

CHARACTERIZATION, REGULATION AND EXPRESSION OF THE MYXOVIRUS
RESISTANCE (MX) GENES IN OVINE ENDOMETRIUM AND IMMUNE CELLS DURING
EARLY PREGNANCY

By

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Abstract

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In early pregnant sheep, conceptus-derived interferon-tau (IFN) acts locally on the endometrium to activate Mx genes expression. We hypothesize that sheep Mx proteins have different regulation during early pregnancy and are important for establishment of pregnancy. The expression Mx genes (oMx1 and oMx2) in the sheep endometrium, peripheral blood leukocytes (PBL) and corpus luteum (CL) during early pregnancy were studied. A second Mx gene (oMx2) was cloned from day 15 pregnant ewe endometrium that resembles human MxB protein. The oMx2 mRNA appeared to be highly expressed by immune cells at the fetal-maternal interface, whereas oMx1 mRNA was expressed throughout endometrium. The oMx1 and oMx2 expression were similar in the endometrium and PBL but different in the CL. The oMx1 appeared to be constitutively expressed in the CL in cyclic and pregnant ewes, whereas oMx2 expression increased in the CL in pregnant ewes only. The cellular localization of the two proteins was different in ovine glandular epithelial cells (oGE). The oMx1 protein was localized in the cytoplasm whereas oMx2 was localized in the cytoplasm and the nucleus. The apparent difference in the expression and localization of the oMx1 and oMx2 suggests that they may have different functions in establishment of pregnancy in sheep.

To understand oMx1 regulation, oMx1 gene promoter was cloned and characterized. Promoter sequence analysis revealed the presence of several binding sites for inflammatory and nuclear receptor transcription factors including ISRE, NF-kappaB, IL-6, AP1, PR and ER. Transfection experiments showed that the 2 proximal ISRE were required for maximal IFN response. In addition, deletion of distal regions of the oMx1 promoter revealed presence of positive and negative regulatory regions. The oMx1 promoter appeared to be constitutively active in the human T47D cell line and could not be induced by IFN. The presence of negative and positive regulatory region and high level of oMx1 promoter activation in T47D cells suggest a very complex regulation of oMx1 gene. These findings are important for understanding the pregnancy-associated regulation of Mx genes. In addition, studying the regulation of Mx genes will help us to decipher their importance for establishment of pregnancy.

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CHAPTER ONE:

Review of Literature

1. Introduction/Rationale

Proper communication between embryo and receptive uterus is crucial for successful pregnancy. The presence of a viable conceptus that can send specific signals to a “pregnancy-ready” uterus that is able to receive those signals and rescue the corpus luteum from luteolysis is an absolute requirement to establish pregnancy. This phenomenon is called maternal recognition of pregnancy and it initiates a very complex series of structural (i.e. endometrial differentiation), transcriptional, and hormonal changes in the pregnant mother. These changes are important to ensure; I). extending the corpus luteum (CL) lifespan; II). nourishing the pre-implantation conceptus(es); and III). readying the uterus for implantation and subsequent placentation. The ovulation and fertilization processes are efficient in most domestic animals. For example, in swine approximately 95% of ovulated ova are fertilized (Pope, 1988). However, the early embryonic loss (i.e., embryonic mortality) is relatively high (~25-30%) (Pope, 1988). Infertility and early embryonic loss are major problems in humans and domestic animals. In humans, it is estimated that early pregnancy loss (EPL) is approximately 25 to 30% (Wang et al., 2003; Wilcox et al., 1988). Early embryonic loss in domestic animals has a large negative impact on animal agriculture, particularly in swine and cattle (Grohn and Rajala-Schultz, 2000; Lucy et al., 2004; Moore et al., 2005). In pigs, the potential litter size is 14 piglets if

the average number of ovulated ova survive till birth, but according to 2001 USDA statistics, the average litter size is only 10.5. This loss results in over \$500 million dollars in losses each year to the swine industry. The financial losses due to infertility in animal agriculture are considered high. Approximately 75% of the embryonic losses take place within the first 30 days of pregnancy (Shahani et al., 1992). A 3% reduction of embryonic loss would result in an additional 3.2 million pigs born/year, 135 thousand sheep/year, 1 million beef calves/year, and 3.7 million gallons of milk/year (Senger, 2003).

There are several factors that contribute to early embryonic mortality. These factors can be grouped into 3 major categories. The first category is related to the reproductive potential of the female and her ability to support the developing embryo to term. Normal ovarian function is an important factor for embryonic survival. For example, the presence of a functional corpus luteum (CL) that is able to produce sufficient progesterone is an absolute requirement for establishment of pregnancy in all domestic species. In cattle, it was reported that smaller follicles and ultimately smaller CL resulted in lower serum progesterone and reduced conception rate (Vasconcelos et al., 2001). Progesterone secreted by CL is essential for enhancing uterine histotroph production (uterine secretions) which is needed to nourish the developing conceptus. In domestic animals, the estrous cycle is dependent on the uterus as source for the luteolysin (PGF2 α). However, in pregnancy, uterus and its glandular secretions are essential for early embryonic development and for placentation (Gray et al., 2002; Spencer and Bazer, 2004).

