spencer et al., 1995; Ing and Tornesi, 1997; Kurita et al., 2000ab). In this study, presumably high levels of follicular estrogen (or exogenous estradiol in ovariectomized animals) coincided with high levels of estrogen and progesterone receptors. Maximal receptor levels can be attributed to estrogen-up-regulated steroid receptor expression, which has been well documented in other estrogen target organs (Cunha and Young, 1992). Expression of ER and PR was low during diestrus, early pregnancy and in OVX-P animals, suggesting that progesterone blocks transcription and/or translation of ER and PR. However, as ER and PR were detected in OVX-E+P cows it appears that estrogen is the dominant regulator of sex steroid transcription in the cow. Estrogen may override the inhibitory effect of progesterone, either directly through estrogen response elements, or indirectly by affecting PR associated cofactors (Kimmins and MacRae, 2000). On the other hand, ovarian steroid free OVX cows had high levels of ER and PR, suggesting that in the absence of ovarian steroids, expression is constitutive. Similar results were observed in

steroid treated ovariectomized sheep (Spencer et al., 1995). This study revealed a unique uterine distribution of the steroid receptors in comparison to other species (Wathes and Hammon, 1993; Spencer and Bazer, 1995; Ing and Tornesi, 1997; Kurita et al., 2000a,b). Detection of ER in the luminal epithelium was limited to estrous cycle days 14 or 16 (discussed in more detail later), and PR were never detected in the luminal epithelium. Until recently it was assumed that hormone action on a cell required expression of the corresponding receptor on that cell. However, studies of epithelial-stromal interactions in the prostate (Cunha and Young, 1991), mammary gland (Cunha et al., 1997), and uterus (Cooke et al., 1997) have shown that the epithelial proliferation response to steroids is mediated by stromal steroid receptors. Using tissue recombinants generated from ER knock-out, PR knock-out and wild type mice, Kurita et al. (2000a, b) demonstrated that estrogen and progesterone regulation of ER and PR in the mouse reproductive tract involves a complex epithelial-stromal paracrine dialogue. In mice, estrogen mediated down-regulation of uterine epithelial PR requires stromal ER α (Kurita et al., 2000a), and progesterone inhibits this effect via stromal PR (Kurita et al., 2000b). Our results suggest that steroid responsiveness of bovine epithelium also relies on stromal estrogen and progesterone receptors. Unlike bovine luminal epithelium, murine epithelium expresses both estrogen and progesterone receptors.

In ruminants, the sex steroids are involved in the regulation of the luteolytic mechanism and the subsequent return to estrus (Mirando, Becker and Whiteaker, 1993; Sheldrick and Flick-Smith, 1993; Leung and Wathes, 1999), although their role in the sequence of events leading up to uterine release of PGF $_{2\alpha}$ remains unclear.

The postulated sequence of events underlying the initiation of PGF_{2α} release in sheep involves auto-down-regulation of PR following extended progesterone exposure, allowing for transcription of ER genes (McCracken et al., 1999). Specifically, Spencer et al. (1995) suggest that the disappearance of PR from the luminal epithelium and the subsequent appearance of ER activates oxytocin receptor gene expression in the sheep. This increased uterine sensitivity to ovarian oxytocin then induces PGF_{2α} release and resumption of cyclicity (Mirando, Becker, and Whiteaker, 1993; Stevenson et al., 1994; Wathes and Lamming, 1995; Ivell and Walther, 1999). In the present study ER were only localized to the luminal epithelium on day 16 (or day 14 in animals having shorter cycles) which is the last day for successful embryo transfer (Betteridge, et al., 1980). By day 16 the bovine conceptus signals its presence to the dam through release of IFN-τ, blocking release of uterine PGF_{2α} (Godkin et al., 1997; Martal et al., 1997; Roberts et al., 1999; Asselin and Fortier, 2000). A change in the ER on day 16, prior to high levels of circulating estradiol, may indicate estrogen-independent activation of the ER via cross-talk between growth-factor mediated signal transduction systems and the ER (Smith, 1998; Weihua et al., 2000). Alternatively, slight increases in estradiol from the emerging dominant follicle and the lack of stromal PR may trigger changes in the ER in the luminal epithelium on cycle day 16. A similar response to low levels of estradiol has been shown to promote oxytocin receptor expression in ovariectomized cows (Lamming and Mann, 1995). Whatever the mechanism, this transient ability to detect ER in the luminal epithelium suggests involvement of ER in the initiation of

luteolytic events in the cow, particularly as ER were not detected in the luminal epithelium of day 16 pregnant animals. Further support for a role of the ER in the release of $\text{PGF}_{2\alpha}$ in the cow is that cultured bovine endometrial epithelial cells are the preferential site of $\text{PGF}_{2\alpha}$ synthesis and secretion (Asselin, et al., 1996). The ability to detect ER on day 16 in luminal epithelium using the AER314 antibody suggests it could serve as a differentiation marker for these cells as they begin to synthesize and secrete prostaglandins.

In the pregnant ewe, $\text{IFN-}\tau$ prevents cyclic increases of ER and oxytocin receptors thus blocking the initiation of the luteolytic mechanism (Spencer and Bazer, 1996). It is likely that $\text{IFN-}\tau$ has the same sort of effect on ER in the bovine uterus as ER were not detected in the luminal epithelium of day 16 pregnant cows. In addition, the glands may be a site of action for $\text{IFN-}\tau$ in the cow as ER were detected in the shallow glands of day 16 pregnant but not in the glands of day 16 cycling animals. In female and male reproductive tracts the ER has been associated with cell proliferation (Buchanan et al., 1998; Tarleton, et al., 1999; Lai et al., 2000), while in pigs, gland adenogenesis requires post-natal glandular expression of $\text{ER}\alpha$ (Tarleton, et al., 1999). In cattle, the presence of ER in the glands may be associated with increased glandular growth in preparation for secretion of histotroph to support pregnancy.

Unlike sheep (Spencer and Bazer 1995), PR were never detected in the luminal epithelium of cycling and pregnant cows, or the glandular epithelium of pregnant cows. Down-regulation of PR in the glands of pregnant cows may be required for uterine receptivity and suggests that glands are a target for $\text{IFN-}\tau$.

Perhaps there is differential action of IFN- τ on steroid receptor transcription in bovine endometrium. In cows IFN- τ may initiate the luteoprotective mechanism by suppressing transcription of PR in the glandular epithelium, thereby allowing for ER expression in the glands, and the subsequent growth promoting effects associated with estrogen responsiveness (Buchanan et al., 1998; Lai et al., 2000). Previous work in sheep provides support for this theory; no PR were localized to the shallow glandular or luminal epithelium during early pregnancy or in ovariectomized ewes treated with IFN- τ (Spencer and Bazer, 1995; Spencer et al., 1995).

In this study, pregnant cattle had reduced PR levels in caruncular tissue compared to intercaruncular endometrium. The lower levels of PR in pregnant bovine caruncles may allow for decreased response to progesterone by decreasing PGF_{2 α} output, thereby increasing the PGE₂:PGF_{2 α} ratio. Indeed, *in vitro* treatment of bovine endometrial cells with IFN- τ increases the PGE₂:PGF_{2 α} ratio in caruncular cells in comparison to intercaruncular cells (Asselin, Drolet, and Fortier, 1998). Differences in the response to the signaling mechanisms associated with early pregnancy are expected between caruncular and intercaruncular sites. The caruncles also exhibit differences in expression of cell adhesion molecules, regardless of pregnancy state (Kimmins et al., 2003a).

In summary, this study reveals both similarities and differences in uterine steroid receptor distribution between sheep and cattle. The changes observed in steroid receptor distribution and expression levels are undoubtedly key to directing uterine cyclicity and the establishment of pregnancy in the cow.

CHAPTER 3 PERIIMPLANTATION EXPRESSION OF INTEGRIN $\alpha_v\beta_3$ AND OSTEOPONTIN ILLUSTRATES DIFFERENCES IN THE UTERINE CELL BIOLOGY OF SHEEP AND CATTLE

3.0 Introduction

Comparative studies of placentation in domestic animals show that implantation is similar (Amoroso, 1952; King et al., 1982; Atkinson et al., 1984; Bowen and Burghardt, 2000). However differences in the molecular properties of receptive endometrium are just now being explored. Placentation in ruminants is categorized as non-invasive as chorionic cell migration into maternal tissue is restricted to the luminal epithelium (King et al., 1980; Wathes and Wooding, 1980). The preimplantation period in the cow and ewe is lengthy in comparison to rodents, carnivores and primates, and coincides with secretion of pregnancy supporting proteins from glandular epithelium, and trophoblast production of the pregnancy recognition factor, IFN- τ (reviewed by Spencer and Bazer, 2002).

In many species, endometrial expression of integrins and their ECM ligands is correlated with embryo attachment and uterine differentiation during the receptive phase and pregnancy (Bowen et al., 1996; Fazleabas et al., 1997; Kimmins and MacLaren, 1999; Apparao et al., 2001; Johnson et al., 2001a; MacIntyre et al., 2002). The most promiscuous integrin in terms of ligand interactions is integrin $\alpha_v\beta_3$, which binds RGD motifs in fibronectin, osteopontin and laminin, among others (Sasaki and Timpl, 2001; reviewed in van der Flier and Sonnenberg, 2001). The affinity of integrins for their ligands is dependent on cell type, changes in integrin activation

states that are mediated by phosphorylation of the cytoplasmic domain (inside out signaling) and ligand availability (outside in signaling) (Jamora and Fuchs, 2002; Miranti and Brugge, 2002).

Osteopontin was first described as a secreted 60-kDa phosphoprotein associated with bone ECM, and as a lymphokine expressed by activated lymphocytes and macrophages (reviewed in Sodek et al., 2000). Expression has since been identified on the epithelium of many tissues such as kidney, breast, and reproductive tract (Brown et al., 1992). Osteopontin expression and distribution differs between cycling and pregnant sheep; little secretion occurs in the glands of cycling animals while secretion increases from glandular epithelium during the periimplantation period (Johnson et al., 1999a). As a secreted protein of the ruminant uterus regulated by progesterone, osteopontin has been proposed to support conceptus growth and act as an adhesive between trophoblast and luminal epithelium via integrin $\alpha_v\beta_3$ (Johnson et al., 1999a; Johnson et al., 2000).

Until now the distributions of integrin $\alpha_v\beta_3$ and its ligand osteopontin have not been studied in the pregnant cow. The distribution of these proteins has been studied in cycling and pregnant sheep, however, the integrin $\alpha_v\beta_3$ results were different from what we observed in cycling cattle. This is surprising since histologically placentation in sheep and cows is similar. The objectives of the present study were to localize the expression of integrin $\alpha_v\beta_3$ in bovine and ovine endometrium during the periimplantation period and to compare the distribution patterns using two monoclonal antibodies that have not been tested in sheep. In addition, the expression of osteopontin during the estrous cycle and periimplantation

period in bovine and ovine endometrium was examined using an antibody to bovine osteopontin.

3.1 Materials and Methods

Animals

See Chapter 2.1 for procedures followed for heifers and tissue collection. Mature Rideau Arcott ewes were synchronized to estrus using intravaginal progesterone pessaries for 14 days followed by observation for estrous behavior in the presence of an intact ram. Ewes were randomly assigned to either cyclic or pregnant status, and mating to rams occurred over a 2-day period. Reproductive tracts were collected on days 13 (n=1) and 17 (n=2) of the cycle, and on day 13 of pregnancy (n=2).

Antibodies

The two monoclonal antibodies specific to human integrin $\alpha_v\beta_3$ were from clone LM609 (Chemicon, Temecula, CA) and clone BV4 (Novus Biologicals, Littleton, CO), and were used for immunohistochemical localisation. Osteopontin was localised using an anti-bovine milk osteopontin antibody kindly provided by Dr. G. Killian (Pennsylvania State University, Almiquist Research Centre, University Park, PA). Horseradish peroxidase conjugated donkey-anti-rabbit IgG (Pierce, Rockford, IL) was used for detection of osteopontin. Negative controls were rabbit IgG (ICN Biomedical Inc., Aurora, ON, Canada) or purified mouse IgG (Chemicon) and were used at the same concentration as primary antibodies. Optimal dilutions of

primary antibodies were empirically determined, and fixation and detection methods were selected based on the conditions that allowed for maximum specific immunostaining.

Immunohistochemistry

Immunoperoxidase staining for integrins was performed on 5 to 8 μm cryostat cross-sections of endometrium. Serial sections were mounted on 3-aminopropyl-triethoxysilane-coated-Superfrost Plus® slides (Fisher Scientific, Whitby Ontario, Canada), then fixed in acetone for 10 min and air dried overnight. Cut sections were stored at -80°C until use. Sections were blocked with 2% w/v BSA in PBS (pH 7.2 to 7.4) for 30 min. Primary antibodies were diluted in 2% w/v BSA in PBS and applied at concentrations of 10 $\mu\text{g}/\text{ml}$ for anti-osteopontin, 0.6 $\mu\text{g}/\text{ml}$ for anti- $\alpha_v\beta_3$ (Chemicon), and 0.2 $\mu\text{g}/\text{ml}$ for anti- $\alpha_v\beta_3$ (Novus Biologicals). All primary antibodies were allowed to bind for 2 h, then the slides were washed in PBS 3 times for 3 min each, and secondary antibody was applied and allowed to react for 45 min followed by washing in PBS. For amplification of integrin $\alpha_v\beta_3$ signal, the Vectastain Elite ABC kit® (Vector laboratories, Burlingame CA) was used. To prevent non-specific staining, avidin (reagent A) was used at half the recommended concentration. Immunostaining was visualised using the chromogen metal enhanced diaminobenzidine (integrin $\alpha_v\beta_3$; Pierce) or 3-amino-9-ethylcarbazole (osteopontin; Sigma, Saint Louis, MO). Slides were counterstained with Mayers Hematoxylin (Electron Microscopy Sciences, Fort Washington, PA) and coated with Aquaperm® mounting media (Fisher). Slides were viewed on a

Leica DMLB microscope (Leica Microsystems, Wetzlar, Germany) and photographed on Ektachrome 160T tungsten film (Eastman Kodak, Rochester, NY). Slides were scanned and images assembled in Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA). All experiments were replicated at least three times for each animal on different days.

Statistical Analysis

Cell compartments within sections were scored by two independent observers on a six point scale (0-negative, 1-very weak, 2-weak but clearly positive, 3-intermediate, 4- high, and 5- very high) for staining intensity. Data were checked for normalcy using the univariate procedure in SASTM (Statistical Analysis Software Version 8, SAS Institute Inc. Cary NC) and log transformed if transformation corrected to a normal distribution. Normal data were analyzed using a general linear models procedure to examine the effect of day on staining intensity, and the Duncan's multiple range test was used to compare population means. Nonparametric statistical comparisons between day of cycle, day of pregnancy or cycle stage (stage 1-metestrus, stage 2-early diestrus; stage 3-late diestrus; stage 4-proestrus and estrus) were made by the Kruskal-Wallis test of populations medians (Sprent, 1993). When significant effects were observed, Mann-Whitney's two sample rank test (Sprent, 1993) was used to test the equality of population medians. The selected alpha level was 0.05.

3.2 Results

Immunohistochemical Localization of Integrin $\alpha_v\beta_3$ in Early Pregnant Bovine Endometrium

At all stages examined, integrin $\alpha_v\beta_3$ was detected in luminal epithelium, endometrial stroma, myometrium and arteriolar smooth muscle (Fig. 8). There was no difference in staining intensity or the distribution pattern in tissue sections treated with antibody to integrin $\alpha_v\beta_3$ from either Chemicon or Novus Biologicals. As previously observed, sections prepared from heifers at day 18 of the estrous cycle showed a band of strong immunoreactivity in the stromal cells underlying the intercaruncular luminal epithelium and presumably in contact with basement membrane matrix, with reduced staining evident in the same region of the caruncles (Fig. 8a). A diffuse weak reactivity was present throughout the stroma. Samples from days 16 and 18 of pregnancy showed a similar pattern of expression to the day 18 cycling heifers (Fig. 8bc). Trophoblast did not express the antigen at any of the stages examined. Expression of integrin $\alpha_v\beta_3$ in luminal epithelium was evident from days 18 to 24 of pregnancy (Fig. 8c-e). As binucleate cell migration proceeded to modify the luminal epithelium, reactivity to integrin $\alpha_v\beta_3$ decreased in the hybrid luminal epithelium and increased in the underlying stroma (Fig. 8ef and Fig. 9). Reactivity scores were always highest in intercaruncular tissues and weak, if any, staining was detected in the caruncles (Fig. 8a-c).

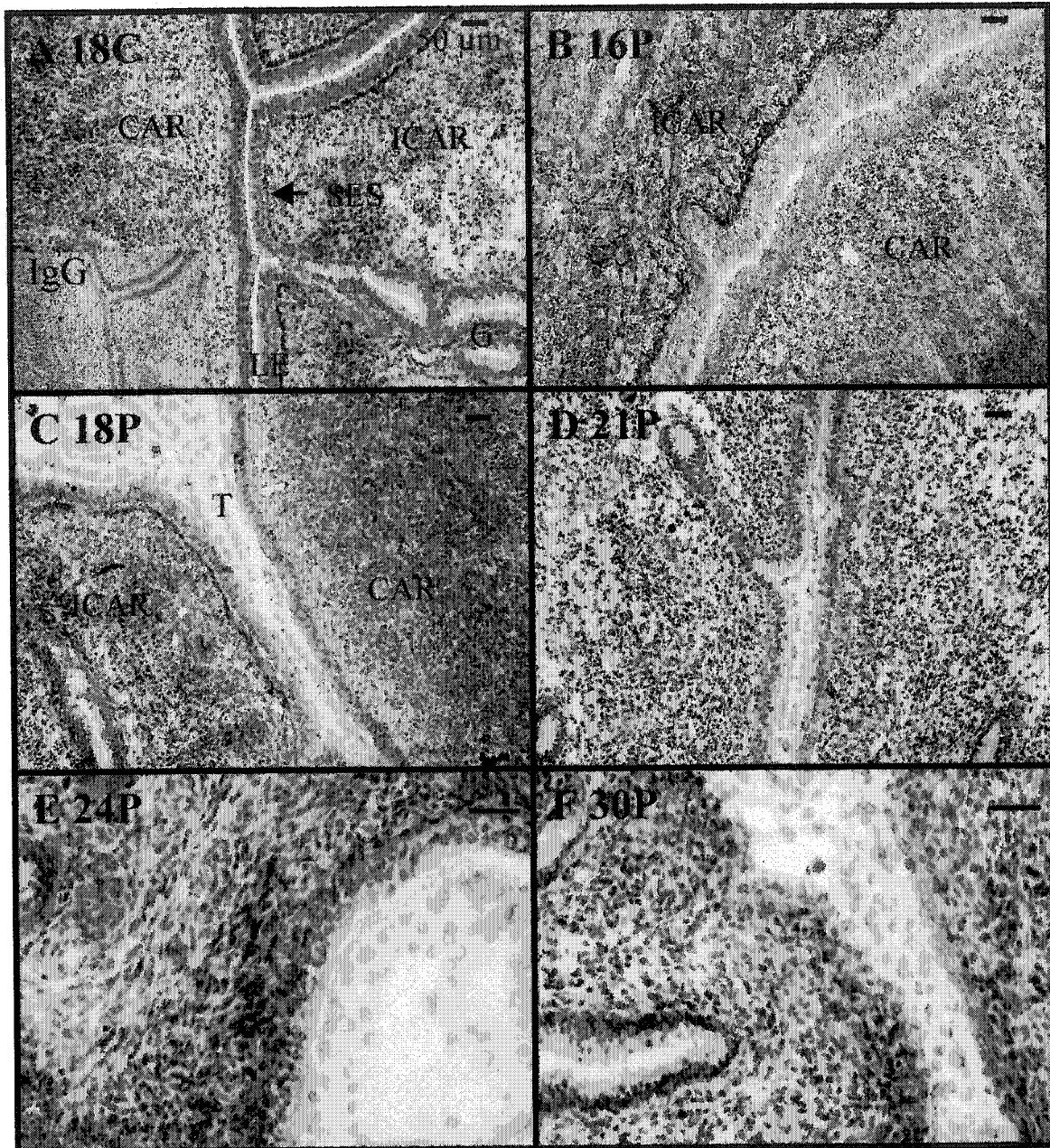


Fig. 8. Immunohistochemical localization of integrin $\alpha_v\beta_3$ in cryostat cross sections of bovine endometrial tissue during the estrous cycle (A day 18C) and at different stages of pregnancy (B day 16P, C day 18P, D day 21P, E day 24P, F day 30P). Positive staining (shown as brown) was strongest in intercaruncular (ICAR) subepithelial stroma (SES) as indicated by the arrow. Note the low reactivity in the caruncles (CAR), glands (G) and trophoblast (T). A negative control treated with mouse IgG is shown as the inset of panel A. Bar = 50 μ m. LE=luminal epithelium.