The second category of embryonic loss is the developmental potentials of the conceptus (the embryo and its associated extra-embryonic membranes). In ruminants, embryos undergo elongation from a spherical form to a tubular and then filamentous form prior to implantation. During this time embryos begin to produce interferon-tau (IFN-tau), the signal for maternal recognition of pregnancy in domestic ruminants (Spencer et al., 2004). Developmentally delayed embryos are unable to produce the IFN-tau in the required amounts and the proper time. This results in endometrial production of PGF2 α and ultimately luteolysis.

The third category of embryo loss includes environmental factors such as diseases, nutrition, and heat-stress. In dairy cows, exposure to different environmental insults such as heat-stress (Drost et al., 1999), high protein diets which results in high concentration of urea in plasma and milk (Canfield et al., 1990), or diseases such as mastitis (Chebel et al., 2004) decreased conception rate. Clearly, these internal and external factors interact to yield either embryonic survival or mortality.

Infertility and early embryonic loss is a complex and multi-factorial problem. Improving fertility in human and domestic animals has been the focus of extensive research by scientists for many decades. However, the role of uterus and its “environment” is an unequivocally important factor for successful establishment of pregnancy. Identifying protein components of this uterine environment that are expressed during early pregnancy and understanding their regulation will enhance our knowledge about key players in the establishment of pregnancy. This ultimately, will lead to developing new strategies to reduce early embryonic loss and improve fertility

in domestic animals and humans. Improving food animal fertility will help to meet growing nutritional demands for human population.

The Mx proteins are large GTPases that belong to the dynamin superfamily of mechanochemical enzymes (Praefcke and McMahon, 2004). Mx proteins are induced by type I IFN and by viruses, and are found in most mammals, birds and fish as primary components of the innate response to viral infection (Haller and Kochs, 2002). In most species, there are at least 2-3 Mx proteins (products of gene duplication), for example there is human MxA and MxB and the rodent orthologs Mx1 and Mx2. The Mx proteins share conserved tripartite GTP binding domains, dynamin signature motif and leucine zipper motif (Haller and Kochs, 2002). Despite relatively high levels of Mx protein sequence conservation between species, their antiviral activities differ between and within species (Lee and Vidal, 2002). The antiviral properties of Mx proteins depend on their cellular localization, species, and type of viruses. In humans, MxA is a cytoplasmic protein and has antiviral activity against a broad array of viruses (Frese et al., 1995; Pavlovic et al., 1990), while MxB protein is localized to the cytoplasm and the nucleus but has not been shown to possess antiviral activity (Melen et al., 1996).

Ovine Mx1 (oMx1) is an interferon stimulated gene that is expressed in the sheep endometrium during the estrous cycle and is strongly up-regulated during early pregnancy in both the endometrium and peripheral blood leukocytes (Ott et al., 1998; Yankey et al., 2001). Our results showed that estrogen and progesterone are required for oMx1 protein induction by IFN in the ovine endometrium (Ott et al., 1999).

Therefore, the focus of this dissertation is first, to identify and characterize unknown Mx proteins that are expressed in the sheep endometrium and peripheral blood leukocytes and CL during early pregnancy and second, to study the regulation of the ovine Mx1 (oMx1) promoter by interferon tau (IFN-tau) and by steroids. This work will enhance our understanding of how these antiviral proteins are regulated in sheep endometrium and shed more light on their importance for establishment of pregnancy. Studying gene regulation in the endometrium and immune cells during early pregnancy is instrumental to decipher the complex feto-maternal communication and maternal immunotolerance. In addition, identifying antiviral genes in domestic animals is vital to combat economically-devastating viral infection such as vesicular stomatitis virus and bovine viral diarrhea.

2. Reproductive Cyclicity in ewes and gilts

Reproductive cycle in sheep (*Ovis aries*) and swine (*Sus scrofa*) is defined as the cyclic events of female receptivity to accept the male for mating. This occurs during estrus or “heat”, and the estrous cycle is defined as the period between one estrus and the next estrus period. The estrous cycle is controlled by intricate endocrine interactions between the hypothalamus, pituitary gland, and ovary. Although, the hypothalamo-pituitary-ovarian hormone axis controls the ovarian cycle, the estrous cycle in domestic farm animals is uterine-dependent. This means that the uterus is the source for prostaglandin-F_{2α} (PGF; luteolysin) and hysterectomy during the cycle results in extended CL function. This is in contrast to humans that possess a uterine-independent ovarian cycle and hysterectomy does not extend CL lifespan

(Bazer, 1998). In domestic farm animals, the estrous cycle is repeated until the end of the breeding season (in season animals like sheep and horses) or establishment of pregnancy (in aseasonal breeders like cattle and swine).

2.1. Estrous cycle

Gilts are polyestrous animals which have estrous cycle duration of 17-25 days (average 21 days and cycle throughout the year if pregnancy is not established. Unlike gilts, ewes are short-day breeders with estrous cycle lengths ranges from 15-19 days (average 17 days). Ewes have multiple estrous cycles beginning in later summer and continuing through early spring in the Northern hemisphere. Seasonal breeding patterns are considered important for neonatal survival. Ewes conceive in the Fall/winter and lamb in the spring when there is an ample food supply; an advantageous factor for survival.