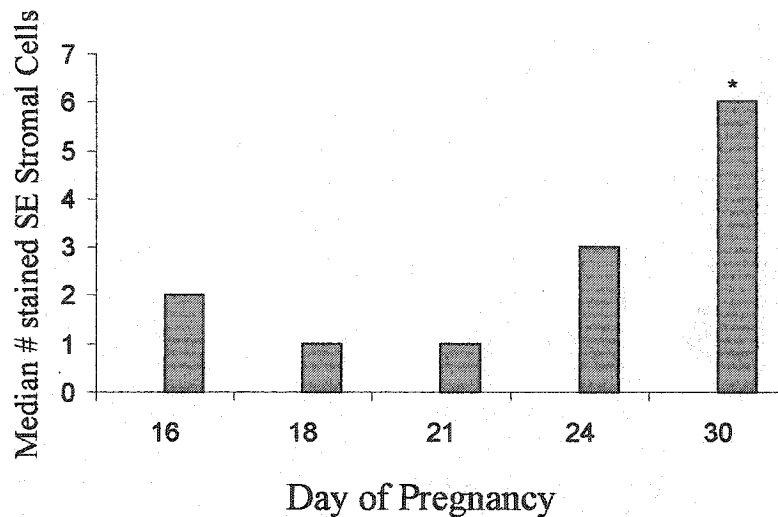


Fig. 9. Effect of pregnancy stage on the number of subepithelial (SE) stromal cells showing positive reactivity to anti- $\alpha_v\beta_3$ antibody. Acetone fixed cryostat cross sections from three cows each at days 16, 18, 21, 24 and 30 of pregnancy were used for immunolocalization of integrin $\alpha_v\beta_3$. The number of stained SE stromal cells extending away from the luminal epithelium were counted by two independent observers and analyzed to determine if there was an effect of pregnancy. Asterisk denotes significantly different from other days of pregnancy ($P < 0.05$).

Immunohistochemical Localization of Integrin $\alpha_v\beta_3$ in Ovine Endometrium.

Both monoclonal antibodies to integrin $\alpha_v\beta_3$ showed similar distribution patterns and reacted strongly with glandular epithelium and blood vessels in ovine endometrium, but did not react with luminal epithelium or trophoblast (Fig. 10). For all ewes examined, reactivity was strongest in the deep glandular epithelium and absent from superficial glands ($P < 0.05$; Fig. 10bc). Endometrium collected from one ewe on day 13 of pregnancy showed a distribution pattern that was similar to cows; there was differential staining between caruncular and intercaruncular regions (Fig. 10e). Staining in this animal appeared reduced in the subepithelial stroma regions of the caruncles in comparison to intercaruncular subepithelial stroma. Like other ewes examined there was strong reactivity in the deep glandular epithelium (data not shown).

Osteopontin Distribution in Cycling Bovine Endometrium

Osteopontin localized to the smooth muscle of the blood vessels and to the uterine epithelium of bovine endometrium throughout the estrous cycle (Fig. 11a-c). The pattern of staining suggested apical secretion of the phosphoprotein and reactivity was strongest in glandular epithelium in comparison to the luminal epithelium ($P < 0.05$; Fig. 11 and Fig. 12a). An effect of cycle stage was observed on luminal epithelium, where reactivity ranged from weak to moderate and was least evident in sections collected from late luteal phase animals ($P < 0.05$; Figs. 11b and 12a). There was no influence of cycle stage on osteopontin scores in blood vessels or glands ($P > 0.05$).

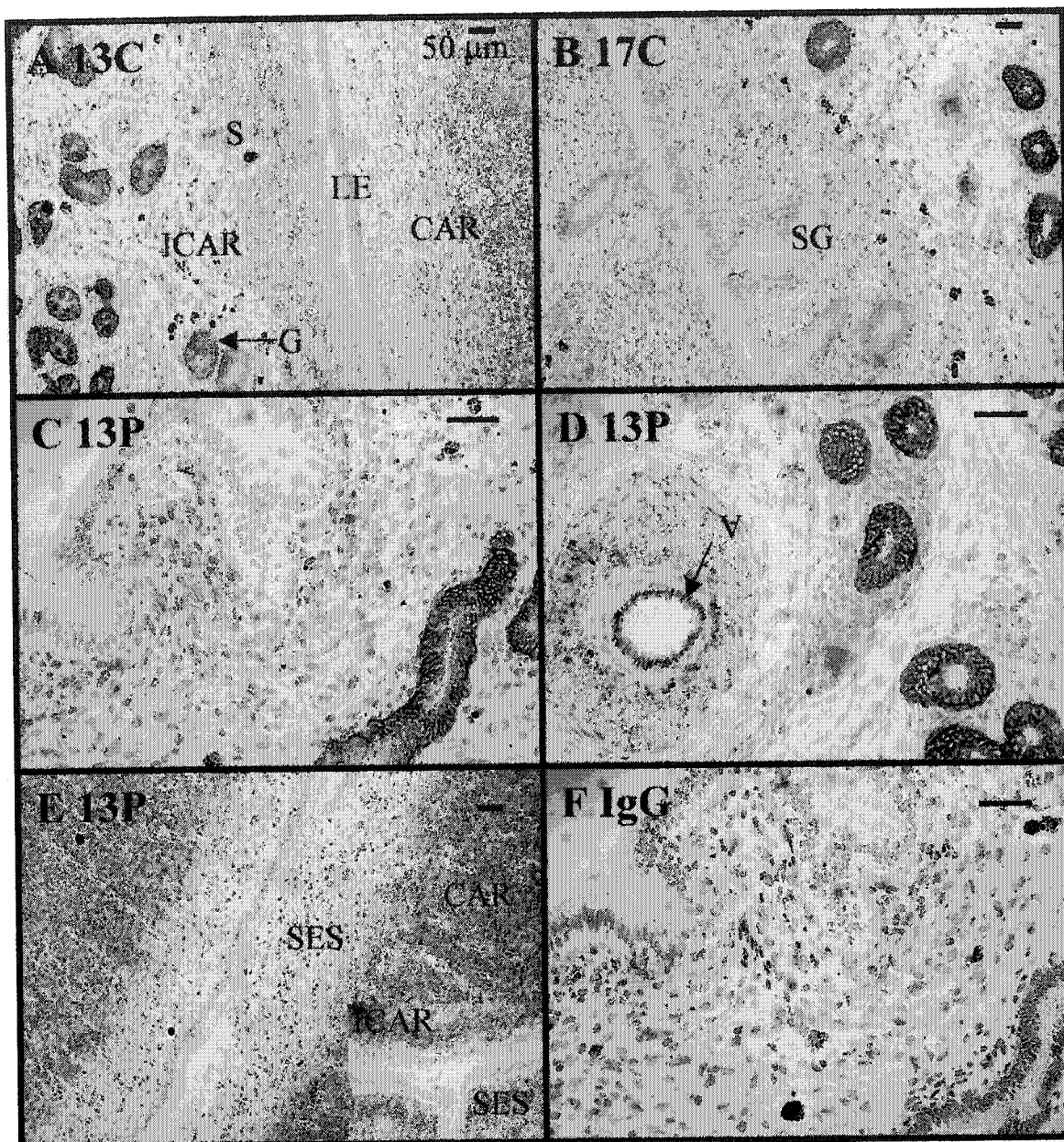


Fig. 10. Immunohistochemical localization of integrin $\alpha_v\beta_3$ in cryostat cross sections of ovine endometrial tissue during the estrous cycle (A day 13C, B day 17C) and at day 13 of pregnancy (C-E day 13P). Positive staining (brown) was strongest in the glands (G) and blood vessels (V), while no reactivity was detected in the superficial glands (SG) or luminal epithelium (LE). Note the low reactivity in caruncular (CAR) subepithelial stroma shown in E versus the intercaruncular region (ICAR) shown in the inset photo. A negative control treated with mouse IgG is shown in panel F. Bar =50 μ m.

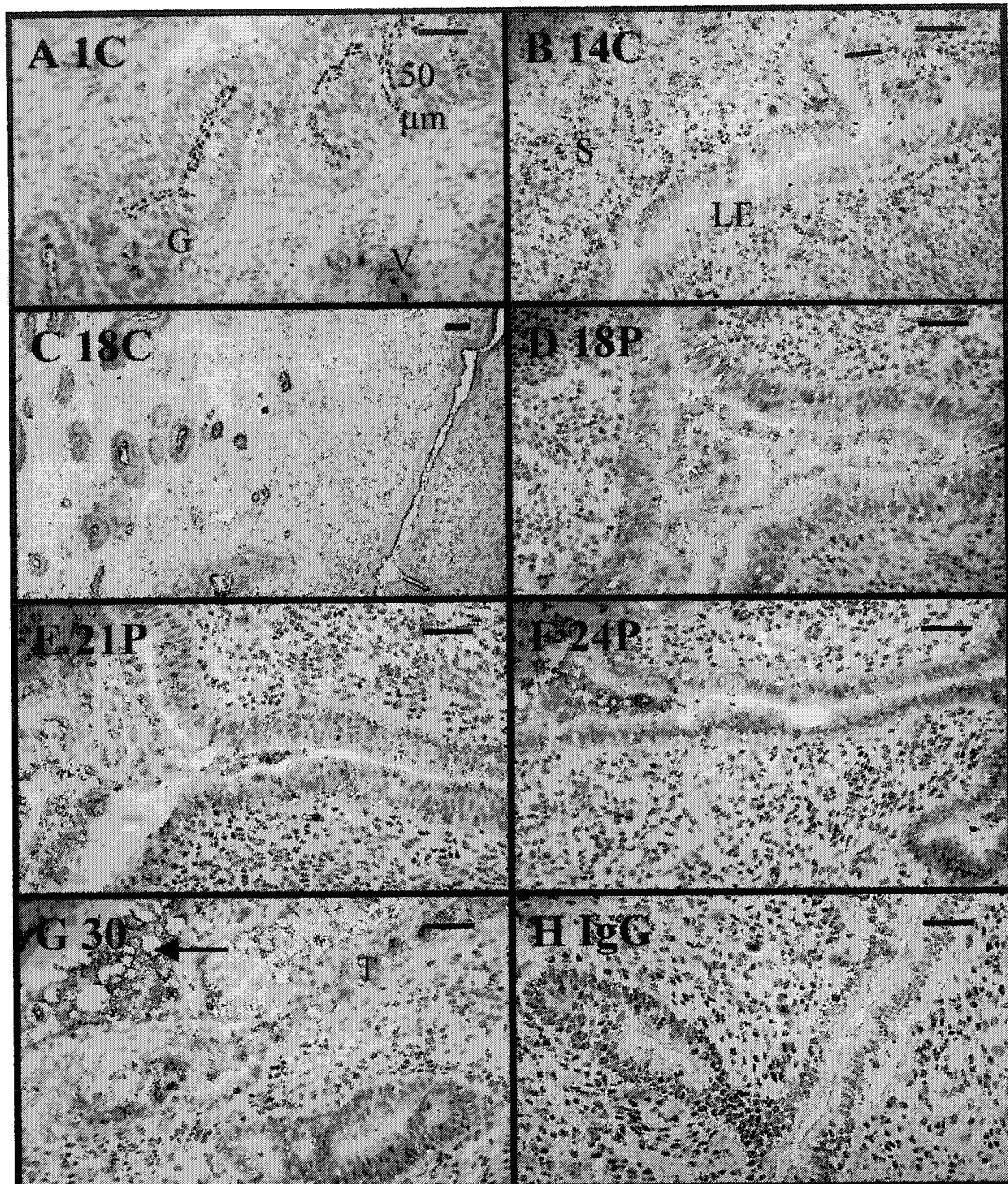


Fig. 11. Immunohistochemical localization of osteopontin in cryostat cross-sections of bovine endometrial tissue during the estrous cycle (A day 1C, B day 14C, C day 18C) and early pregnancy (D day 18P, E day 21P, F day 24P, G day 30P). Osteopontin (positive reactivity is red) was detected at the apical surface of glandular (G) and luminal epithelium (LE), associated with smooth muscle of blood vessels (V) and on extraembryonic membrane (arrow) luminal to the trophoblast (T). Reduced staining of endometrial epithelium was apparent in sections collected during late diestrus (C). A representative control treated with rabbit IgG is shown in H. Bar =50 μ m.

Osteopontin Distribution in Early Pregnant Bovine Endometrium

The distribution of osteopontin was similar in cryosections prepared from pregnant animals to those prepared from cyclic animals, although reactivity scores for the apical surface of the luminal and glandular epithelium decreased with advancing pregnancy (Fig. 11d-g). Staining was lowest in glandular epithelium in samples from day 30 of pregnancy in comparison to other pregnancy stages examined ($P < 0.05$; Fig. 11g and Fig. 12b). In many sections from day 24 and day 30 pregnant animals extraembryonic membrane, probably allantois, expressed moderate to high levels of osteopontin (Fig. 11e-g).

Distribution of osteopontin in ovine endometrium

Similar to the staining patterns observed in cows, in sheep osteopontin was immunolocalized to the apical surfaces of uterine epithelium, the smooth muscle of blood vessels, and when present, trophoblast (Fig. 13). No difference in reactivity was observed between ewes regardless of pregnancy status ($P > 0.05$).

3.4 Discussion

In pregnant bovine endometrium reactivity scores for integrin $\alpha_v\beta_3$ were always highest in the stroma underlying the luminal epithelium of intercaruncular tissues, and weak or no staining was detected in the caruncles or stroma underlying the glands. This concurs with our previous study of cycling cattle (Kimmins and MacLaren, 1999). In cows, integrin $\alpha_v\beta_3$ may be involved in constraining

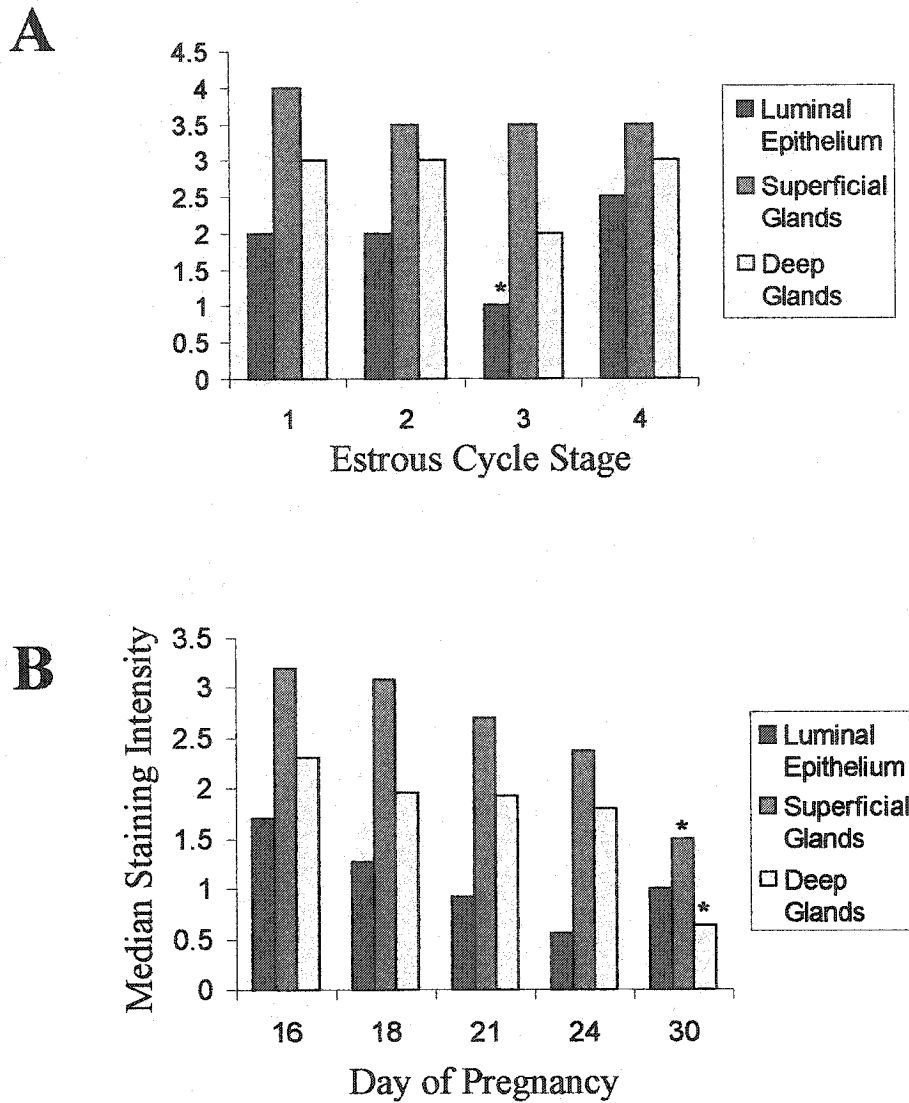


Fig. 12. Estrous cycle (A) and pregnancy (B) effects on osteopontin levels in bovine endometrium. Tissue sections from stages 1 (metestrus), 2 (early diestrus), 3 (late diestrus), and 4 (proestrus/estrus) of the estrous cycle, and from early pregnancy (days 16, 18, 21, 24 and 30) were scored by two independent observers for staining intensity of the luminal epithelium, superficial glands and deep glands. Osteopontin secretion by luminal epithelium decreased in late diestrus. As pregnancy progressed apical secretion of osteopontin in the glands decreased. Asterisk means significantly different from other stages ($P < 0.05$).

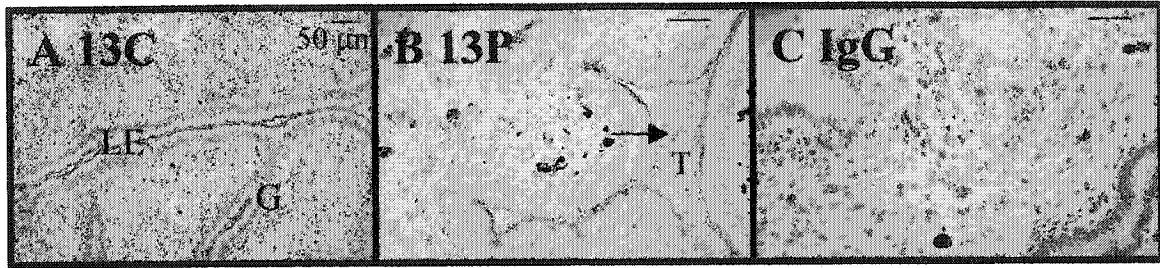


Fig. 13. Immunohistochemistry for detection of osteopontin in endometrial tissue collected from cycling (A day 13C) and pregnant (B day 13P) sheep. Moderate to strong staining for osteopontin, shown in red, was detected on glands (G), luminal epithelium (LE) and extraembryonic membrane (arrow). A representative control treated with rabbit IgG is shown in C. Bar =50µm.

trophoblast and endometrium in the intercaruncular regions, since the growth of both tissues to form the chorionic villi and intervening maternal septae occurs only in the caruncles. We hypothesize that intercaruncular subepithelial stromal integrin $\alpha_v\beta_3$ is involved in regulating the behavior of the overlying epithelium and adjacent trophoblast. Such a signal from stroma underlying the uterine epithelium is logical, since the epithelium itself is modified rapidly after attachment by trophoblast binucleate cell migration and fusion with maternal epithelium to form giant cells (Wathes and Wooding, 1980). This migration is maximal about day 24, then subsides to the extent that trinucleate, rather than giant cells, form and there is some regeneration of the luminal epithelium (Wathes and Wooding, 1980). The observation in the current study that this is preceded by increased expression of stromal integrin $\alpha_v\beta_3$ provides support for a role of this integrin in constraining invasion.

Integrin $\alpha_v\beta_3$ was absent from the subepithelial stromal region in sections from all but one ewe in this study and in all sheep endometrium examined by Johnson et al. (1999a; 2001). This difference between sheep and cattle is interesting and may be attributable to the slightly more invasive attachment process that occurs in sheep in comparison to cattle. Unlike in cattle, in sheep there is significant degeneration of luminal epithelium from migrant trophoblast binucleate cells that results in a syncytium that persists throughout pregnancy (King, 1993; Guillomot, 1995). In addition, cytoplasmic processes penetrate the basal lamina of fetomaternal hybrid epithelium to contact the underlying stroma (Guillomot, 1995).

In cattle, the transient disappearance of subepithelial integrin $\alpha_v\beta_3$ on day 16 of the cycle, but not day 16 of pregnancy suggests a possible role for this integrin in cycle regulation. Our more recent studies have shown that this temporary downregulation can be mediated by estrogen (Kimmins et al., 2003b), a known stimulator of the luteolytic mechanism (Thatcher et al., 1986), although high levels of estrogens as are observed at estrus are associated with high levels of endometrial integrin $\alpha_v\beta_3$ expression.

In this study integrin $\alpha_v\beta_3$ was absent from bovine trophoblast, was expressed at moderate intensity in the glandular epithelium at all periods examined, and in the luminal epithelium until it was modified by binucleate cell migration. In contrast, in ovine endometrium, glandular epithelium exhibited a strong pericellular expression but no reactivity was observed in the luminal epithelium. The pattern of integrin $\alpha_v\beta_3$ expression we observed in ovine endometrium was not only different from cows, but also from what has previously been reported for the ewe for the individual α_v and β_3 integrin subunits (Johnson et al., 2001a).

This earlier study in sheep immunolocalized the integrin α_v and β_3 subunits to the apical surfaces of uterine epithelium and trophoblast. Although the integrin α_v subunit has many potential partners for heterodimer formation, the β_3 subunit is only known to dimerize with α_v in non-immune cell lineages (Gille and Swerlick, 1996). Since monomeric integrin subunit protein is not normally expressed at the cell surface (Gille and Swerlick, 1996; van der Flier and Sonnenberg, 2001), this alone does not explain differences in distribution patterns between studies. It is noteworthy that no differences in the distribution pattern or levels of integrin subunits α_v and β_3

were observed between cycling and pregnant sheep in the study by Johnson et al., (2001a). Since in most species studied to date a hormone dependent distribution has been observed this is unusual (Lessey et al., 1992; Sutherland et al., 1993; Fazleabas et al., 1997; Kimmins and MacLaren, 1999). Furthermore, we were unable to demonstrate specific immunoreactivity-reactivity in sheep or bovine endometrium using the same integrin subunit α_V targeting antibody used by Johnson et al., (2001a).

Moderate but consistent osteopontin expression was observed in uterine epithelium and trophoblast of sheep and cattle. It has been proposed that osteopontin or another matrix molecule could act as a “bridge” for integrin $\alpha_V\beta_3$, connecting trophoblast and uterine epithelium during embryo attachment (Coutifaris et al., 1998; Garlow et al., 2002; Johnson et al., 1999ab; Lessey, 2002). Integrin $\alpha_V\beta_3$ is thought to be the functionally most important receptor for osteopontin in bone and vascular tissue (Faccio et al., 2002; Takano et al., 2000), and recent experiments with the Ishikawa endometrial cell line suggest that this is also true for human endometrial cells (Apparao et al., 2001). Since in this study integrin $\alpha_V\beta_3$ was not detected on both trophoblast and the apical surface of the luminal epithelium of either sheep or cattle, another integrin may be anchoring the bridge. The integrins $\alpha_V\beta_1$, $\alpha_V\beta_5$, $\alpha_4\beta_1$, $\alpha_8\beta_1$ and $\alpha_9\beta_1$, as well as CD44, can bind osteopontin (Sodek et al., 2000; Marcinkiewicz et al., 2000). Bovine trophoblast and uterine epithelium express β_1 integrins (MacLaren and Wildeman 1995; MacIntyre et al., 2002), but the alpha subunits associated with β_1 in these tissues have not been fully characterized. We have not been able to detect the integrin α_4 subunit at the fetomaternal interface

(MacLaren, unpublished), although it has been so localized in other species with surface implantation (Bowen et al., 1996). The pattern of expression of osteopontin was similar in bovine and ovine endometrium to what has been observed in other species (von Wolff et al., 2001; Apparao et al., 2001). However, unlike what has been observed in those species, the scores for osteopontin reactivity in epithelium decreased during the luteal phase in cyclic heifers. Although the human and murine promoters for osteopontin possess progesterone response elements, the role of progesterone is complex in endometrium. The downregulation we observed occurred when progesterone levels are high but expression of progesterone receptors in epithelium is low (Kimmins and MacLaren, 2001; Robinson et al., 2001). Likewise, sheep infused with progesterone showed increased osteopontin mRNA in the glands, however no progesterone receptors were expressed by these cells (Johnson et al., 2000). These experiments suggest that progesterone may act in concert with local modulators of osteopontin expression, which include hepatocyte growth factor, transforming growth factor β 1 and epidermal growth factor (Apparao et al., 2001; Malyankar et al., 1997; Lessey, 2002), via a stromal mediated paracrine mechanism to modulate osteopontin expression during the estrous cycle.

In conclusion, integrin $\alpha_v\beta_3$ in the subepithelial stromal cells of bovine endometrium may have a role in the epithelial-stromal signaling events that regulate remodeling of pregnant epithelium and trophoblast during attachment in the cow. The different patterns of expression observed in sheep endometrium indicate that at the molecular level, the signaling mechanisms between the epithelium and stroma to form the synepithelial placenta of sheep differs from those in cows. The unmatched

expression of integrin $\alpha_v\beta_3$ and osteopontin at the fetomaternal interface of sheep and cows suggest that unlike in primates and rodents, in ruminants these proteins are not acting in concert to facilitate embryo attachment.

CHAPTER 4 THE EFFECTS OF ESTROGEN AND PROGESTERONE ON PROSTAGLANDINS AND INTEGRIN BETA 3 (β_3) SUBUNIT EXPRESSION IN PRIMARY CULTURES OF BOVINE ENDOMETRIAL CELLS

4.0 Introduction

We and others have described the patterns of integrin expression in cycling and pregnant cattle (Kimmins and MacLaren, 1999) sheep (Johnson et al., 2001a; Kimmins et al., 2003a), pigs (Bowen et al., 1996), humans (Lessey et al., 1996) and mice (Sutherland et al., 1993 Illera et al., 2000). Expression of bovine integrin $\alpha_v\beta_3$ is altered during the estrous cycle and pregnancy, suggesting a role for the ovarian steroids and/or local uterine or embryonic factors in its regulation (Kimmins and MacLaren, 1999; Kimmins et al., 2003a). In cattle, it is most strongly expressed in intercaruncular subepithelial stroma from day 0 to 15 of the estrous cycle, and then is temporarily down-regulated on day 16 in cyclic (Kimmins and MacLaren 1999) but not pregnant endometrium (Kimmins et al., 2003a). Expression resumes to previous levels on day 17.

We have shown by immunohistochemistry that reactivity to ER antibody changes transiently in the luminal epithelium on day 16 of the cycle (Kimmins and MacLaren 2001). This suggests that regulation of integrin $\alpha_v\beta_3$ in bovine endometrium may be a direct or indirect result of ER action. Day 16 of the estrous cycle is significant as it marks initiation of luteolysis in the non-pregnant heifer. From day 6 to day 17, the uterine environment is dominated by ovarian progesterone produced by the corpus luteum. However, about day 16, there is a small rise in follicular estrogen which stimulates oxytocin signaling, and in turn causes

endometrial epithelial cell secretion of $\text{PGF}_{2\alpha}$, the luteolytic signal (Campbell et al., 1995; Kim and Fortier, 1995; Asselin et al., 1996; Martal et al., 1996; Bazer et al., 1997; Roberts et al., 1999).

Although there is an increasing body of information supporting a role for integrins in establishment of pregnancy there is little information regarding their regulators in the ruminant uterus. To test our hypothesis that the ovarian steroids may be involved in the cyclic regulation of integrin $\alpha_v\beta_3$ and prostaglandins, endometrial cells were isolated and treated with either estrogen, progesterone or both. Prostaglandin levels and the presence of ER and PR were examined in these cultures to relate these values to the expression of integrin subunit β_3 .

4.1 MATERIALS AND METHODS

Isolation of Intercaruncular and Caruncular Epithelial and Stromal Cells

For each experiment, three or four uteri from nulliparous mixed beef breeds, determined to be between days 1-3 of the cycle by the presence of a corpus hemorrhagicum, were collected at the local abattoir and transported back to the laboratory on ice. Epithelial and stromal cells were isolated from endometrium using a modification of methods described in Fortier et al. (1988). Briefly, excess tissue was removed from the horns which were dipped in 90% ethanol then washed in sterile Hanks balanced salt solution (HBSS) with antibiotic-antimycotic (Life Technologies; Burlington, ON, Canada). Perimetrium and myometrium were removed from the horns, which were then inverted to expose the uterine lining, and the caruncular and intercaruncular tissues were separated. Each horn was cut into

three equal size pieces, which were placed separately in 50 ml polypropylene tubes for digestion in HBSS with 0.3% trypsin (Roche Molecular Biochemicals, Laval, QC, Canada) and 1X antibiotic-antimycotic (Life Technologies). All tissues were digested overnight at 4 °C, followed by a 1 h incubation at 37 °C. After the first digestion, for retrieval of epithelial cells the horns were gently massaged with a glass rod to free any remaining epithelial cells, washed and transferred to a new tube for the stromal digest. For the stromal digest horns were incubated at 37 °C for 2 h in HBSS with 0.03% trypsin and 0.064% collagenase (ICN Biomedicals Inc., Aurora, ON, Canada). Following each digest the cells were retrieved by a series of washes and centrifugation at 500 g for 10 min. Cells were resuspended in 300 ml of phenol red-free RPMI-1640 (Life Technologies) supplemented with 1X antibiotic-antimycotic and 10% charcoal stripped fetal calf serum (Life Technologies). Epithelial cells were pre-plated for 8 hrs, and then the medium containing any unattached cells was plated in 25 cm² Corning culture flasks (Fisher Scientific, Whitby, ON, Canada). This effectively removed any contaminating stromal cells from the epithelial cell population. Stromal cells were plated for 3 h then washed 1X with HBSS to remove epithelial cells. Thereafter the medium was changed for all cultures every 48 h. Cells were grown at 37 °C in 95% air-5% CO₂. Purity of cultured cells was established to be $\geq 90\%$ by visual examination of the cell morphology and by positive (epithelial cells) or negative (stromal cells) reaction to anti-cytokeratin antibodies (Sigma Chemical Co., St Louis, MO).

Estrogen and Progesterone Treatment of Cultures

For experiment 1, monolayers of caruncular epithelial and stromal, and intercaruncular epithelial and stromal cells were grown to 90% confluence then treated with either control medium (C), water soluble estradiol-17 β (E, 0.1 nM; Sigma), progesterone (P, 1.0 nM; Sigma), or estrogen and progesterone together (E+P, P-E+P). Treatment regimes for experiment 1 are shown in Fig. 14. In experiment 2, ICAR stromal cells were treated at 100% confluence for 48 h with either control medium (C), estradiol-17 β (E, 1.0 nM; Sigma), progesterone (P, 10.0 nM; Sigma) or estrogen and progesterone (E+P). Doses of estradiol and progesterone were based on levels previously shown to stimulate (estrogen treated) or suppress (progesterone) ER in monolayers of cultured bovine endometrial cells (Xiao and Goff, 1999). Each treatment was replicated three times. At termination, medium was collected for measurement of PGE₂ and PGF_{2 α} by enzyme immunoassay (EIA) as described by Asselin et al. (1996). The interassay and intraassay coefficients of variation were 16% and 10 % respectively (n=12). Total cellular RNA was harvested with Trizol™ (Life Technologies) reagent according to the manufacturer's directions. RNA integrity was visually assessed on denaturing agarose gels.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The levels of integrin subunit β_3 and presence of ER α and PR in cell cultures were assessed by RT-PCR. Total RNA (1 μ g) was reverse transcribed using Superscript II™ (Life Technologies) and random primers (200 ng; Roche).

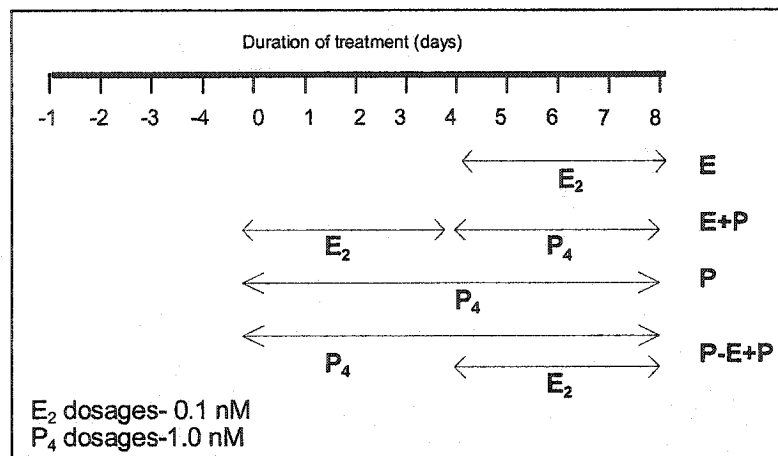


Figure 14. Experiment 1 treatments. Epithelial and stromal cells from caruncular and intercaruncular endometrium were isolated by enzymatic dispersion and cultured as monolayers. Cells were grown to 90% confluence in RPMI-1640, supplemented with 1X antibiotic-antimycotic, and 10% charcoal stripped fetal calf serum. Cultures were then treated in triplicate with estradiol-17 β (0.1 nM) and/ or progesterone (1.0 nM).

The 20 μ l reaction was incubated at 37 $^{\circ}$ C for 50 min, and 65 $^{\circ}$ C for 15 min in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 0.01 M DTT and 1 mM dNTPs (Roche). The PCR was performed using intron flanking primers to ensure any contaminating DNA, if amplified, would be a different size than the expected product. A list of primer sequences and annealing temperatures are shown in Table 1. Polymerase chain reaction was performed using 3 μ l of the RT reaction, 0.2 mM dNTPs, 1 U Taq DNA polymerase, 3 μ l 10X PCR buffer (Roche) in a total volume of 30 μ l. The identity of the PCR products was confirmed by sequence analysis after cloning into the pGEM-T vector (Amersham Pharmacia Biotech, Baie d'Urfé, PQ, Canada). There were no competing PCR products for integrin subunit β_3 or PR. A secondary transcript, presumably another isoform, was observed in ER α PCR

reactions. Known amounts of integrin subunit β_3 mRNA were amplified to ensure PCR was in the linear range (Fig. 15a). Negative controls of non-reverse transcribed samples were included in each set of reactions. Equal amounts of PCR products were run on a 1.2% agarose gel stained with ethidium bromide and the amount of integrin subunit β_3 cDNA was quantified by measuring the pixel intensity emitted from the correct size band using the Gene Genius Bioimaging System (Fisher Scientific). Each of the replicate samples underwent RT-PCR analysis at least three times.

Table 1.

Target	Genbank accession number	Primer sequence (5' - 3')	Product size (bp)	Annealing temperature
Integrin β_3	AF239959	GTGATCACTTCTCCTGTGTCC GTGTCCCATTGGCTCTG	638	56 C
ER α	X98006	ACAGATCCAAGCCAACGAG TCTCCAGGTAATAGGGCACC	952	55 C
PR	Z86041	CGTAAGCCAGAGAATCACT GACTCCTTCATCCGCTGTTC	333	55 C

Northern Blotting

Reverse transcription polymerase chain reaction results were confirmed using Northern blots. Ten μg of total RNA was electrophoresed in denaturing 1.2% agarose, 2.2 M formaldehyde gels in 1X morpholinepropanesulfonic acid (MOPS, 0.02 M, pH 7.0, 2 mM sodium acetate, 1mM EDTA pH 8.0) with appropriate markers for 4 h at 4 V/cm. Gels were blotted onto a positively charged nylon membrane (Roche) using downward capillary transfer in alkaline buffer for 1 h.

Biotin labeled riboprobes were prepared via in vitro transcription (Roche). Blots were prehybridized at 65 °C for 30 min prior to the addition of the riboprobe. The blot was probed overnight at 65 °C, then washed at 65 °C 3 times for 20 min in 2X SSC/0.1% SDS (1X SSC= 150 mM sodium chloride and 15 mM sodium citrate pH 7.0). Hybridized probe was detected using chemiluminescence performed according to the North2South™ kit protocol (Pierce Chemical Company, Rockford, IL). Intensity of integrin subunit β_3 mRNA and 18S rRNA bands were measured using the Gene Genius Bioimaging System (Fisher Scientific) and data were expressed as ratios of 18S rRNA band intensity. Each set of samples was replicated once with similar results.