The estrous cycle is controlled by hormonal inter-play between pituitary, hypothalamus, and ovary which is called hypothalamo-pituitary-ovarian axis. Gonadotropin-releasing hormone (GnRH) is a neuropeptide hormone that is secreted from the hypothalamus to stimulate the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary. The Gonadotropin FSH and LH are glycoprotein hormones that are secreted from the anterior pituitary. FSH and LH hormones travel through blood circulation to act on the ovary and are required for follicular development and the subsequent ovulation and corpus luteum (CL) formation. Depending on the dominant ovarian structure, the reproductive cycle can be divided into 2 major phases; follicular phase and luteal phase (Senger, 2003).

2.2. Follicular phase

This phase of the estrous cycle is named follicular phase because of the dominant structure in the ovary is the developing follicle(s). The follicular phase constitutes about 20% of the estrous cycle and is divided into two stages, proestrus and estrus. Proestrus starts after the CL regression (Luteolysis) and ends at ovulation and occurrence of estrus (heat). Corpus luteum (CL) is the source of progesterone (P4) and therefore, CL regression results in a rapid decline in progesterone concentrations in the circulation. Progesterone has a negative feedback effect on GnRH production from the hypothalamus, consequently a decline in P4 results in an increase in GnRH secretion (Kasa-Vubu et al., 1992). Increased GnRH secretion leads to stimulation of the anterior pituitary to release FSH and LH. Released FSH and LH are transported in the blood and act on the ovary, to support the follicular growth, ovulation and CL function (hence the name gonadotropins).

Folliculogenesis describes the dynamics of follicular growth, recruitment, selection and dominance of the follicle(s). Follicular growth is accompanied by a gradual increase in estradiol (E2) production from the growing follicles. Maximal production of estradiol (E2) is achieved during the dominance phase of folliculogenesis. Estradiol has a positive feedback action on the hypothalamic surge center (preovulatory center) resulting in high frequency and high amplitude release of GnRH. This “surge” of GnRH stimulates a surge of LH that ultimately results in ovulation of the dominant follicle(s) (Adams, 1983; Baird and McNeilly, 1981). Beside estrogens positive feedback on the hypothalamus, it has other effects including

stimulating female receptivity to the male (heat/estrus), and morphological and physiological changes in the female reproductive tract. Increasing levels of E2 and decreasing levels of progesterone during proestrus leads to morphological changes in uterine, cervical and vaginal tissues (Adams, 1986; DiAugustine et al., 1988). Uterine gland growth and increased mucus secretion by the cervix and vagina is consequence of increased E2 levels (Senger, 2003).

2.3. Estrus

Estrus is defined as the period of female sexual receptivity to the male. During proestrus the increasing levels of E2 production by the dominant follicle(s), leads to LH surge and ovulation (Baird and McNeilly, 1981). Ovulation results from increased vascularization, histamine and prostaglandins induced smooth contractions, and collagenase activity to weaken the follicle wall (Krishna et al., 1989; Murdoch et al., 1986). Estrus duration in sheep is about 18-48 hours. Ovulation occurs 24 to 30 hours after the onset of estrus. In pigs, estrus lasts about 12-96 hours and ovulation occurs 36-44 hours after the onset estrus. Interestingly, cattle ovulate after the end of estrus during metestrus. The ovulated follicle undergoes remarkable cellular and structural changes to form the corpus luteum (CL) which is the main ovarian structure in the luteal phase.

2.4. Luteal phase

The luteal phase of the estrous cycle starts after ovulation and continues until corpus luteum regression (luteolysis). The luteal phase constitutes approximately 80%

of the estrous cycle and includes two stages; metestrus and diestrus. After ovulation and due to rupture of small blood vessels, blood clots are formed in the ovulated follicles (called the corpus hemorrhagicum or CH). The CH is a transitional ovarian structure that is made of blood clots, cell debris and mixture of theca and granulosa cells. Angiogenesis (new blood vessel growth) in the corpus hemorrhagicum (CH) helps in the transformation of the transient structure (CH) into a functioning endocrine organ, the corpus luteum (Bazer, 1998). This process of transformation, known as luteinization, begins shortly after ovarian follicle rupture and is characterized by remarkable changes in the composition and function of theca interna and granulosa cells (Murphy, 2000). Following ovulation the theca and granulosa cells form a mixture of cells that develop into thecal-derived Small and granulosa-derived Large luteal cells (Hoyer and Niswender, 1985). During metestrus, which lasts about 4 days in sheep, both estrogen and progesterone levels are low. However, the newly established CL starts to secrete progesterone immediately, and it is detectable in the peripheral circulation by about day 4 of the cycle. Maximal CL size and maximal progesterone secretion occur between day 7 and 14.