Immunohistochemistry

Integrin $\alpha_v\beta_3$ protein was localized as previously described in chapter 3.1. Briefly, treated cells were fixed in acetone for 10 min, washed in PBS, blocked in 2% BSA in PBS and incubated with the monoclonal anti-human $\alpha_v\beta_3$ from Chemicon (AB1976, Temecula, CA) for 2 h, followed by signal amplification using the Vectastain Elite ABC kit® (Vector Laboratories, Burlingame, CA). Immunoreactivity was visualized using 3-amino-9-ethylcarbazole (Sigma).

For immunofluorescent localization of ER α and PR, treated cells were fixed in 4% paraformaldehyde in PBS, permeabilized with 1:1 acetone and methanol, followed by washing 3 times for 5 min in 0.2% Triton X-100 in PBS and blocking with 2% BSA in PBS for 1 h. Cells were incubated overnight with 0.4 μ g/ml of either the PR targeting antibody (MS-192, Neomarkers Inc., Union city, CA), ER α

targeting antibody (MS-168, Neomarkers Inc.), or the control, 0.4 $\mu\text{g/ml}$ purified mouse IgG (Chemicon). For immunofluorescent localization of cytokeratin, cells were fixed in acetone, washed in PBS, blocked with 2% BSA, then incubated for 2 h with 1.8 $\mu\text{g/ml}$ of anti-cytokeratin (Sigma), or the control 1.8 $\mu\text{g/ml}$ of purified mouse IgG (Chemicon). Following incubation with primary antibodies cells were incubated for 1 h with a biotinylated anti-mouse secondary (Vector Laboratories). Immunoreactive protein was detected using a fluorochrome-conjugated streptavidin probe (Alexa-488, Molecular Probes Inc.) incubated for 30 min. Staining was preserved in Fluoromount G (Electron Microscopy Sciences). Representative images were recorded using a Leica DMLB microscope (Leica Microsystems, Wetzlar, Germany) and photographed on Kodak Royal Gold film (Eastman Kodak, Rochester, NY).

Data Expression and Statistical Analysis

Prostaglandin data from experiment 1 were not normally distributed and were therefore transformed logarithmically to meet normality criteria. Effects of steroid treatment on integrin subunit β_3 expression and prostaglandin production were determined by analysis of variance using the general linear models procedure in SASTM (Statistical Analysis Software Version 8, SAS Institute Inc. Cary NC). Main effects of cell type, steroid treatment and their interactions were determined. When significant effects of treatment or cell type were observed, the Tukey-Kramer least square means test was used to determine differences between means. For analysis of

RT-PCR data, day of the reaction also was included as a factor in the model and found to be insignificant ($P \geq 0.05$). The selected α error rate was $P \leq 0.05$.

4.2 RESULTS

RT-PCR

The β_3 subunit dimerizes with α_v only and its synthesis limits the assembly of integrin $\alpha_v\beta_3$ (Cao et al., 1993). The ability of estrogen and progesterone to regulate integrin β_3 subunit mRNA expression was investigated using RT-PCR to amplify a cDNA product of 637 bp. In experiment 1, the integrin β_3 subunit mRNA was consistently detected in intercaruncular stromal cells but not in caruncular epithelial, caruncular stromal or in intercaruncular epithelial cells. In experiment 1, treatment of intercaruncular stromal cells with estrogen alone resulted in the lowest level of expression in comparison to other steroid supplemented treatments as measured by relative pixel intensity of specific RT-PCR products ($P \leq 0.01$) (Fig. 15b). Estrogen treated cultures showed 32-36% lower pixel intensity levels of β_3 cDNA than progesterone, or estrogen + progesterone treated cultures. There was no difference in levels of integrin subunit β_3 mRNA between progesterone treated, and estrogen + progesterone treated ICAR stromal cells ($P \geq 0.05$).

In experiment 2, only intercaruncular stromal cells were isolated and treated. Like in experiment 1, estrogen suppressed integrin subunit β_3 mRNA levels in experiment 2; estrogen treated cultures had 47% lower β_3 cDNA pixel intensity values in comparison to untreated controls ($P \leq 0.05$). Integrin β_3 PCR product levels were not different between controls, progesterone, and estrogen + progesterone

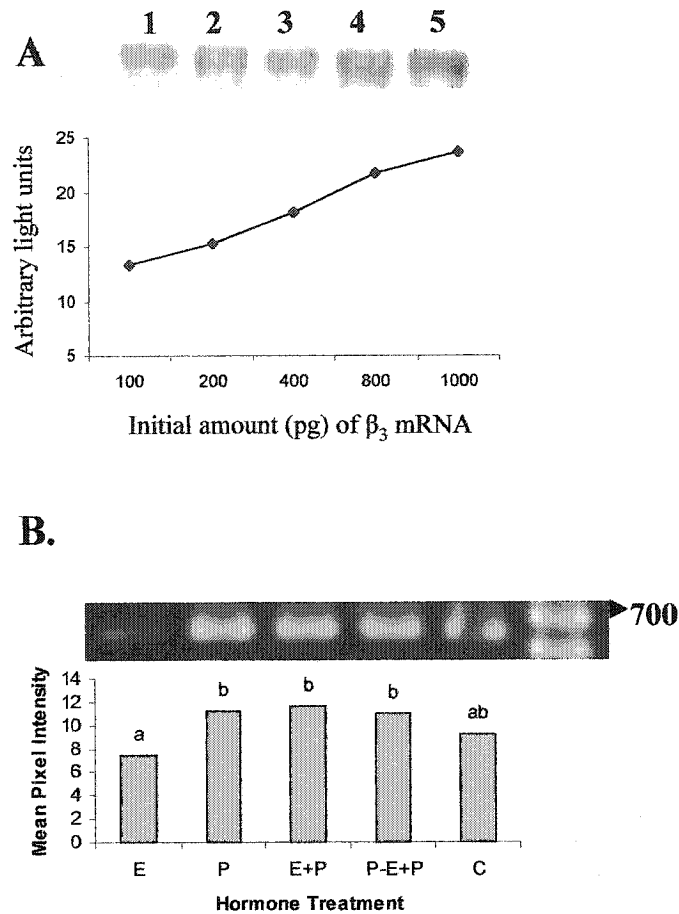


Figure 15. Reverse-transcriptase PCR of integrin β_3 subunit mRNA. **(A)** A standard curve using known concentrations of β_3 mRNA was used to establish that the PCR was within the linear range. One hundred to 1000 pg of integrin subunit β_3 mRNA was reversed transcribed and PCR amplified. Five μ l of PCR product were run on a 1.2 % agarose gel, stained with ethidium bromide and band intensity was measured using the Gene Genius Bioimaging system. There was a strong linear relationship between amount of integrin subunit β_3 mRNA and the 638 bp PCR product band intensity ($R^2=0.97$). **(B)** Shown are experiment 1 RT-PCR results for integrin subunit β_3 mRNA from intercaruncular stromal cultures treated with E_2 (E; 0.1 nM) or P_4 (P; 1.0 nM), or E+P or P-E+P or control media (C) . Statistical analysis was performed using ANOVA followed by Tukey's means comparison test; significance was set at $P \leq 0.05$. Estrogen treatment of intercaruncular stromal cells downregulated integrin subunit β_3 ($P < 0.05$).

treated cells ($P \geq 0.05$) (Fig. 16). Cells in experiments 1 and 2 under all treatments expressed ER α and PR as indicated by the presence of specific PCR products (Fig. 17).

Northern Blots

Northern blot analysis of cultured endometrial cells revealed a single 2.4 kilobase (kb) transcript for bovine integrin subunit β_3 . This transcript size corresponds to the published coding sequence for the bovine integrin subunit β_3 of 2.364 bp (Neff et al., 2000). Levels of integrin subunit β_3 transcript were lower in estrogen treated intercaruncular stromal cells than controls ($P \leq 0.05$) (Fig. 18). Confirming RT-PCR results, caruncular epithelial, caruncular stromal and intercaruncular epithelial cells were found to contain low to undetectable levels of integrin subunit β_3 .

Immunocytochemical Localization of Integrin $\alpha_v\beta_3$, Estrogen, and Progesterone Receptors

Moderate levels of estrogen and progesterone receptor proteins were detected in subpopulations of all cell types regardless of treatment, including intercaruncular stromal cells (Fig. 17). Estrogen treated intercaruncular stromal cells showed reduced staining for integrin $\alpha_v\beta_3$ in comparison to other treatments (Fig. 19).

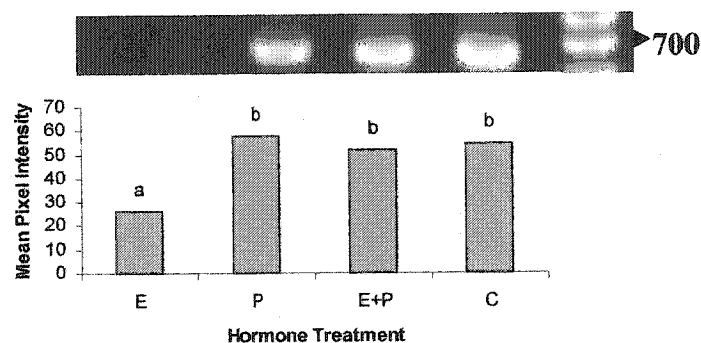


Figure 16. Experiment 2 RT-PCR results for integrin subunit β_3 mRNA from cultured intercaruncular stromal cells treated with E_2 (1.0 nM) and P_4 (10 nM) for 48 h. Statistical analysis was performed using ANOVA followed by Tukey's means comparison test; significance was set at $P \leq 0.05$. Estrogen treatment of intercaruncular stromal cells downregulated integrin subunit β_3 ($P < 0.05$).

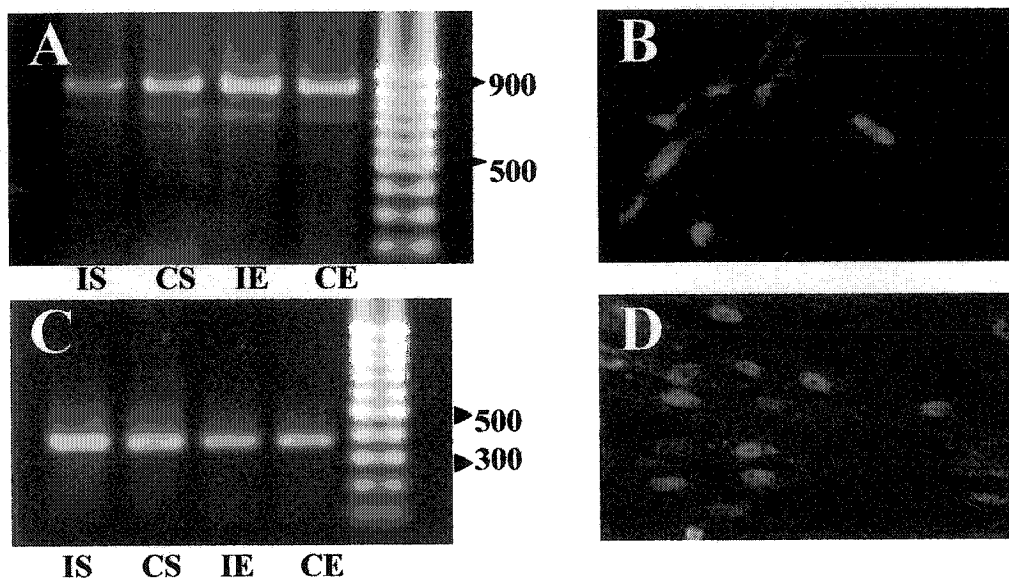


Figure 17. Detection of mRNA and protein for $ER\alpha$ and PR in bovine endometrial cell monolayers by RT-PCR and immunohistochemistry. (A) Primary (952bp) and secondary (750 bp) transcripts for $ER\alpha$ were detected in intercaruncular stroma (IS), caruncular stroma (CS), intercaruncular epithelium (IE) and caruncular epithelium (CE). (B) ER protein localized to nuclei of endometrial cells in all cultures (intercaruncular stroma shown). (C) A single PR transcript of the expected size (333 bp) was detected by RT-PCR in all four endometrial cell types. (D) Nuclei of endometrial cells reacted with PR antibodies (intercaruncular stroma shown).

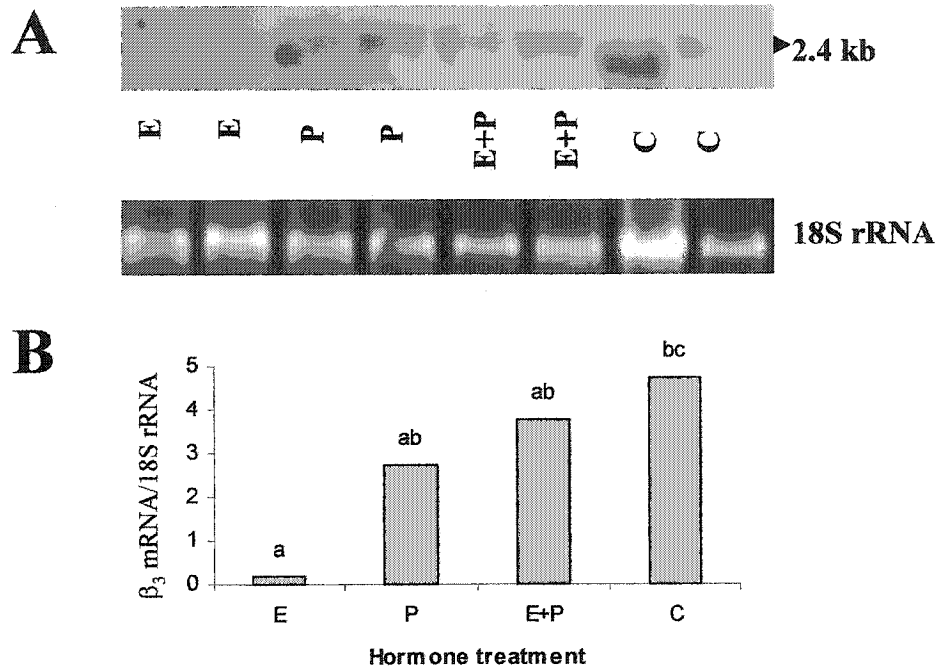


Figure 18. Northern blot analysis of integrin β_3 subunit expression in intercaruncular stromal cells from experiment 1. A single 2.4 kb transcript of the integrin β_3 subunit was detected (**A**). Shown on the graph (**B**) are the mean ratios of the integrin β_3 subunit mRNA to the 18S rRNA band intensities as measured in arbitrary light units by the Gene Genius Bioimaging System. Bars not sharing the same letter are significantly different ($P < 0.05$). Note the decreased mRNA levels in E_2 treated cells in comparison to the controls.

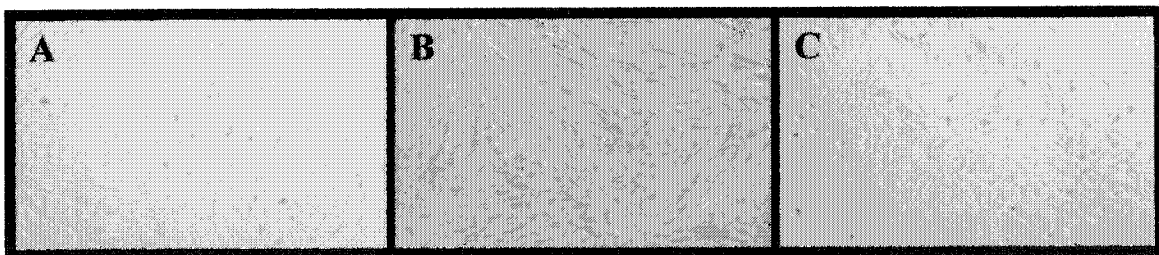


Figure 19. Immunocytochemical staining of intercaruncular stromal cells for integrin $\alpha_v\beta_3$. Estrogen treated cells (**A**) showed reduced reactivity to anti- $\alpha_v\beta_3$ in comparison to controls (**B**). Control slides treated with Mouse IgG (**C**). Magnification 100X.

Prostaglandin Accumulation in Response to Steroid Treatments

Media from stromal cells had the greatest levels of PGE₂ and contained approximately 8 times more PGE₂ than media from epithelial cells (mean raw values of 3907 µg/ml versus 475 µg/ml of supernatant respectively) ($P \leq 0.05$), with intercaruncular stromal cells secreting the most ($P \leq 0.01$). Epithelial cells accumulated approximately 2 fold more PGF_{2 α} than stromal cells (mean raw values of 590 µg/ml versus 365 µg/ml of supernatant respectively) ($P \leq 0.05$), and intercaruncular epithelial cells accumulated more PGF_{2 α} than caruncular epithelium ($P \leq 0.01$). Steroid treatment of epithelial and stromal cells in experiments 1 and 2 (results not shown) had no effect on the levels of PGF_{2 α} accumulated ($P > 0.05$) (Fig. 20).

In experiment 1, endometrial cells (excluding caruncular stromal cells) treated with estrogen (0.1 nM) secreted less PGE₂ in comparison to controls and other treatments ($P \leq 0.01$) (Fig. 20). In contrast in experiment 2, short term estrogen (1.0 nM) and progesterone (10.0 nM) treatment of intercaruncular stromal cells had no effect on prostaglandin production (results not shown).