The CL secretes high levels of progesterone during diestrus. Progesterone acts on different components of reproductive system including hypothalamus (Scott et al., 2000), pituitary (Meikle et al., 2004), uterus (Meikle et al., 2004; Spencer and Bazer, 2002) and mammary gland (Chappell et al., 1997). Progesterone from the CL suppresses GnRH pulse frequency, behavioral estrus, and LH and FSH levels. Suppression of the gonadotropins inhibits ovulation of the developing Graafian follicles. Progesterone plays a fundamental role in changing the estrogen-primed

endometrium during early pregnancy to provide an optimum environment for embryo development and survival (Bazer and First, 1983; Spencer and Bazer, 2002). Endometrial glands undergo extensive hyperplasia and hypertrophy during early pregnancy which is required for histotroph secretion. Progesterone down-regulates its receptor in luminal and glandular epithelia, a process necessary for triggering progesterone-induced secretory proteins and for glandular differentiation and remodeling (Spencer et al., 1999). Histotroph contains proteins, ions, enzymes and carbohydrates that are essential for nourishing the developing conceptus (Bartol et al., 1999; Bazer and First, 1983; Gray et al., 2000; Gray et al., 2002). In addition, progesterone causes uterine quiescence throughout pregnancy thus preventing fetal expulsion.

2.5. Luteolysis

Luteolysis or CL regression is an essential process in reproductive cycles and allows a female to return to estrus, and increases the chances for pregnancy. Cattle, sheep and swine are spontaneous ovulators with uterine-dependent luteolytic mechanisms (Bazer, 1998). The uterine endometrium is the source of $\text{PGF}_2\alpha$ (McCracken et al., 1999) which is transported from the uterine vein to the ovarian artery by a counter-current exchange mechanism (McCracken et al., 1972). The hormonal control of luteolysis is quite fascinating with four major hormonal mediators; progesterone, estrogen, oxytocin and $\text{PGF}_2\alpha$.

During late diestrus and in the absence of a proper embryonic signal, the endometrium releases pulses of $\text{PGF}_2\alpha$ in response to oxytocin. Prostaglandin $\text{F}_2\alpha$

pulses (5 pulses per 24 h period in sheep) cause functional and structural CL regression which is marked by increase in PGF-2 alpha metabolites and rapid decline of progesterone levels (Zarco et al., 1988). In sheep the endometrium is the source of PGF₂ α ; primarily the luminal epithelium (LE) and superficial glandular epithelium (sGE) (Gray et al., 2000). Endometrial LE and sGE cells express oxytocin receptors (OTR) (Wathes and Lamming, 1995) and the cyclooxygenase 2 (COX-2) enzyme an important and rate limiting enzyme for prostaglandins synthesis (Charpigny et al., 1997). Steroid hormones (estrogen and progesterone) play important role in luteolysis. Progesterone increase lipid stores and activates enzymes necessary for PGF₂ α production (Spencer et al., 2004). After estrus, ovarian E₂ stimulates expression of uterine estrogen receptors (ER α), progesterone receptors (PR) and oxytocin receptors (OTR) (Spencer and Bazer, 1995; Wathes and Hamon, 1993). During luteal phase progesterone downregulates ER α and OTR expression in the endometrium, a phenomenon called the “progesterone block” (McCracken et al., 1984). The mechanism by which progesterone downregulate ER alpha expression is not well defined, but suppression of OTR by progesterone is may be a consequence of ER alpha downregulation (Fleming et al., 2006). Continuous exposure of the uterus to P₄ (8-10 days) results in downregulation of PR in endometrial LE and sGE by days 11-12 after estrus (Spencer and Bazer, 1995). Progesterone receptor downregulation removes the block on ER α and OTR expression and results in rapid increases in ER α expression, followed by OTR expression on day 13 and 14, respectively (Hixon and Flint, 1987; Spencer and Bazer, 2002). Oxytocin released from the Large luteal cells (Wathes and Denning-Kendall, 1992) and the posterior pituitary (Hooper et al., 1986)

binds to OTR and results in activation of phospholipase C (PLC). Phospholipase C hydrolyzes phosphatidyl inositol 4,5-bisphosphate (PIP₂) to yield the second messenger, inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG). Diacylglycerol activates Ca²⁺-dependent protein kinase C (PKC), whereas IP₃ promote release of intracellular Ca²⁺ from the endoplasmic reticulum. Activated PKC along with Ca²⁺ activate phospholipase A₂ (PLA₂) which releases arachidonic acid (AA) substrate from membrane phospholipids. The released AA is converted by cyclooxygenase to prostaglandin G₂ (PGG₂) and PGH₂ intermediates. Prostaglandin synthase (PGFS) converts PGH₂ to PGF₂α (Bazer, 1998; Flint et al., 1986; McCracken et al., 1999; Silvia and Raw, 1993, 1993).