4.3 DISCUSSION

This is the first study to examine steroidal regulation of integrin subunit β_3 using primary cultures of endometrial cells from a species with non-invasive placentation. In these experiments estrogen down-regulated integrin subunit β_3 mRNA levels in intercaruncular endometrial stroma. In several cell lines, estrogen treatment and ER α signaling have reduced expression of integrin $\alpha_v\beta_3$; for example,

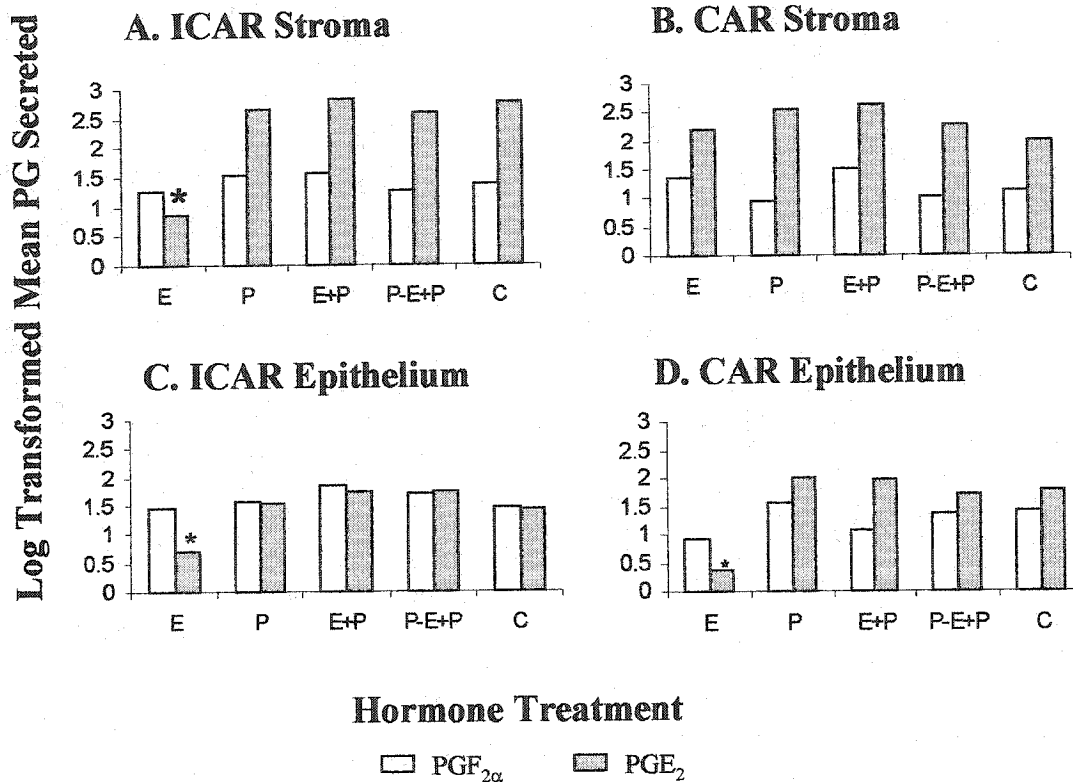


Figure 20. Effect of steroid treatment on prostaglandin accumulation in cultured monolayers of bovine endometrial epithelial and stromal cells from intercaruncular and caruncular regions in experiment 1. Data are presented as the least square means of the log transformed values and were analyzed by ANOVA followed by Tukey's means comparison to determine the effect of treatments. There was no effect of treatment on PGF_{2α} synthesis in any cell type ($P \geq 0.05$). Estrogen decreased PGE₂ synthesis in intercaruncular stromal cells, intercaruncular epithelium and caruncular epithelium ($P \leq 0.05$). Asterisk denotes difference in least square means in hormone treatment

bone resorption by cultured osteoclasts was blocked by treatment with estradiol, which caused a reduction in the number of cells that expressed integrin $\alpha_v\beta_3$ (Sarma et al., 1998). Human endothelial cells that overexpress ER α have lower levels of integrin $\alpha_v\beta_3$ (Ali et al., 2001). Similar to results presented here, Castlebaum et al. (1997) and Widra et al. (1997) showed suppression of integrin $\alpha_v\beta_3$ by estrogen in endometrial adenocarcinoma cells. In contrast, Grossinsky et al. (1996) and Sillem et al. (1997) were unable to demonstrate steroid regulation of integrin $\alpha_v\beta_3$ in primary cultures of human cells.

Relative to control cultures, there was a less pronounced effect of estrogen on integrin subunit β_3 expression in experiment 1 than in experiment 2. However in experiment 1, estradiol concentration was 10X lower and the time in culture longer resulting in a slowing of growth which could correlate with a loss of expression, as suggested by the lower mean pixel intensities detected in these cultures. A similar phenomenon was documented in a culture system employing embryonic fibroblasts, where proliferating cells expressed integrin subunit β_3 , but at confluence expression was lost (Bates et al., 1991). Alternatively, the inhibitory effect of estrogen may have lessened due to downregulation of its receptors after the longer exposure periods of experiment 1 (Xiao and Goff 1999).

The results presented here are consistent with what we know of integrin $\alpha_v\beta_3$ distribution and regulation in the bovine uterus. Expression of integrin subunit β_3 mRNA was low in epithelial cells and in caruncular stromal cells. In vivo, expression of integrin $\alpha_v\beta_3$ is greatly reduced in the caruncles in comparison with intercaruncular regions and little integrin $\alpha_v\beta_3$ is present in luminal epithelium

(Kimmins and MacLaren, 1999). There is no cyclic regulation in these tissues and none was shown in culture. Apparently these cells maintain their phenotype in culture as low integrin $\alpha_v\beta_3$ expressers.

Our prostaglandin data confirm previous studies; *in vitro* the luteolytic prostaglandin $\text{PGF}_{2\alpha}$ is preferentially synthesized by epithelial cells and the luteoprotective PGE_2 by stromal cells (Betts and Hansen 1992; Kim and Fortier, 1995; Asselin et al., 1996). The 0.1 nM estradiol-17 β dose administered to endometrial cells over a four day period decreased PGE_2 synthesis (experiment 1). However, estrogen administered at a 10 fold higher dose for 2 days (experiment 2) had no effect, which is in agreement with prior studies (Asselin et al., 1996). This suggests changes in steroid sensitivity of cells with time in culture and/or estradiol dose.

The ruminant luteolytic mechanism is estrogen dependent. Destruction of growing follicles prevents luteolysis and exogenous administration of estradiol enhances pulsatile release of $\text{PGF}_{2\alpha}$ (Villa-Godoy et al., 1985; Thatcher et al., 1986). However, in the present study steroid treatment of epithelial cells did not affect levels of $\text{PGF}_{2\alpha}$. It has previously been shown that cultured epithelial cells increase $\text{PGF}_{2\alpha}$ production in response to estrogen in the presence of oxytocin (Asselin et al., 1996). Perhaps the dose used in the current study was too low to elicit an effect or the exposure period was too long. However, estrogen treatment did decrease PGE_2 secretion in the long-term cultures in experiment 1. A possible explanation could be that stromal cells are more sensitive to steroid regulation in terms of prostaglandin synthesis than epithelial cells. Alternatively epithelial cells may require an

instructive signal from the underlying stroma or require oxytocin to increase $\text{PGF}_{2\alpha}$ production.

Integrin regulation is the result of complex cell-cell dialogue involving growth factors, steroids, and components of the ECM (Kim and Yamada, 1997). In cycling cows, expression of integrin $\alpha_v\beta_3$ is strongest in a subpopulation of stromal cells in contact with the ECM of the luminal epithelium. These stromal cells are likely to be involved in a paracrine feedback loop with the overlying epithelium. On cycle day 16, the luminal epithelium begins to synthesize and secrete prostaglandins, an event that may be influenced by stromal cell expression of integrin $\alpha_v\beta_3$ or vice versa. Asselin et al. (1998) showed that intercaruncular endometrium was the preferred target of oxytocin stimulated prostaglandin synthesis, consistent with our observed pattern of integrin $\alpha_v\beta_3$ and prostaglandin regulation. Interaction between prostaglandins, estrogens and integrin $\alpha_v\beta_3$ has been well documented in tissue culture models of bone resorption. Osteoclast expression of integrin $\alpha_v\beta_3$ is increased by PGE_2 and decreased by estrogen (Holt and Marshall 1998; Duong et al., 2000). Furthermore, integrin $\alpha_v\beta_3$ activation stimulates cyclooxygenase-derived prostaglandin synthesis in coronary arterioles (Hein et al., 2001). Our *in vivo* and *in vitro* results to date suggests that there is coincidental regulation of estrogen, integrin $\alpha_v\beta_3$ and prostaglandins in bovine endometrium, although the nature of this relationship is not yet clear.

In vitro models can not precisely simulate the *in vivo* environment, however they do allow for study of the individual parameters of complex systems. In our culture system, like *in vivo*, intercaruncular stromal cells were the most sensitive to

estrogen effects in terms of integrin subunit β_3 regulation and prostaglandin production. The role of estrogen-mediated downregulation of integrin $\alpha_v\beta_3$ and its relationship to endometrial prostaglandin synthesis remains to be determined.

CHAPTER 5 PROSTAGLANDINS BUT NOT INTERFERON-TAU AFFECT INTEGRIN $\alpha_v\beta_3$ EXPRESSION IN BOVINE ENDOMETRIAL CELLS

5.0 Introduction

The signaling pathways that establish and maintain pregnancy in the bovine uterus involve a multitude of factors that include IFN- τ , prostaglandins, growth factors, cytokines, and steroid hormones (Hansen et al., 1999; Demmers et al., 2001; Mann and Lamming, 2001). There is a growing body of evidence suggesting membrane receptors such as integrins are not only regulated by these paracrine and endocrine factors, but that they act in concert to enhance or block their signaling cascades (Coutifaris et al., 1998; Bowen and Hunt, 2000; Puri et al., 2000; Miranti and Brugge, 2002; Schwartz and Ginsberg, 2002). In cattle, early embryonic loss is highest during the preimplantation period, days 8 to 17, and coincides with maternal recognition of pregnancy (Sreenan and Diskin, 1986).

The principal prostaglandins produced in the bovine uterus are the luteolytic PGF $_{2\alpha}$ and the luteoprotective PGE $_2$ (Hansen et al., 1999; Mann and Lamming, 2001; Thatcher et al., 2001). On day 16 of the estrous cycle, in the absence of a conceptus, low levels of estrogen from the pre-ovulatory follicle trigger a signaling cascade that initiates oxytocin induced episodic pulses of PGF $_{2\alpha}$ lasting for 36 hours, causing luteolysis and a return to estrus (reviewed in McCracken et al., 1999). Interferon-tau blocks luteolysis by preventing pulsatile PGF $_{2\alpha}$ secretion and may modify the prostaglandin ratio in favor of PGE $_2$ (Roberts et al., 1992; Godkin et al., 1997; Hansen et al., 1999). There is a developing concept that production of PGE $_2$ is important for maternal recognition of pregnancy. An interesting possibility is that

stromal cell synthesis of PGE₂ is increased by IFN- τ , which may act to alter or stabilize the levels of key enzymes in prostaglandin synthesis (Asselin et al., 1997ab; Xiao et al., 1998; Charpigny et al. 1997; Charpigny et al., 1999; Asselin and Fortier, 2000).

In pregnancy, expression of integrin $\alpha_v\beta_3$ increases in intercaruncular subepithelial stroma from days 24-30 as attachment proceeds (Kimmins et al., 2003a). Changes in integrin $\alpha_v\beta_3$ expression in the non-pregnant animal coincide with endocrine and paracrine events associated with the return to estrus (Kimmins and MacLaren 1999). Specifically, epithelial cells basally secrete PGF_{2 α} (Asselin et al., 1996; Newton et al., 1998), which may affect underlying stromal cell expression of integrin $\alpha_v\beta_3$ as these cells are in close contact with the epithelium via integrin mediated adhesion to the extracellular matrix (Jamora and Fuchs, 2002). In contrast, on day 16 of pregnancy, IFN- τ may sustain integrin $\alpha_v\beta_3$ levels in the subepithelial intercaruncular stroma directly or by modulating cell production of prostaglandins.

The rate limiting subunit in integrin $\alpha_v\beta_3$ heterodimer assembly is integrin subunit β_3 (Cao et al., 1993), and in numerous cell systems it can be regulated by estrogen, progesterone, prostaglandins, growth factors and nonsteroidal anti-inflammatory drugs (reviewed in Kim and Yamada, 1997; Simón et al., 1997; Somkuti et al., 1997; Duong et al., 2000; Dormond et al., 2001). Based on our previous studies the hypothesis was that prostaglandins and IFN- τ may regulate integrin $\alpha_v\beta_3$ during luteolysis and pregnancy recognition in the bovine uterus. Objectives of the present study were to determine whether IFN- τ , PGE₂ and PGF_{2 α} affect the levels of integrin subunit β_3 mRNA in primary cultures of bovine

intercaruncular stromal cells using semi-quantitative RT-PCR and immunohistochemistry.

5.1 Materials and Methods

Isolation of Intercaruncular Stromal Cells

Intercaruncular stromal cells were isolated by enzymatic dispersion from 3 heifer per experiment determined to be at the metestrus stage of the cycle by the presence of a corpus hemorrhagicum. Refer to chapter 4.1 for cell isolation procedures.

Treatment of Cultures

Monolayers of intercaruncular stromal cells were grown to 100% confluence which takes four to six days, then treated with either control medium, PGF_{2α} (14 nM; Sigma) PGE₂ (14nM; Sigma), or recombinant ovine IFN-τ (50 ng μl⁻¹) (kindly provided by Drs. F. W. Bazer and T. E. Spencer, Texas A & M University, College Station, Texas) for 48 h. All treatments were carried out in triplicate and the experiment was replicated once. At termination, total cellular RNA was harvested with Trizol™ (Life Technologies) reagent according to the manufacturers directions.

Semi-Quantitative RT-PCR

Levels of integrin subunit β₃ mRNA were measured by semi-quantitative RT-PCR as described in chapter 4.1. Each sample was analysed four times on four separate days.

Immunohistochemistry

Integrin $\alpha_v\beta_3$ was immunolocalized in cultures of intercaruncular stromal cells as described in chapter 4.1. At least three coverslips per treatment were examined.

Statistical Analysis

Effects of treatment on integrin subunit β_3 expression were determined by analysis of variance using the general linear model procedure in SASTM and the Tukey-Kramer least square means test to determine differences between treatments (Statistical Analysis Software Version 8, SAS Institute Inc. Cary, NC). For analysis of RT-PCR data, day of the reaction was included as a factor in the model and found to be insignificant ($P \geq 0.05$). The selected α error rate was $P \leq 0.05$.

5.2 Results

Semi-Quantitative RT-PCR

Stromal cells from the intercaruncular regions of the uterus were grown as monolayers to confluence and treated with either $\text{PGF}_{2\alpha}$, PGE_2 , or $\text{IFN-}\tau$ to determine if these uterine paracrine factors could regulate integrin subunit β_3 . Prostaglandins stimulated integrin subunit β_3 expression in intercaruncular stromal cells. Analysis of total RNA by RT-PCR from $\text{PGF}_{2\alpha}$ and PGE_2 treated cells showed higher levels of β_3 mRNA in comparison to controls as measured by relative pixel intensity of the specific RT-PCR products ($P < 0.05$) (Fig. 21). In contrast, expression of integrin subunit β_3 in $\text{IFN-}\tau$ treated cells did not differ from the basal levels

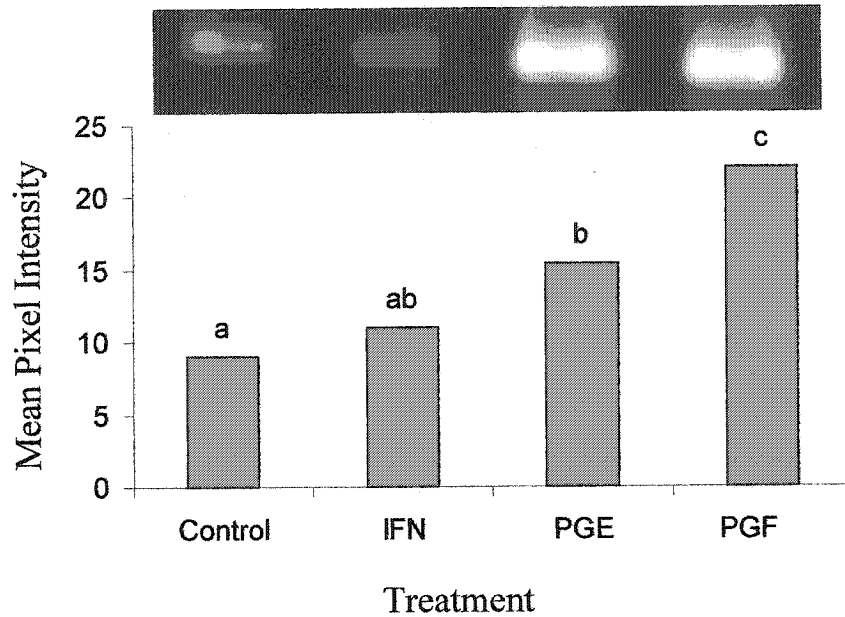


Figure 21. Reverse-transcriptase PCR of integrin β_3 subunit mRNA from cultured intercaruncular stromal cells treated with control media, IFN- τ ($50 \text{ ng } \mu\text{l}^{-1}$), PGE $_2$ ($5 \text{ } \mu\text{g ml}^{-1}$) or PGF $_{2\alpha}$ ($5 \text{ } \mu\text{g ml}^{-1}$) for 48 h. Statistical analysis was performed using ANOVA followed by Tukey's means comparison test; significance was set at $P \leq 0.05$. Prostaglandin treatment of intercaruncular stromal cells increased integrin subunit β_3 in comparison to controls ($P < 0.05$).

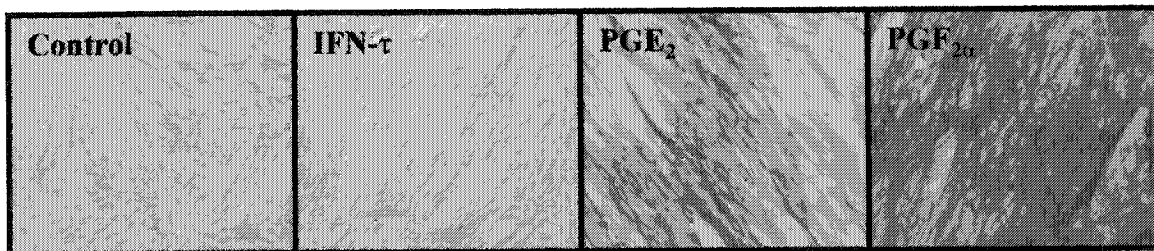


Figure 22. Immunocytochemical staining of intercaruncular stromal cells for integrin $\alpha_v\beta_3$. Prostaglandin treated cells showed increased reactivity to anti- $\alpha_v\beta_3$ in comparison to control and IFN- τ treated cells. For comparative background staining refer to the mouse IgG control shown in Figure 19. Magnification 100X.

observed in the controls as shown by the similar levels of pixel intensity ($P>0.05$) (Fig. 21).

Immunocytochemical Localization of Integrin $\alpha_v\beta_3$

Treatment of intercaruncular stromal cells with $\text{PGF}_{2\alpha}$ and PGE_2 increased integrin $\alpha_v\beta_3$ protein in comparison to controls as indicated by the intense cytoplasmic staining (Fig. 22). In contrast no difference in staining intensity was observed between IFN- τ treated cells and controls (Fig. 22)

5.3 Discussion

This study demonstrates that prostaglandin treatment in cultured intercaruncular stromal cells stimulates integrin subunit β_3 expression. Recently a link has been established between cancerous tumor growth, integrin $\alpha_v\beta_3$ and COX-2, the rate limiting enzyme in arachidonic acid metabolism to prostaglandins. Treating endothelial cells with nonsteroidal anti-inflammatory drugs to block COX-2 suppressed integrin $\alpha_v\beta_3$ and inhibited tumor growth (Dormond et al., 2001). This indicates that by blocking COX-2 and subsequent prostaglandin production, upregulation of integrin $\alpha_v\beta_3$ is prevented.