The exact mechanism by which PGF₂α causes CL destruction is not well understood. The luteolytic actions of PGF₂α have been attributed to: decrease in luteal blood supply; reduction of LH receptors; uncoupling of LH receptor from adenylyl cyclase; activation of protein kinase C (PKC); influx of high levels of calcium and/or activation of cytotoxic cascade (Bazer, 1998). Luteolysis is characterized by both functional and structural events. As the source of P₄, CL regression is marked by a drop in the plasma P₄ level (i.e. functional regression) and then followed by luteal cell death (structural regression) (Hoyer, 1998). In ewes, PGF₂α administration resulted in decrease of serum progesterone within 5 hours (functional regression), an event precede luteal cell death (structural regression) which occurred after 12 hours (McGuire et al., 1994). In pigs, luteolysis also occurs late in diestrus; following progesterone stimulation of endometrium for 10-12 days. Removal of uterus (hysterectomy) extends the estrous cycle and CL lifespan (Bazer, 1998). In pigs

PGF2 α is the uterine luteolysin (Bazer, 1998). Administration of exogenous PGF2 α into the anterior uterine vein before day 12 of the cycle does not cause luteal regression (Krzymowski et al., 1978). Therefore, the pig CL is refractory to PGF2 α -induced luteolysis until 12-13 day of the cycle. Corpus luteum insensitivity to exogenous PGF during this period of the cycle is due to low numbers of PGF2 α receptors (Gadsby et al., 1990). Gadsby et al. (1990) found that the number of high-affinity PGF2 α receptors was low before days 12 and increased starting on days 13, 14 to their highest levels at 16-17. Exogenous administration of OT decreased interestrus intervals by a uterine-dependent mechanism (Prince et al., 1995). Unlike ewes, CL in pigs contain very low levels of OT and the neurohypophysis is considered the major OT source (Choy and Watkins, 1988). In pigs, endometrium secretes OT and hence considered a primary source of OT (Trout, 1995; Vallet et al., 1998). The concentration of 13,14-dihydro-15-keto-PGF2 α metabolite PGFM (the major stable metabolite of PGF2 α) was higher in cyclic than pregnant gilts on day 14 and 16 post-estrus after intravenous OT administration (Carnahan et al., 1996). However, gilts exposed to intrauterine OT infusion showed a decrease in plasma PGFM concentrations and delayed decline in progesterone during late diestrus (Sample et al., 2004). These findings suggest that uterine-derived OT has antiluteolytic effect and may be involved in PGF2 α redirection during early pregnancy.

Corpus luteum regression marks the end of luteal phase and the start of a new cycle. However, CL can be rescued by conceptus-produced antiluteolytic signals; a process is called maternal recognition of pregnancy.

3. Maternal recognition of pregnancy in ewes and gilts

The phenomenon of maternal recognition of pregnancy can be defined as the result of reciprocal communication between the conceptus and the mother to ensure prolonged CL lifespan and establishment of pregnancy. In domestic farm animals this is achieved by conceptus secretion of an antiluteolytic hormone that is perceived by a pregnancy-permissive uterus as a signal to block luteolysis. Synchrony between conceptus and uterus is essential. In other words the conceptus must be in the right place (i.e. receptive uterus) at the right time (window of receptivity) for successful maternal recognition of pregnancy. The signal of maternal recognition of pregnancy is different among species. For example, conceptus-produced chorionic gonadotropin (CG), E2 and IFN-tau are the embryonic signals in humans, pigs and sheep/cattle, respectively. Human chorionic gonadotropin is a polypeptide hormone that is secreted from the trophoblast and acts on the CL directly to stimulate P4 production (Bazer, 1998). In pigs, conceptus-produced E2 and exocrine secretion of uterine PGF into the lumen are thought to be the critical factors in rescuing the CL (Bazer and Thatcher, 1977). In ruminants, CL rescue by IFN-tau is mediated by its action on the endometrium to abolish PGF 2α pulses (Bazer, 1998). Regardless of the type of signal, the main objective of this embryonic signal is to rescue CL from luteolysis and maintenance of P4 secretion.

In sheep, cows, and goats, IFN-tau has antiluteolytic effects by acting on the endometrium in paracrine manner to prevent luteolytic pulses of PGF 2α . In these species, the embryo undergoes elongation from a spherical form to a tubular and then filamentous coincident with the onset of IFN-tau production (Roberts et al., 1999; Spencer et al.,

2004). Interferon secretion by conceptuses corresponds is apparently developmentally regulated. For example, conceptus IFN-tau secretion (ng/uterine flushing) during transition from spherical to tubular to filamentous is 312ng, 1380ng, and 4455ng, respectively (Spencer et al., 1996). Interferon-tau is a subclass of Type I interferons and has antiluteolytic, antiviral, antiproliferative, immunomodulatory activities (Bazer et al., 1994; Tuo et al., 1993). In sheep, conceptus mononuclear trophoctoderm cells synthesize and secrete IFN-tau between Days 10 and 21 with maximal production on days 14-16 of the cycle (Roberts et al., 1999).

The mechanism of IFN tau action on the uterus has been the focus of much research over the past 20 years. Interferon tau apparently acts via a Type I IFN receptor to activate the interferon stimulated genes (ISGs) (Stewart et al., 2001). The antiluteolytic effect of IFN-tau does not carried out by maintaining endometrial progesterone receptor (PR) (Spencer and Bazer, 1995). Antiluteolytic action of IFN-tau is achieved by suppression of endometrial ER and OTR expression (Fleming et al., 2001; Spencer et al., 1995) which in turn prevents endometrial luteolytic PGF 2α production. Interferon tau does not suppress OTR expression directly, but suppresses ER gene transcription and therefore precludes OTR expression by estrogen (Fleming et al., 2006).