In cultured bovine uterine cells, intercaruncular stromal cells are the primary producers of endometrial PGE_2 and epithelial cells predominantly produce $\text{PGF}_{2\alpha}$ (Betts and Hansen, 1992; Danet-Desnoyers et al., 1994; Kim and Fortier, 1995; Asselin et al., 1997c; Kimmins et al., 2003b). The $\text{PGF}_{2\alpha}$ upregulation of integrin subunit β_3 that occurred in these cultures suggests that the temporary downregulation

observed in cycling animals on day 16 occurs prior to luteolytic $\text{PGF}_{2\alpha}$ production. Cellular changes leading up to luteolysis are mediated by estrogen, and concurrent with decreased integrin $\alpha_v\beta_3$ and include upregulation of COX-2 and a change in ER in luminal epithelium (Boos, 1998; Kimmins and MacLaren 2001; Arosh et al., 2002). Estrogen is required for initiation of luteolysis (Thatcher et al., 1986) and in culture estrogen treated intercaruncular stromal cells downregulate integrin subunit β_3 (Kimmins et al., 2003). We suggest that on estrous cycle day 16, integrin $\alpha_v\beta_3$ is downregulated by the small rise in estrogen from the pre-ovulatory follicle. The subsequent basal secretion of $\text{PGF}_{2\alpha}$ by epithelial cells (Asselin et al., 1996; Newton et al., 1998) on day 17 reverses the negative estrogen effect so expression of integrin $\alpha_v\beta_3$ resumes. Furthermore, integrin $\alpha_v\beta_3$ may have a role in the generation of the luteolytic mechanism as its downregulation is coordinated with initial cellular events that precede luteolysis. Downregulation of integrin $\alpha_v\beta_3$ could alter the ECM, signaling of growth factor receptors and/or sequestering of growth factors in the matrix (Jamora and Fuchs, 2002; Schwartz and Ginsberg, 2002). Any of these actions could in turn alter the epithelial cell phenotype permitting epithelial cell differentiation into the $\text{PGF}_{2\alpha}$ secreting phenotype.

Interferon-tau has been localized exclusively to trophoblast cells of unattached bovine embryos from days 11-24 (Morgan et al., 1993), and alters the uterine environment to one favoring pregnancy through interaction with endometrial receptors (Godkin et al., 1984; Hansen et al., 1999). In this experiment, expression of integrin subunit β_3 was not altered by treatment with IFN- τ , but was increased by treatment with PGE_2 . It may be that maintenance of integrin $\alpha_v\beta_3$ on day 16 of

pregnancy in the subepithelial stroma is an indirect action of IFN- τ , if in pregnant cows IFN- τ causes a shift in prostaglandin production to increase the PGE₂:PGF_{2 α} ratio. In these experiments, IFN- τ did not affect integrin subunit β_3 expression directly. Perhaps this is due to an inability of IFN- τ to affect prostaglandin synthesis in culture. In studies by Meyer et al. (1996), and Danet-Desnoyers et al. (1994), epithelial and stromal cells treated with bovine IFN- τ secreted levels of PGF_{2 α} and PGE₂ that were not different from controls. In contrast, intrauterine infusion of IFN- τ attenuated PGF_{2 α} secretion in explant cultures (Meyer et al., 1996). Experiments *in vitro* that have shown effects of IFN- τ on prostaglandin levels (Salamonsen and Findlay 1990; Newton et al., 1998) used tissue from which the cells or explants were previously exposed to progesterone. Since COX-2 expression is upregulated by progesterone and correlates with increased PGE₂ (Charpigny et al., 1997; Charpigny et al., 1999), perhaps an effect of IFN- τ on integrin subunit β_3 expression would have occurred had the cells been pre-exposed to progesterone.

In conclusion the results suggest a further role for PGE₂ in maternal recognition of pregnancy given that maintained expression of integrin $\alpha_v\beta_3$ in the subepithelial stroma is required for pregnancy establishment in cattle, and the possibility that integrin $\alpha_v\beta_3$ has a role in the events preceding luteolysis.

CHAPTER 6 THE EFFECTS OF ESTROGEN, ITS ANTAGONIST ICI 182, 780 AND INTERFERON- TAU ON THE EXPRESSION OF ESTROGEN RECEPTORS AND INTEGRIN $\alpha_v\beta_3$ ON CYCLE DAY 16 IN BOVINE ENDOMETRIUM

6.0 Introduction

Day 16 of the bovine estrous cycle is critical as it is the last day for embryo transfer and marks the generation of the luteolytic signal in the absence of a viable conceptus (Betteridge 1980; McCracken et al. 1999). We have identified two potential molecular markers of the day 16 uterine environment: the adhesion and signalling molecule, integrin $\alpha_v\beta_3$ and the estrogen receptor (ER) (Kimmins and MacLaren 1999; Kimmins and MacLaren 2001).

In comparison to other domestic animals (Bowen et al., 1997; Johnson et al., 2001), preferential expression of integrin $\alpha_v\beta_3$ in the stromal endometrium is unique to cattle (Kimmins and MacLaren, 1999). There is little expression in luminal epithelium and the stroma of the caruncles, the endometrial sites where the maternal component of the placenta will develop. Its expression is strongest in the periluminal stroma in cells in contact with the basal lamina of luminal epithelium. Downregulation of integrin $\alpha_v\beta_3$ in subepithelial stroma occurs on day 16 of the estrous cycle, but not pregnancy (Kimmins and MacLaren 1999; Kimmins et al., 2003a). This downregulation coincides with the onset of luteolysis and a transient change in the ER in the luminal epithelium (Kimmins and MacLaren 1999; Kimmins and MacLaren 2001). In support of estrogen regulation of integrin $\alpha_v\beta_3$ in bovine endometrium, cultured endometrial cells change their expression of the rate limiting

integrin subunit, β_3 , in response to estrogen (Cao et al., 1993; Somkuti et al., 1997; Kimmins et al., 2003b).

In sheep, infusion of IFN- τ blocks upregulation of ER α and oxytocin receptor expression in the luminal epithelium, a mechanism that is thought to suppress luteolysis (Spencer and Bazer, 1996). In cattle, the effects of IFN- τ on ER α in the luminal epithelium at maternal recognition of pregnancy are unknown. There are differences in the uterine location and timing of ER α expression between sheep and cows, and between research groups (Spencer and Bazer 1995, Kimmins and MacLaren 1999; Robinson et al., 2001). The results reported by Robinson et al. (2001) show a different ER α protein distribution during the bovine estrous cycle and pregnancy than what we have previously reported. In their study ER α protein was low to undetectable in the luminal epithelium throughout the estrous cycle and pregnancy. In contrast, using a different ER α targeting antibody (AER314), we detect ER α in the luminal epithelium only on day 16 of the estrous cycle, and not in endometrium from pregnant cows. The differences in results between our study and Robinson et al. (2001) are likely due to differences in antibody reactivity and/or fixation and detection methods.

The anti-estrogen, ICI 182, 780 is classified as a pure antiestrogen due to its lack of estrogen-like activity. ICI 182,780 acts by binding ER, which causes disassociation of receptor associated proteins, resulting in impaired receptor dimerisation, and increased receptor degradation (Wakeling 2000). *In utero* ICI 182, 780 blocks the trophic action of estrogen in rodents, women and pigs (Tarleton et al.,

1999; Wakeling, 2000). The effects of ICI 182, 780 in bovine endometrium have not yet been tested.

In the present study our objectives were to investigate the following hypotheses: 1) transient changes in ER α in the luminal epithelium and downregulation of integrin $\alpha_v\beta_3$ on day 16 of the estrous cycle are dependent on estrogen action, 2) at the time of maternal recognition of pregnancy IFN- τ alters ER α in the luminal epithelium and 3) IFN- τ prevents downregulation of integrin $\alpha_v\beta_3$ on day 16 of pregnancy.

6.1 Materials and Methods

Animals

This study was performed using 12 beef heifers of mixed breeds of similar age and weight (1.5-2 years; 520 \pm 31 kg) and exhibiting estrous cycles of 18-20 days. Heifers were checked for estrus twice daily throughout the experiment and trained for tie stalls one month prior to uterine infusions to adjust them to handling and confinement. Animals were synchronized to estrus using Estrumate® (500 μ g cloprostenol, Schering Canada Inc.; QUE, Canada), according to the synchronization schedule shown in Figure 23. All procedures performed were in accordance with the guidelines of the Canadian Council on Animal Care and were reviewed and approved by the Nova Scotia Agricultural College Animal Care and Use Committee.

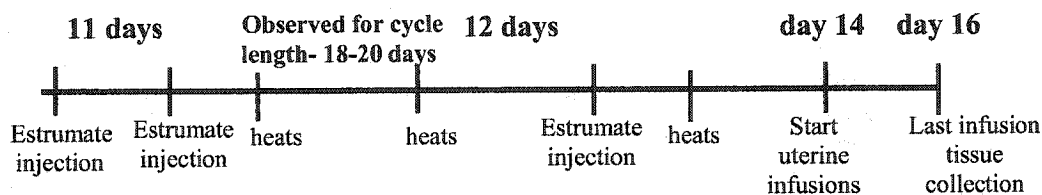


Figure 23. Synchronization schedule, heat detection and treatment regimes for heifers used in the study. Heifers were injected with Estrumate[®] 11 days apart and observed for heats after the second injection. All heifers were observed in standing heat within the same twelve hour period then observed for one cycle length of 19 ± 1 days. Twelve days after non-induced estrus, heifers were injected a third time and observed for heats. At 6 pm, fourteen days following standing heat treatment began. Heifers received four uterine infusions 12 hr apart of either saline (control), IFN- τ , estrogen or ICI 182,780. At 2 pm on day 16 of the estrous cycle, heifers were slaughtered and tissues were collected for analysis.

Treatments

Following induced estrus, 3 heifers per treatment were randomly assigned to receive uterine infusion of a control solution (0.1% BSA in saline), or estradiol-17 β (117 ng/dose; Sigma; Saint Louis, MO), or ICI 182, 780 (3mg/dose, Tocris; Avonmouth, Bristol, United Kingdom) or recombinant ovine IFN- τ (0.25 mg/dose=biological activity of 5×10^7 antiviral units/day; kindly provided by Drs. T. E. Spencer and F.W. Bazer, Texas A & M University, College Station, TX), from days 14 to 16 of the estrous cycle. The dose of estradiol was based on physiological levels, where the maximal venous-arterial difference in estradiol-17 β was multiplied by the average daily uterine arterial blood flow measured for cows on days 13-17 of

the estrous cycle (Ford and Chenault, 1981). The dose used for ICI 182, 780 was based on an effective mg/kg dose administered to sheep via intrauterine infusion (Robertson et al., 2001). The dose for IFN- τ was selected based on previous studies in sheep (Spencer and Bazer 1995) and increased to account for the greater uterine size of cows. Recombinant ovine IFN- τ has been shown to be as effective as recombinant bovine IFN- τ in preventing luteolysis (Meyer et al., 1995). Treatments were delivered every 12 h beginning at 6 pm on cycle day 14 via intrauterine infusion of both horns using 12 gauge Foley® catheters (Agtech; Manhattan, KS), which were positioned immediately anterior to the cervix prior to delivery of the 5 ml treatment volume. Following expulsion of the treatment, the horns were gently massaged to ensure even distribution throughout the uterus. This volume and delivery mechanism were optimized prior to the experiment using reproductive tracts obtained from the local abattoir and in vivo using cull heifers.

Tissue Collection

Uteri were collected within 30 min postmortem and morphological observations were made on the ovaries and reproductive tract. Data on ovarian morphology according to the methods of Ireland (1979) were recorded to confirm cycle stage. For each heifer uterine, cervical and corpus luteum weight and length were recorded. Transverse sections 1 cm³ were dissected from the uterine horn ipsilateral to the corpus luteum and snap frozen in liquid nitrogen then stored at -80°C prior to cutting of cryostat cross-sections for immunohistochemical analysis. Intercaruncular endometrium was dissected from the horn ipsilateral to the corpus luteum and snap frozen in liquid nitrogen for use in RNA analysis.

Northern Blotting

For analysis of levels of integrin β_3 mRNA total RNA from each heifer was analyzed by Northern blot which was performed as described in chapter 4.1.

Immunohistochemistry

Estrogen receptor- α and integrin $\alpha_v\beta_3$ distribution were localized in cryostat cross-sections of endometrium from each heifer as previously described in chapters 2.1 and 3.1 respectively. Immunohistochemistry experiments were replicated at least three times for each animal on different days. To determine effects of treatment on levels of integrin $\alpha_v\beta_3$ protein in uterine tissue, signal intensity within uterine epithelium, stroma and subepithelial stroma was scored by two observers on a six point scale (0-negative, 1-very weak, 2-weak but clearly positive, 3-intermediate, 4-high, and 5- very high).

Data Expression and Statistical Analysis

Data were checked for normalcy using the univariate procedure in SASTM (Statistical Analysis Software Version 8, SAS Institute Inc. Cary NC) and analyzed using a general linear models procedure to examine the effect of treatment on reproductive tract characteristics, level of integrin $\alpha_v\beta_3$ protein, and integrin subunit β_3 mRNA. When significant effects of treatment were observed differences between means were determined using the Tukey-Kramer least square means test. The selected α error rate was $P \leq 0.05$.

4.2 Results

Effects of Treatment on Uterine Characteristics

From visual examination all uteri were similar among treatments in terms of size, tone or color. Although not statistically significant there was a tendency for heavier uterine weights from estrogen-treated heifers ($P=0.1$). No differences were observed between uterine, cervical or corpus luteum measurements of weight and length ($P>0.05$). Gross uterine observations are summarized in Table 2.

Treatment	Uterine Weight (g)	Uterine Length (cm)	Cervix Length (cm)	Corpus luteum Weight (g)
Control	171±6	23±3	9±3	5±0.4
IFN- τ	187±8	23±4	8±1	5±0.4
Estrogen	275±19	25±7	8±1	4±1
ICI 182,780	189±43	22±3	7±1	5±1

Mean \pm standard error

Table 2. Uterine characteristics of heifers treated with either the saline control, interferon-tau (IFN- τ), estrogen or ICI 182, 780 from days 14 to 16 of the estrous cycle.

Effect of Treatment on Uterine Distribution of the Estrogen Receptor and Integrin

$\alpha_v\beta_3$

In this study, reactivity to ER α antibody was used to measure the effects of treatments and as a marker of the day 16 uterine environment. It was expected that if ICI 182, 780 had agonist activity the uterine distribution of ER between estrogen and ICI 182,780 treated heifers would not differ. In sections from ICI 182, 780 treated animals little immunofluorescence was detected throughout the endometrium (Fig. 24). In contrast, strong immunofluorescent signals were present in the glands and stroma in sections from estrogen treated heifers (Fig. 24). Immunofluorescent

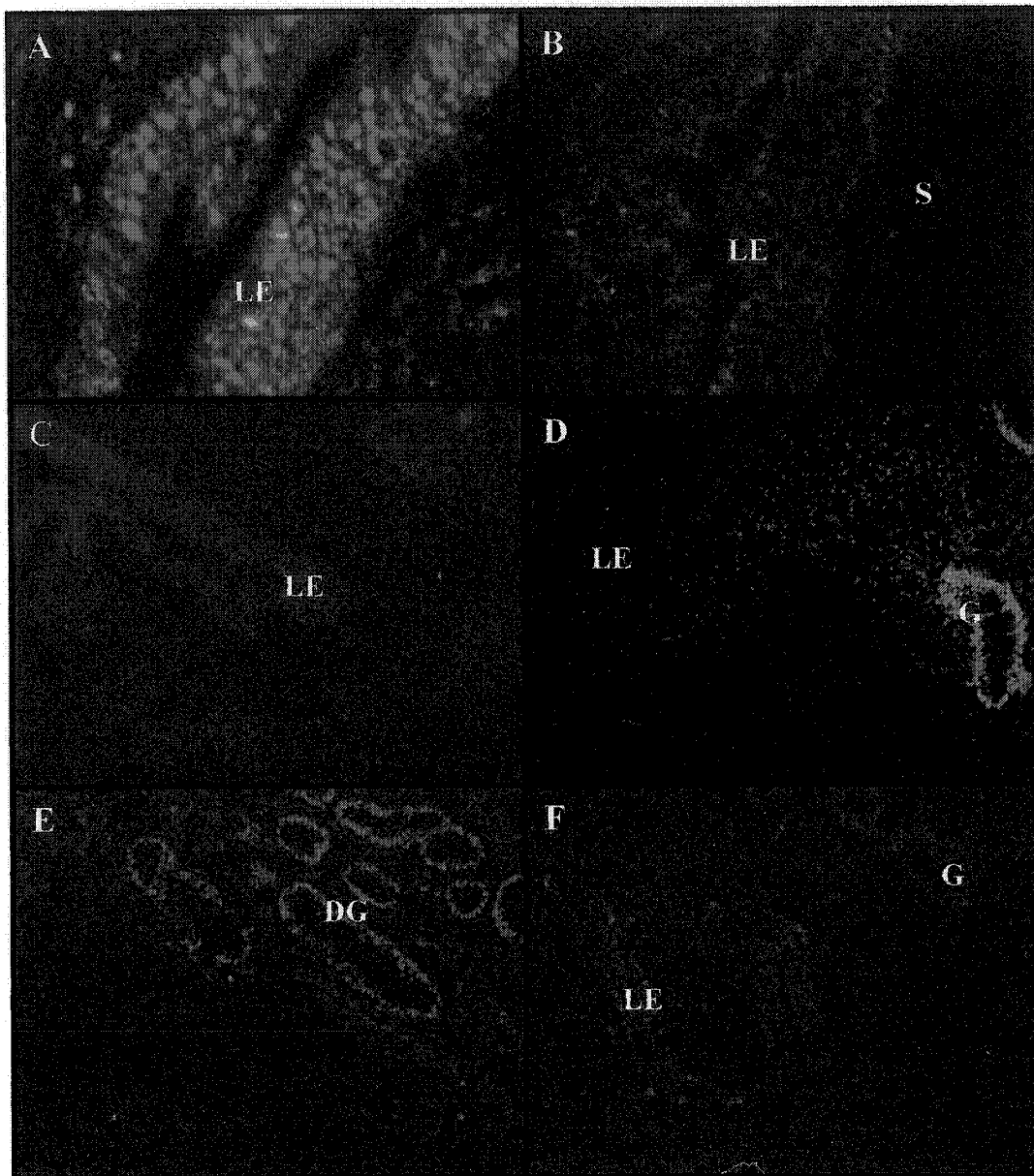


Figure 24. Immunofluorescent localization of the estrogen receptor in cryostat cross-sections of endometrium obtained on day 16 of the estrous cycle after uterine infusion with either, the saline control (A), interferon-tau (B), ICI 182, 780 (C), or estrogen (D and E). A tissue section treated with mouse IgG in place of primary antibody is shown in F. Positive nuclear reactivity was present in the luminal epithelium (LE) of control heifers only. In sections from estrogen treated animals reactivity was detected in the stroma (S) and glands (G). Magnification for A and B 200X and C-F 100X.

reactivity in the luminal epithelium was present only in sections from control heifers, in agreement with results from our previous study (Fig. 24). Reactivity to anti-ER α in sections from heifers infused with the IFN- τ was present in the deep glandular epithelium of some sections and there was no reactivity in the shallow glands or luminal epithelium (Fig. 24).