During early pregnancy, conceptus and IFN-tau do not influence endometrial COX2 expression (Charpigny et al., 1997; Kim et al., 2003). Therefore, IFN-tau exerts its antiluteolytic effects by inhibiting ER (directly) and OTR (indirectly), hence, diminishing the peak frequency and amplitude of uterine PGF 2α secretion.

Maternal recognition of pregnancy in pigs is rather fascinating. In 1977, Bazer and Thatcher proposed the endocrine-exocrine theory of maternal recognition of

pregnancy in pigs (Bazer and Thatcher, 1977). The theory states that during early pregnancy uterine PGF₂ α secretion is directed away from the uterine vasculature (endocrine) toward the uterine lumen (exocrine). In cyclic pigs, during late diestrus and in the absence of conceptus signal, PGF₂ α is secreted toward uterine blood vessels, and transported from the uterine vein to ovarian artery by a counter-current exchange mechanism resulting in luteal regression. Redirection of secretion of from endocrine to exocrine mode results in extension of CL lifespan. Concentration, frequency and amplitude of PGF₂ α pulses in the utero-ovarian vein are lower in pregnant and estrogen-induced pseudopregnant gilts than in cyclic gilts (Frank et al., 1977; Knight et al., 1977; Moeljono et al., 1977; Spencer et al., 2004). Uterine flushes from pregnant and pseudopregnant gilts contain higher concentration of PGF than those from cyclic gilts (Zavy et al., 1980). The switch of PGF secretion from endocrine to exocrine direction occurs between day 10 and 12 of pregnancy, a time associated with high E₂ secretion by the elongating conceptuses (Bazer, 1989). Conceptus-secreted or exogenous E₂ induces calcium release into uterine lumen followed by re-uptake by endometrial and/or conceptus tissues (Geisert et al., 1982). Calcium release and re-uptake cycles by endometrium are temporally associated with endocrine-exocrine switch in pregnant and estrogen-induced pseudopregnant gilts (Bazer, 1989). Treatment with a calcium ionophore induced luminal-direction (exocrine) secretion of PGF from endometrium from day 14 cyclic gilts, suggesting that calcium cycling may be involved in this process (Mirando, 1988). In addition, E₂ and prolactin interact to cause PGF directional secretion toward the uterine lumen (Mirando, 1988). It is thought that prolactin enhance calcium movement across the uterine epithelium and into the uterine lumen (Bazer,

1992). More recently, the endocrine/exocrine theory has been challenged. Krzymowski et al., (2004) suggested that protection of the CL from luteolysis occurs as a result of retrograde transport of PGF by the mesometrium (Krzymowski and Stefanczyk-Krzymowska, 2004). The broad ligament contains lymphatics, and arterial and venous vessels which are in close anatomical association and provide an ideal system for transporting PGF into the uterine artery (Krzymowski and Stefanczyk-Krzymowska, 2004). This retrograde transport of PGF toward uterus results in lower PGF levels available in the ovarian area to cause luteal regression.

4. Interferons (IFNs)

Interferons (IFNs) are a large family of cytokines produced in some form by virtually every cell in the body. Interferons were first characterized by Isaac and Lindenmann in the late 1950 as factors that interfered with viral replication (Isaacs and Lindenmann, 1957). Interferons play crucial roles in fighting viral infection, regulating cell growth and differentiation and in host immune system modulation (Bazer et al., 1994; Bonjardim, 2005; Haller et al., 2006). In ruminants, early embryos produce a unique IFN, IFN- τ , which has antiviral properties and is the signal for pregnancy recognition in those species (Bazer et al., 1994).

4.1. Classification of interferons

Interferons can be classified according to their activation mechanism/ cellular source into two major types; Type I IFN and Type II and IFN-like cytokines (Pestka et al., 2004). Type I IFNs include 7 subclasses; IFN- α , IFN- β , IFN- ω , IFN- τ , IFN- ϵ , IFN- δ , and IFN- κ . In contrast, Type II IFN consists of only one member, IFN- γ .

Interferon-like cytokines have been discovered; limitin (found only in mice) and interleukin-28A (IL-28A), IL-28B and IL-29 found in humans and other species (Pestka et al., 2004). IFN- α , IFN- β , IFN- ω , IFN- ϵ , IFN- κ , IL-28A, IL-28B and IL-29 are found in human, but IFN- τ , IFN- δ , and limitin are found in ruminants, pigs and mice, respectively (Lefevre et al., 1998; Pestka et al., 2004; Roberts, 1989). With the exception of IFN- κ , type I IFN are coded by an intronless multigene family located on chromosome 9 and 4 in humans and mice, respectively (van Pesch et al., 2004). The interferon- γ gene contains three introns and is located on human 12 and on mouse chromosome 10 (Samuel, 2001). There are 14 subtypes of IFN- α genes and only a single gene for IFN- β , IFN- κ , IFN- ϵ and IFN- γ in the human genome (Pestka et al., 2004). Receptors are unique for each type I and II IFN and IFN-like cytokines. The type I IFN receptor consists of IFN- α R1 and IFN- α R2c chains, while IFN- γ signals through receptor chains IFN- γ R1 and IFN- γ R2 (Pestka et al., 2004). IFN-like cytokines (IL-28A, IL-28B and IL-29) use a special receptor complex consisting of IL-10R2 and IL-28R1 chains (Pestka et al., 2004).