For all treatments, integrin $\alpha_v\beta_3$ expression was detected in the stroma, myometrium and arteriolar smooth muscle. In tissue sections from all animals, reactivity was low in the caruncles (Fig. 25). Uterine tissue sections from control animals had the lowest scores in the subepithelial stroma for integrin $\alpha_v\beta_3$ in comparison to sections from ICI 182,780 and estrogen treated heifers ($P < 0.05$) (Fig. 25a, b). Immunostaining for integrin $\alpha_v\beta_3$ was low in subepithelial stromal cells in sections from IFN- τ treated animals and did not differ from controls ($P > 0.05$) (Fig. 25a, b). The most intense reactivity was observed in sections from estrogen treated heifers on stromal cells in close proximity with the basement membrane of luminal epithelium (Fig 25a, b).

Effect of Treatment on Expression of Integrin Subunit β_3 mRNA

Northern blot analysis revealed a single 2.4 kb transcript for bovine integrin subunit β_3 corresponding to the published coding sequence of 2.364 kb (Neff et al., 2000). In intercaruncular tissue samples levels of integrin subunit β_3 transcript were lowest from heifers infused with saline and IFN- τ in comparison to tissue from estrogen and ICI 182,780 treated heifers ($P < 0.05$) (Fig. 26a, b). Caruncular tissue was not used for mRNA analysis of integrin $\alpha_v\beta_3$ since our previous work shows

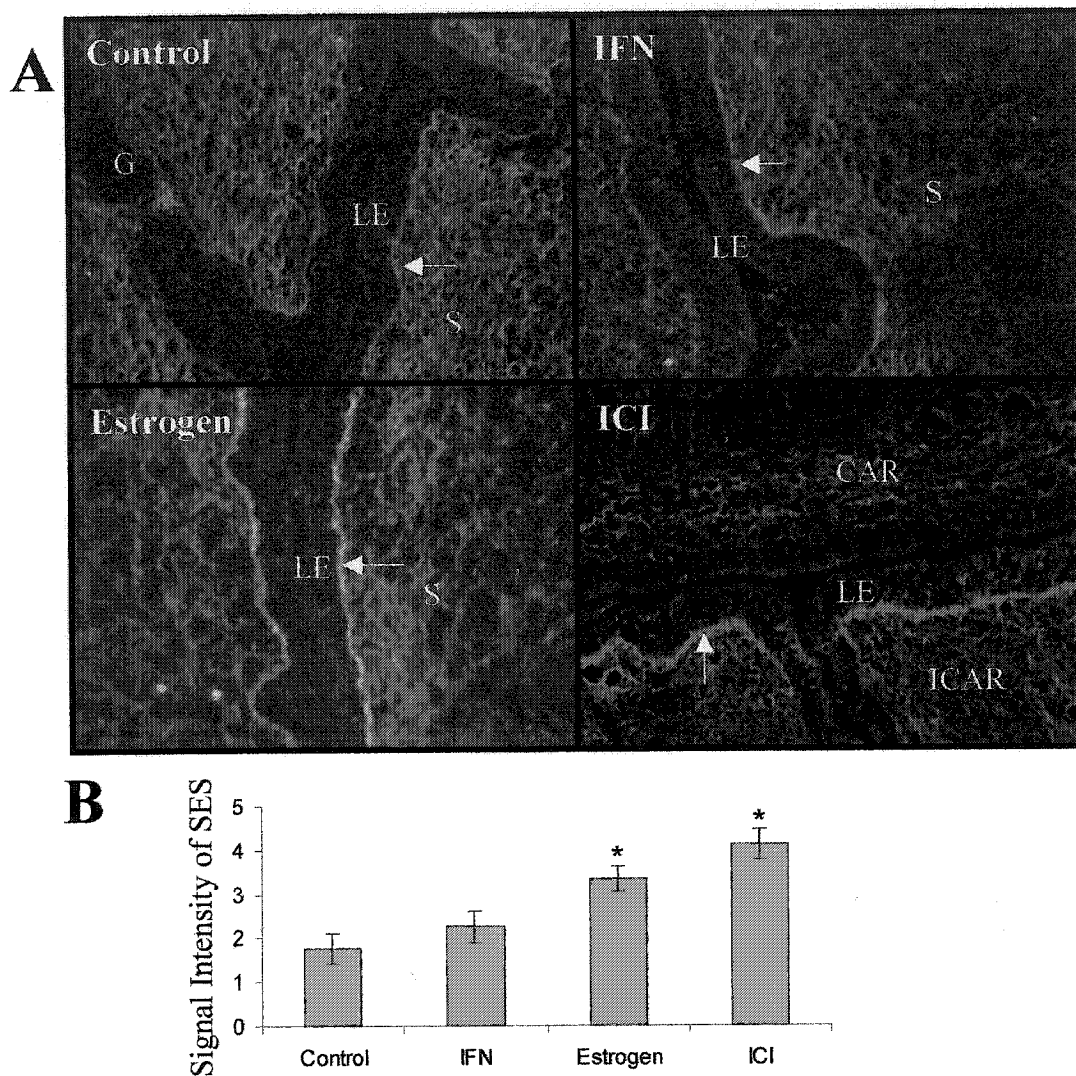


Figure 25. (A) Immunoreactivity to anti- $\alpha_v\beta_3$ in sections from heifers treated with either control, interferon-tau (IFN), estrogen or ICI 182, 780 (ICI). Diffuse staining was present in the stroma (S), low reactivity was observed in the glands (G) and luminal epithelium (LE). Note the strong reactivity of intercaruncular (ICAR) subepithelial stromal cells (arrow) in sections from estrogen and ICI 182, 780 treated animals. Magnification was 200X. (B) Signal intensity of integrin $\alpha_v\beta_3$ in the subepithelial stroma (arrow) was scored on a six point scale (0-negative and 5- very high) and the data were subjected to ANOVA to determine significant effects of treatments and the Tukey-Kramer least square means test to identify differences in comparison to the control. Asterisk indicates means that are significantly different from the control at $P < 0.05$. CAR=caruncular

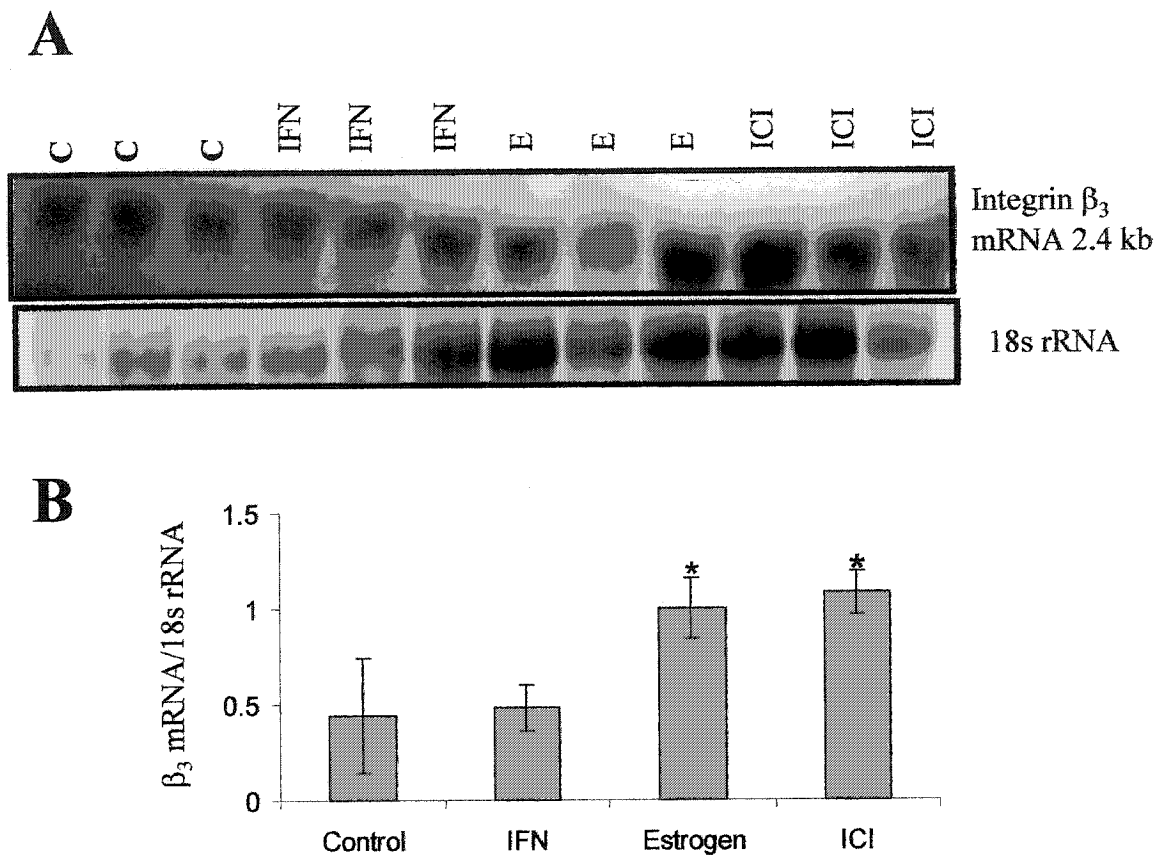


Figure 26. Effect of treatment on expression of integrin subunit β_3 as detected by Northern blot analysis of intercaruncular endometrium. (A) Three animals were used in each treatment group and received uterine infusions of either the saline control (C), interferon tau (IFN), estrogen (E), or ICI 182, 780 (ICI). Ten micrograms of total RNA from each animal was loaded per lane and biotin labeled riboprobes specific to integrin subunit β_3 were used for hybridization to the membrane. A single 2.4 kb transcript was detected. (B) The signal intensity for integrin subunit β_3 mRNA was normalized with the signal of 18S rRNA, and the mean for each treatment group is depicted on the graph. Asterisk indicate means that are significantly different from the control at $P < 0.05$.

expression is low in the caruncles regardless of the day of the cycle or pregnancy state (Kimmins and MacLaren 1999; Kimmins et al., 2003a).

5.3 DISCUSSION

This is the first study to test the anti-estrogen ICI 182, 780 in the bovine uterus. No differences in uterine morphology were observed between treatments, which is not unexpected given the short exposure period, although estrogen-treated heifers tended to have heavier uterine weights. The greatest expression of ER α were detected in sections from estrogen treated heifers in the glandular epithelium and stroma similar to what would be observed in a proestrus animal. Expression of ER α in the stroma, glands and myometrium increased through proestrus and estrus and in ovariectomized estrogen treated heifers (Boos et al., 1996; Robinson et al., 2001; Kimmins and MacLaren 2001), indicating estrogen upregulates its own receptor. Estrogen receptors were low in ICI 182, 780 treated heifers, indicating ICI 182,780 did not have an agonist effect on ER α in bovine endometrium. Confirming prior observations that there are changes in the ER α in the luminal epithelium on day 16 of the cycle (Kimmins and MacLaren 1999), only control heifers had detectable levels of ER α protein in the luminal epithelium. Since ER α were not detected in the luminal epithelium of ICI 182,780 treated heifers, estrogen from the dominant follicle may effect its own receptor in the luminal epithelium, and this action appears to have been blocked by ICI 182, 780 treatment. Similar to the anti-estrogen effect observed in this study, *in vitro* ICI 182, 780 had antagonist action in bovine endometrium (Leung and Wathes 2000). Estrogen treatment of bovine endometrial

explants upregulated oxytocin receptor expression and this effect was blocked by treatment with ICI 182, 780 (Leung and Wathes 2000). In contrast in sheep, uterine infusion of ICI 182, 780 was reported to have partial agonist effects as evidenced by uterine weights, and GAPDH mRNA expression that did not differ from estrogen treated ewes (Robertson et al., 2001). However similar to this study, endometrial expression of ER α were lower in ICI 182, 780 treated sheep than estrogen treated sheep (Robertson et al., 2001).

Estrogen receptor- α were not detected in the luminal epithelium of heifers infused with IFN- τ . Likewise, in sheep, IFN- τ blocks ER α transcription in the luminal epithelium by stimulating binding of IFN regulatory factor 1 to interferon stimulated response elements on the ER α gene (Spencer et al. 1998; Fleming et al. 2001). It was first reported in sheep, that in pregnancy IFN- τ downregulates ER α (Spencer and Bazer, 1996). In cattle it appears that ER α are still present in the luminal epithelium in pregnancy, but expression is reduced (Robinson et al., 2001) and the transcription rate and/or activation state is altered by IFN- τ . Estrogen receptors were not detected in the luminal epithelium of IFN- τ infused heifers on cycle day 16. Perhaps IFN- τ causes binding of a co-repressor to the AF-1 site, binding of the ER to estrogen response elements. This sort of action could mask the epitope recognised by antibody AER314.

The characteristic downregulation of integrin $\alpha_v\beta_3$ in subepithelial stroma on day 16 of the estrous cycle was not observed in heifers treated with ICI 182,780. This may result from antagonist action of ICI 182, 780 preventing endogenous estrogen from binding endometrial ER α . Alternatively, the lack of difference in the

level of integrin $\alpha_v\beta_3$ expression between ICI 182, 780 and estrogen treatment groups, may be due to agonist action of ICI 182, 780 on the integrin β_3 gene. This possibility seems less likely as ICI 182, 780 did not have an agonist effect on endometrial ER. Downregulation of integrin $\alpha_v\beta_3$ on day 16 may occur through ER α directly binding the β_3 gene, or via transactivation where the ER α interacts with another DNA bound transcription factor. The promoter region for bovine integrin subunit β_3 has not been sequenced. While there are no estrogen response elements in the avian (Cao et al., 1993) and mouse (McHugh et al., 2001) integrin subunit β_3 promoters, both contain AP-1 and SP-1 binding sites. Many examples exist of ER interacting with the AP-1 and SP-1 transcription factors to confer estrogen responsiveness (Qin et al., 1999; Vyhldal et al., 2000; Klinge, 2001; Zampieri et al., 2002) and a similar mechanism might occur here. In light of recent publications it is possible that ER action on integrin $\alpha_v\beta_3$ is indirect and may be a consequence of HOXA10 transcription factor signaling. Primary cultures of human endometrial cells upregulate HOXA10 mRNA in response to estrogen and progesterone (Taylor et al., 1998) and in turn reduced expression of HOXA10 is associated with reduced levels of integrin subunit β_3 mRNA (Daftary et al., 2002).

Heifers receiving intrauterine infusion of estradiol had the greatest integrin $\alpha_v\beta_3$ mRNA and protein in comparison to control heifers. These results confirm what we have observed in cycling animals at estrus and suggest that at high levels, estrogen is a positive regulator of integrin $\alpha_v\beta_3$ and at low levels such as would occur on day 16, a negative regulator. The negative effects of estrogen on day 16 could be acting in concert with other endometrial conditions established following long-term

progesterone exposure to downregulate integrin $\alpha_v\beta_3$. We have not observed a biphasic effect of estrogen on integrin $\alpha_v\beta_3$ in monolayer culture of intercaruncular stromal cells (Kimmins et al., 2003b). Integrin $\alpha_v\beta_3$ regulation in the subepithelial stroma is likely to involve an epithelial-stromal dialogue given that the cells of interest are in contact with the basal lamina. In other species it has been shown that the steroid responsiveness of epithelium and stroma is co-dependent in endometrium (Kurita et al., 2000).

Uterine infusion with IFN- τ did not prevent downregulation of integrin $\alpha_v\beta_3$ in intercaruncular subepithelial stroma on day 16 of the estrous cycle but did influence ER in the luminal epithelium. Clearly downregulation of integrin $\alpha_v\beta_3$ in the subepithelial stroma is not a function of ER action in the luminal epithelium. Pregnancy does prevent downregulation of integrin $\alpha_v\beta_3$. Therefore there must be other trophoblast factors or endometrial dialogue not induced by treatment with IFN- τ that are important for integrin $\alpha_v\beta_3$ regulation. In cyclic ewes after four days exposure to IFN- τ , the IFN- τ signaling molecules, signal transduction and activation of transcription 1 and 2, were observed in the subepithelial stroma (Choi et al., 2001). Based on these results and the observation that IFN- τ begins to be produced by trophoblast around day 12 of pregnancy (Martal et al., 1997), the exposure period in this experiment may have been too brief to elicit expression of epithelial or stromal interferon stimulated genes that may be required for maintenance of integrin $\alpha_v\beta_3$ in the subepithelial stroma.

To summarize, on day 16 of the estrous cycle ICI 182 780 blocked detection of ER in the luminal epithelium and the transient downregulation of integrin $\alpha_v\beta_3$ in

bovine endometrium. Intrauterine infusion of IFN- τ suppressed changes in ER protein reactivity in the luminal epithelium but did not affect integrin $\alpha_v\beta_3$ expression, suggesting IFN- τ may not prevent integrin $\alpha_v\beta_3$ downregulation during early pregnancy.

CHAPTER 7 IMPLICATIONS AND DISCUSSION

7.0 Variation in Estrogen and Progesterone Receptors in Ruminant Endometrium: Significance and Roles in Luteolysis

In general, based on the results presented here, and the results of others, there is consensus on the location and roles of steroid receptors in sheep and bovine endometrium during the estrous cycle. However, there are some differences in the uterine location and timing of expression of ER and PR between sheep and cows and research groups. The results of Robinson et al. (1999, 2001) show a slightly different ER protein distribution in cows than we have reported. In their studies using a different ER targeting antibody, low to undetectable levels of ER protein were detected in the luminal epithelium throughout the estrous cycle with no increase in staining observed on day 16 of the estrous cycle (Robinson et al., 2001). The differences in results between our study and Robinson et al. (2001) are likely due to differences in antibody reactivity and/or fixation and detection differences.

Schuler et al. (2002) localised ER in bovine placentomes using four ER-targeting antibodies. Two of the antibodies recognised the ER C-terminal and positively reacted with maternal luminal epithelium, while the two N-terminal targeting antibodies, including the same antibody used in this study (AER314) did not. Interestingly antibody AER314 did react with calf uterine epithelium (Schuler et al., 2002). Early postnatal calf uterine epithelium would be in a continued state of differentiation that is likely to require functional ER (Bartol et al., 1999). The antibody AER314 targets the AF-1 domain that undergoes a conformational change when bound by estrogen, caused by its association with the AF-2 site located in the

C-terminus (MacGregor and Jordan, 1998). It is interesting that this antibody binds ER α in the luminal epithelium only on day 16, suggesting that there is an alteration in ER α conformation that may be associated with its activation and the differentiation of these cells. The changes in anti-ER α binding we report could be due to receptor phosphorylation in the N-terminus (MacGregor and Jordan, 1998), estrogen binding, and/or ER cofactor association (Kimmins and MacRae 2000; Nephew et al., 2000). All of these factors could alter the conformation of the receptor and therefore the availability of the epitope of interest. The antibody AE314 is unique and highly useful in its recognition of a temporary change in the ER α and indicates that it could be used as a marker of the onset of luteolysis.

McCracken et al. (1999) proposed that like sheep, in cattle a transient up-regulation of ER in the luminal epithelium is required to up-regulate oxytocin receptors for production of luteolytic PGF $_{2\alpha}$. Our work indicates that there may be a change in the ER α activation state specific to estrous cycle day 16 that facilitates luteolysis. A possible action of ER α in generation of luteolysis could be activation of the IGF receptor to increase COX-2 (Dipopolo et al., 2000), and epithelial cell production of PGF $_{2\alpha}$. It was recently shown that estrogen can enhance IGF-I signalling through direct interaction between ER α and IGF-I receptor (Kahlert et al., 2000).