Type I IFN α and β are produced from leukocyte (α) or somatic cells (α and β) in response to viral infections (Bonjardim, 2005; Samuel, 2001), however, ruminant IFN- τ is uniquely secreted by the conceptus trophoderm during a short period of early pregnancy. The expression of IFN- τ is thought to be developmentally regulated as it is not induced by viral infection (Bazer, 1989; Bazer and Johnson, 1991; Roberts et al., 1999). Interferon- α and β are secreted by most cells in culture in response to viral challenge, however, IFN- γ is secreted by immune cells in response to antigenic or mitogenic activation (Samuel, 2001). Interferon- γ is produced by

certain specialized immune cells, including natural killer (NK) cells, CD8⁺ cytotoxic suppressor T cells, and CD4⁺ T helper 1(Th1) cells (Bach et al., 1997; Bonjardim, 2005). Interestingly in pigs, the conceptus secretes both IFN- α and IFN- γ , with the latter making up the larger proportion (75%) of the secreted interferons (Bazer et al., 1994). In addition, IFN- δ is secreted by the pig conceptus trophoctoderm and is the shortest type I IFN (Lefevre et al., 1998). Intrauterine injection of pig conceptus-secretory proteins (Bazer et al., 1994) or IFN- δ and IFN- γ (Lefevre et al., 1998) had no effect on interestrus interval, plasma progesterone or timing of CL regression. Therefore, conceptus-derived interferons do not appear to serve as pregnancy recognition signals in pigs as they do in ruminants. Therefore the role of conceptus-produced IFN during the periimplantation period in swine remains an enigma.

Ruminant IFN- τ is a unique subclass of type I IFN family that is secreted by conceptus trophoctoderm. The uniqueness of IFN- τ compared to other type I IFN is due to 1; its lack of viral induction, 2; its massive production by conceptus and 3; its low toxicity (Roberts et al., 1999). Although, IFN- τ possesses antiviral activity, it is not induced by viruses (Roberts et al., 1999). The first evidence that products of conceptus-origin were responsible for CL maintenance in ruminants was from the work of Moore and Rowson in the 1960s and of Martal and associates in the late 1970s (Martal et al., 1979). They show that injection of homogenates or extracts from 15-16 day old trophoblasts extended the CL lifespan. The IFN- τ is secreted by conceptus between day 10-21 of pregnancy and was first named ovine trophoblastin and then ovine trophoblast protein-1 (oTP-1) and finally named as ovine IFN- τ (Bazer, 1989; Bazer et al., 1996; Roberts et al., 1999). Interferon-tau is an

antiluteolytic cytokine that is required for establishing pregnancy in ruminant ungulates including sheep, goats, cows, deer and giraffe (Bazer et al., 1996; Ott et al., 1993; Roberts et al., 1999). Ovine IFN- τ is approximately 18,000 Dalton in molecular weight and consists of several non-glycosylated isoforms, whereas bovine IFN- τ (bIFN- τ) has molecular masses of 22,000 and 24,000 Dalton with N-glycosylated isoforms (Bazer et al., 1996; Demmers et al., 2001). Most type I IFNs contain 166 amino acids with exception of IFN- ω and IFN- τ which have 172 amino acids (Bazer et al., 1996; Demmers et al., 2001; Roberts et al., 1999). Similar to omega, tau interferon contains 6 extra amino acids at the carboxyl terminus which are not required for its biological activities (i.e. antiluteolytic and antiviral properties) (Ealy et al., 1998). Like IFN- α , ruminant IFN- τ genes are intronless and have different isoforms which probably arose from multiple genes (Bazer et al., 1996). A significant biological function of these isoforms is not known, because each oIFN- τ isoform is able to extend CL lifespan when injected into uterine lumen (Ealy et al., 1998; Martal et al., 1990; Ott et al., 1993). Recently, ovine IFN- α isoforms (α 1-4) have been cloned from sheep lymphocytes which share less than 50% sequence identity with IFN- τ (Green et al., 2005). Intrauterine administration of equivalent antiviral units (AVU) of either oIFN- α 1 or - τ extended the estrous cycle to 21 (IFN- α 1) vs. 33 (oIFN- τ) days compared to control (Green et al., 2005).

4.2. Role of interferons in innate and acquired immunity

Since their discovery by Isaac and Lindenmann in the late 1950 (Isaacs and Lindenmann, 1957), IFNs are being subjected to very extensive research to elucidate

their roles in host immune defense. The first response of immunocompetent organisms to a pathogen challenge is an inflammatory reaction which includes secretion of chemokines and/or cytokines. These inflammatory mediators attract or activate the cellular components of the innate arm of the immune system such as neutrophils, macrophages, natural killer (NK) cells and dendritic cells (DC) (Malmgaard, 2004). The cells of the innate immune system, although not pathogen-specific, initiate and coordinate inflammatory processes to combat the invading pathogens. The objectives of these inflammatory mechanisms are either to destroy or contain pathogens until more pathogen-specific destruction occurs by the adaptive immune system (Malmgaard, 2004). The cells of the innate immune system are not completely non-specific; indeed these cells do not attack self antigens, but their activation is mediated by the interactions of foreign organisms/products and the host cell.