It was first reported in sheep that in pregnancy IFN- τ downregulates ER α (Spencer and Bazer, 1996). In cattle it appears that while ER α protein at low levels is still present in the luminal epithelium, expression is reduced (Robinson et al., 2001), and the activation state is altered by IFN- τ ; ER α were not detected in the

luminal epithelium of IFN- τ infused heifers on cycle day 16 (Kimmins et al., 2003c). Perhaps IFN- τ causes binding of a co-repressor to the AF-1 site, preventing AF-1 and AF-2 interaction, and thus binding of the ER to estrogen response elements. This sort of action could mask the epitope recognised by antibody AER314.

In sheep, Spencer and Bazer (1995) report the disappearance of PR mRNA and protein from the luminal epithelium on days 11 and 13 of the estrous cycle. This pattern of steroid receptor regulation supported the theory first postulated by McCracken et al. (1984) that progesterone blocks expression of ER α and oxytocin receptor in the luminal epithelium of sheep up until the initiation of luteolysis. In cattle, we did not detect PR in the luminal epithelium at any time during the estrous cycle, a result confirmed by Robinson et al. (2001). In contrast, Boos et al. (1996) used an antibody that recognized a different epitope and detected PR in the luminal epithelium on day 8, but not on any other day of the estrous cycle examined.

It is unlikely that in cows PR in the luminal epithelium are responsible for blocking ER activation in the luminal epithelium during the luteal phase, given their brief appearance on day 8 only of the bovine estrous cycle. An alternative hypothesis is that PR in the stromal region prevents ER α activation in the luminal epithelium throughout the estrous cycle, except during development of the luteolytic mechanism. Support for stromal control of epithelial cell steroid-responsiveness in the uterus via steroid receptor expression has been demonstrated by Cunha and colleagues and there are many elegant studies in mammary gland biology illustrating stromal control of epithelial cell differentiation (see references in Hennighausen and Robinson, 2001).

Detection of ER α in the luminal epithelium was limited to days 14 and 16 of the estrous cycle (Kimmins and MacLaren, 2001), although more day 16 tissues were positive for ER α than day 14 (4 vs 2). Perhaps this altered ER α reactivity in the luminal epithelium could be used to account for the range in cycle length observed in cows. A cow with a shorter cycle length of 18 days would be expected to show expression of ER α in the luminal epithelium around day 14 (Kimmins and MacLaren, 2001). On the whole there was little variation in the results from tissues collected on day 16 of the estrous cycle, especially considering the number of animals used. This is a reflection of careful heat observations and the uniform group of beef heifers used. All heifers were healthy, showed good signs of heat, and were similar in age and weight. Furthermore, beef heifers are more likely to have two waves of follicle development compared with three for dairy cows (Ahmad et al., 1997; Ireland et al., 2000). Therefore the animals used would have similar hormone profiles and similar times for luteolysis.

The role and/or requirement of estrogen in the onset of luteolysis is often debated in the literature for the following reasons: 1) the first waves of follicle development in the luteal phase do not initiate luteolysis and, 2) oxytocin treatment given without estrogen can stimulate release of PGF $_{2\alpha}$ in vitro (Bogacki et al., 2002). Both of these points should be considered with the following experimental evidence. Cows having a 21 day estrous cycle will typically have waves of follicle development on days 2, 10 and 16 (Ireland et al., 2000). McCracken and co-authors (1984) suggested that in sheep, follicular estrogen does not elicit pulsatile release of PGF $_{2\alpha}$ during early waves of follicle development because ER action on the luminal

epithelium is blocked by epithelial progesterone receptors. This hypothesis concurs with work in cattle, but estrogen effects on the luminal epithelium may be prevented by expression of PR in the periluminal stroma, not the epithelium (Kimmins and MacLaren, 2001; Robinson et al., 2001). Once PR are downregulated late in diestrus, estrogen binds its receptor in luminal epithelium and can initiate luteolysis. Supporting the notion that estrogen is required for activation of its receptor at luteolysis, cattle infused with the anti-estrogen ICI 182,780 from days 14 to 16 of the estrous cycle did show reactivity to anti-ER AER314 in the luminal epithelium (Kimmins et al., 2003c).

7.1 Integrin $\alpha_v\beta_3$ and Insulin-Like Growth Factor: Potential Functions in Luteolysis and Maternal Recognition of Pregnancy

Knowledge of the cellular and endocrine processes that underlie uterine secretion of $\text{PGF}_{2\alpha}$ at luteolysis, and PGE_2 production at maternal recognition of pregnancy has progressed. However, the nature of the relationship between the luminal epithelium and subepithelial stroma is complex, and which paracrine factors contribute to epithelial cell differentiation within the uterus remain poorly defined. The recently identified estrous cycle regulation of integrin $\alpha_v\beta_3$ and IGF-I in the subepithelial stroma of bovine endometrium correlates with differentiation of luminal epithelium into $\text{PGF}_{2\alpha}$ secreting cells (Keller et al., 1998; Kimmins and MacLaren, 1999; Kimmins et al., 2003c; Robinson et al., 2000). Presently we do not know the connection, if any, between integrin $\alpha_v\beta_3$, the IGF system, luteolysis and maternal

recognition of pregnancy, but the temporal link and information from other cell systems support the hypothesis that they are involved in these processes.

Insulin-like growth factors are single chain polypeptides that are homologous to proinsulin. They are synthesized and secreted *de novo* and promote cell proliferation and differentiation within the immediate tissue environment. Biological action of IGFs occurs through type I and II tyrosine kinase receptors and through IGF binding proteins. Generally IGF binding proteins compete with IGF receptors, and thereby attenuate IGF action, however they can have the opposite effect and increase IGF availability for receptor binding (Butler and Leroith, 2001). Insulin-like growth factors, receptors, and binding proteins have been detected in the uterus and placenta of sheep and cattle (Geisert et al., 1991; Cann et al., 1998; Robinson et al., 2000). Insulin-like growth factor I has been localized to the subepithelial stroma, while IGF-I receptor and IGF binding protein I have been detected in the luminal epithelium (Robinson et al., 2000). In other cell systems IGF-I receptor and integrin $\alpha_v\beta_3$ are known to have cooperative signaling roles (Maile et al., 2002), and both proteins have been linked to prostaglandin production via COX-2 (Guan et al., 1998; Di Popolo et al., 2000; Dormond et al., 2001; Hein et al., 2001). Cyclooxygenase-2 is upregulated by IGF-I in human colon carcinoma cells (Di Popolo et al., 2000), and a functional link has been established between integrin $\alpha_v\beta_3$ and COX-2 signaling (Dormond et al., 2001).

In cows, but not sheep, expression of IGF-I is restricted to a band of stromal cells directly beneath the luminal epithelium, in a similar pattern to integrin $\alpha_v\beta_3$. Furthermore, in tissue from day 16 cycling cows, expression of IGF-I mRNA in this

region reached a cycle low which preceded the proestrus increase beginning on day 17 (Robinson et al., 2000). Similar patterns of IGF-I protein expression were observed by Ohtani and coworkers (1996) in cyclic heifers, although the downregulation was observed in day 14-15, rather than day 16-17, animals, consistent with the shorter estrous cycle generally observed in heifers compared to cows. Again, similar to what was observed for integrin $\alpha_v\beta_3$ in the current study, Robinson et al. (2000) found that pregnant cows were more likely to express stromal IGF-I mRNA on day 16 than non-pregnant animals, although the results were not conclusive.

Perhaps integrin $\alpha_v\beta_3$ binds IGF binding protein I via its RGD site (Jones et al., 1995), and the downregulation of integrin $\alpha_v\beta_3$ on day 16 promotes epithelial differentiation into the $\text{PGF}_{2\alpha}$ phenotype. Such a mechanism could free IGF binding protein I from integrin $\alpha_v\beta_3$, leading to IGF-I interaction with its receptor, increased COX-2 mRNA (Dipolo et al., 2000), and thus epithelial cell synthesis of $\text{PGF}_{2\alpha}$. In support of IGF-I involvement in luteolysis, phorbol 12,13-dibutyrate stimulated production of $\text{PGF}_{2\alpha}$ in cultured bovine endometrial cells (BEND), was augmented by the addition of IGF-I (Badinga et al., 2002). Conversely at maternal recognition of pregnancy, integrin $\alpha_v\beta_3$ in the subepithelial stroma bound to IGF binding protein I, could suppress IGF-I action on the luminal epithelium. A weakness of this hypothesis is that the above proposed events would be a better fit with known events *in vivo* if IGF-I was increased on day 16 of the estrous cycle and decreased on day 16 of pregnancy. Further work is required to determine what link if any exists between

IGF-I and integrin $\alpha_v\beta_3$ and their relationship to initiation of luteolysis and the pregnancy recognition processes in cattle.

7.2 Cultured Endometrial Cells

Experimental systems using cell lines, tissue recombinants, explants, co-cultures, and ECM-like biomatrices, have been used to study endometrial cells in an environment separate from the influence of the entire maternal reproductive system (Munson et al., 1990; Sheldrick et al., 1993; Binelli et al., 2000; Kurita et al., 2000 ab). These studies allow for the tight control of system inputs and for separation of the influence of the various tissue components.

The cells of primary interest in the uterus are epithelial and stromal cells. When cultured there is no morphological variation across species; attached epithelial cells appear cuboidal and stromal cells appear spindle shaped (Munson et al., 1988; Julian et al., 1992; Sillem et al., 1997). Studies that have separated endometrial cells have been used to investigate uterine receptivity and other hormonally regulated events such as decidualization. Many research groups have found that epithelial cells grown as monolayers were slow to attach and proliferate. However if the cells are plated at high concentrations this can be overcome (Kimmins et al., 2003b). A further difficulty often mentioned when working with epithelial cells was the discovery that under some experimental conditions monolayers were unable to respond to hormone treatment unless polarized (reviewed by Glasser and Mulholland 1993). Based on our results and those of others, polarization as a requirement for hormonal receptivity does not seem necessary. Many research groups have elicited

dose dependent responses to steroid treatments using non-polarized epithelial monolayers (Davidson et al., 1995; Asselin et al., 1996; Briand et al., 1999; Xiao and Goff, 1999; Kimmins et al 2003b).

While data obtained from epithelial and stromal monolayers have established that each cell type can respond differently to hormonal cues, extrapolation of results to events *in vivo* is problematic specifically in the case of steroid and growth factor elicited responses. To date much of the work in the field of maternal recognition of pregnancy, including work presented here, suggests that there is a complex paracrine dialogue between the epithelium and underlying stroma. For example, IGF-I is expressed exclusively in the stroma while its receptor is present in the luminal epithelium. Furthermore the progesterone responsiveness of the luminal epithelium is probably dependent on stromal PR as none were detected in the luminal epithelium. Therefore while cultured monolayers of epithelial and stromal cells can respond to steroids the receptors are not likely to be regulated in a manner such as occurs *in vivo*. This suggests that a co-culture system would be appropriate when studying the events surrounding maternal recognition of pregnancy.

7.3 Endocrine and Paracrine Regulation of Integrin $\alpha_v\beta_3$ in Bovine Endometrium

Based on the knowledge accumulated from the experiments described herein, a model of endocrine and paracrine factors that may regulate integrin $\alpha_v\beta_3$ on day 16 of the estrous cycle, and block its downregulation during pregnancy is proposed (Figure 27).

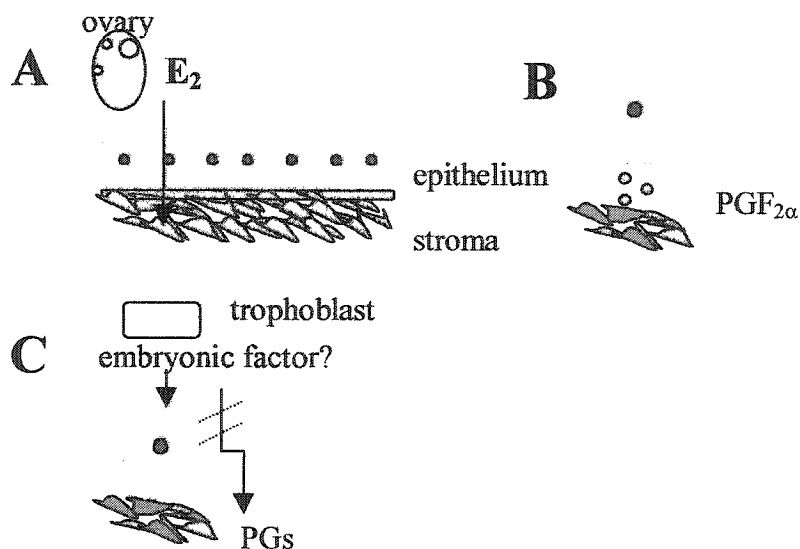


Figure 27. Model of integrin $\alpha_v\beta_3$ regulation in bovine endometrium. The collective results indicate that downregulation of integrin $\alpha_v\beta_3$ on day 16 of the bovine estrous cycle (A) occurs prior to initiation of luteolysis, and is mediated by the preovulatory rise in estrogen from the last wave of follicle development. Downregulation of integrin $\alpha_v\beta_3$ may permit differentiation of the luminal epithelium into the PGF_{2 α} secreting phenotype by altering epithelial-ECM interactions. On day 17 of the estrous cycle (B) PGF_{2 α} from the luminal epithelium stimulates stromal cell expression of integrin $\alpha_v\beta_3$ in a paracrine manner. Maintenance of integrin $\alpha_v\beta_3$ in intercaruncular subepithelial stromal cells in pregnant bovine (C) endometrium could be a downstream effect of IFN- τ signaling resulting in increased stromal and/or epithelial cell production of prostaglandins (PGs), or due to another as yet unidentified factor produced by the embryo or endometrium in pregnancy. Orange cells are integrin $\alpha_v\beta_3$ positive and pink are negative.

7.4 Future Directions

This research identified some interesting possibilities for the functions of integrin $\alpha_v\beta_3$ and ER in bovine endometrium. Understanding their roles in endometrium is worthy of further study as the results suggest they are likely to

function in epithelial cell differentiation during the critical periods, luteolysis, maternal recognition of pregnancy and embryo attachment. The main objectives of continued research should be: 1) confirming that they contribute to epithelial cell differentiation, 2) investigate the intracellular signaling pathways in epithelium affected by their regulation and, 3) determine if integrin $\alpha_v\beta_3$ affects trophoblast invasion.

To determine if there is a connection between the activation state of integrin $\alpha_v\beta_3$ and IGF signaling in bovine endometrium, explants of bovine endometrium could be treated with the following factors, the disintegrin echistatin which blocks $\alpha_v\beta_3$, IGF-I, and anti-IGF-I receptor. The following treatment combinations should be used: echistatin+IGF, IGF+anti-IGF-receptor, and IGF. Measurements of prostaglandin secretion and COX-2 mRNA would give an idea of the involvement of integrin $\alpha_v\beta_3$ and IGF-I in prostaglandin secretion. Immunoprecipitation with an antibody that recognizes the phosphorylated form of the IGF-receptor would show whether the activation state of integrin $\alpha_v\beta_3$ affects IGF-I signaling in bovine endometrium.

The distribution of ER α in bovine endometrium during the estrous cycle suggests that we are detecting an altered conformation that may relate to its activation state. To characterize differences in the ER α in the luminal epithelium due to pregnancy or cycle stage, immunoprecipitation experiments should be carried out using uterine scrapings from day 16 pregnant, and non pregnant heifers. It is expected that closely associated proteins bound to the ER α would co-precipitate.

This would permit identification of ER α associated proteins and could be used to determine differences in ER α signaling.

We hypothesized that, based on the intense expression of integrin $\alpha_v\beta_3$ in the intercaruncular subepithelial stroma, it may be involved in constraining villus development. This could be tested using an *in vitro* model where caruncular and intercaruncular endometrium are separated and epithelial and stromal cells from each region are collected and co-cultured on a matrigel surface and overlaid with trophoblast. It is expected based on the tissue culture model of Arnold et al., (2001) that these cells will form a 3-D culture; stromal cells will invade into the matrigel while the epithelial cells will remain on the matrigel surface and become polarized. Intercaruncular and caruncular cells would be treated or not treated with anti- $\alpha_v\beta_3$. After allowing for a period of attachment, differences in the degree of placental development between the caruncular and intercaruncular cell types would be examined. Based on our results we know that intercaruncular and caruncular cells maintain their *in vivo* phenotype as moderate and low integrin $\alpha_v\beta_3$ expressors respectively. If the hypothesis is correct, and precluding any other specialized attachment factors produced by the caruncular endometrium, villus development would be expected in all caruncular cultures and the intercaruncular cultures treated with anti- $\alpha_v\beta_3$.

A rapidly developing area in the field of maternal recognition of pregnancy is the connection between diet and an embryo-receptive environment. Dietary fats can affect uterine and ovarian function (Mattos et al., 2000; Robinson et al., 2002). For example dairy cows fed a diet high in polyunsaturated fatty acid had increased levels

of IGF-I, $\text{PGF}_{2\alpha}$ and estradiol (Robinson et al., 2002). It is highly likely that such a diet would also affect endometrial expression of steroid receptors and integrin $\alpha_v\beta_3$. Combining nutritional trials with integrin and steroid receptor localization and expression studies could identify opportunities to manipulate their expression to enhance reproductive performance.

7.5 Conclusions

The most enlightening aspects of this work within the current knowledge of maternal recognition of pregnancy and embryo-uterine interactions in the ruminant are: 1) In cows, estrogen receptors and estrogen are likely to be involved in luteolysis and maternal recognition of pregnancy. The role of the ER in maternal recognition of pregnancy in cows was recently dismissed in the literature by Mann and colleagues (et al., 1999) based on their recent work. The results from this research indicate that the mechanisms are complex and will hopefully stimulate further investigation of this topic. 2) Integrin $\alpha_v\beta_3$ is not expressed at the apical surface of the luminal epithelium or in the trophoblast of sheep or cows. Therefore this adhesion molecule is not likely to facilitate embryo attachment. This distribution of integrin $\alpha_v\beta_3$ is unique among species studied to date and contradicts previous localization studies in sheep. The different distribution patterns observed between sheep and cows for integrin $\alpha_v\beta_3$ is new evidence that embryo attachment, and stromal-epithelial interactions are probably different in these species despite similar placental anatomy. This strengthens the argument that there is species-specific gene expression in sheep and cows during the estrous cycle and pregnancy and that results

from one species are not necessarily applicable to the other. 3) The effects of estrogen and IFN- τ on integrin $\alpha_v\beta_3$ mRNA and protein that were observed in culture were replicated *in vivo*. Therefore the culture system used appears to be a valuable tool for studying uterine parameters that are involved in maternal recognition of pregnancy. The downregulation of integrin $\alpha_v\beta_3$ by estrogen and its upregulation by prostaglandins has delineated the sequence of events that determine its regulation during the estrous cycle and pregnancy.

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