Interferons play fundamental tasks as initiators, regulators and effectors of both innate and humoral (adaptive) immune response to viral challenge (Bonjardim, 2005). Indeed, IFN- α/β is produced quite rapidly by host cells upon sensing of viral invasion (Haller et al., 2006). Viral factors such as envelope glycoproteins, CpG DNA, dsRNA interact with cellular pattern-recognition receptors (PRRs), such as mannose receptors, toll-like receptors (TLRs), and cytosolic receptors, to elicit IFN- α/β production (Bonjardim, 2005; Smith et al., 2005). Secreted IFN- α/β binds to their receptors in the same cell (autocrine) or neighboring cells (paracrine) to activate antiviral responses. Cellular antiviral status is increased by activation of early (IRF3, IRF7) and delayed antiviral genes by type I IFNs (Bonjardim, 2005; Haller et al.,

2006; Samuel, 2001). Type I IFN stimulate large numbers of antiviral genes (interferon stimulated genes, ISGs) including, the double-stranded RNA-activated protein kinase (PKR), the 2',5-oligoadenylate synthetases (2'OAS), Mx proteins, RNase L, RNA-specific adenosine deaminase (ADAR) and many others (Bonjardim, 2005).

Interferons essential roles in host immunity are not restricted to the immediate response to intruding microbes (innate response), but equally extend to trigger an acquired (adaptive/humoral) immunity (Kadowaki et al., 2000). For example, IFN- β is required for maturation of the professional antigen presenting cells (APC), the dendritic cells (DCs), and subsequent stimulation of ISGs (Kadowaki et al., 2000; Le Bon and Tough, 2002). Activation of DCs is characterized by production of proinflammatory cytokines, IL12 and tumor necrosis factor-alpha (TNF- α). IL12 stimulates T helper 1 (Th1) cell differentiation and IFN- γ secretion; characteristics of cellular immunity. In addition, DCs are capable of processing and presenting viral antigenic fragments through major histocompatibility complex (MHC) class II to T cells, which sequentially trigger an adaptive immune response (Le Bon and Tough, 2002). The intricate cross-talk between the innate and acquired immune system is mediated by IFNs, a mechanism that was developed to fight the continually immune-evading pathogens.

4.3. Induction of interferons

Host cell invasion by microbes elicits, by themselves or through their by products, cell sensory system to induce IFN production. Cells respond to the presence

of invading organism either viral (Haller et al., 2006; Samuel, 2001) or non-viral including bacteria, protozoa, and helminthes (Bogdan et al., 2004; Mattner et al., 2004; Ritter et al., 2004; Schleicher et al., 2005; Schleicher et al., 2004; von Loewenich et al., 2004) through the interaction between the organisms or their secreted factors or replication by-products and cellular PRRs such as mannose receptors, and toll-like receptors (TLRs) to elicit IFN- α/β production (Bogdan et al., 2004; Bonjardim, 2005; O'Neill, 2006; O'Neill et al., 2003; Smith et al., 2005). Pathogen associated molecular patterns (PAMPs) including viral glycoproteins, double-stranded RNA (dsRNA), single-stranded RNA (ssRNA), and bacterial lipopolysaccharide (LPS), CpG oligonucleotides, GU-rich single-stranded RNA interact with cellular PRRs (Bogdan et al., 2004; Malmgaard, 2004; O'Neill, 2006; O'Neill et al., 2003).

The induction of IFN- α/β during viral challenge occurs by the interaction of host PRRs and viral PAMPs through extracellular and endosomic (intracellular) receptors (Malmgaard, 2004). Extracellular lectin receptors, particularly the mannose receptors (MR), were found to be important in IFN-alpha synthesis by DCs in response to herpes simplex virus (HSV), vesicular stomatitis virus (VSV) and human immunodeficiency virus-1 (HIV-1) (Milone and Fitzgerald-Bocarsly, 1998). Toll-like receptors (TLRs) are growing class of PRRs which are either extracellular transmembrane or intracellular receptors (Ahmad-Nejad et al., 2002; Matsumoto et al., 2003). The TLRs signaling cascades through four different toll/IL-1 receptor (TIR) domain-containing adaptors which include MyD88; TIR domain-adaptor protein (TIRAP); TIR domain inducing IFN- β (TIRF), also called TIR-containing

adapter molecule (TICAM)-1; and TRIF-related adapter molecule (TRAM) (O'Neill, 2006; O'Neill et al., 2003; Oshiumi et al., 2003). The interaction between dsRNA and TLR-3-TRIF results in activation of IRF3 and NF- κ B which induce IFN- β expression (Fitzgerald et al., 2003). The best characterized PRR intracellular receptor is PKR. Double-stranded RNAs (dsRNAs), by-products of viral replication cycle, bind to PKR which results in activation of NF- κ B and subsequent induction of IFN- α/β (Malmgaard, 2004). Another recently identified intracellular PRR is RNA helicase retinoic acid inducible gene1 (RIG-1) which can bind to viral dsRNA and activate IFN- β expression in a similar fashion of PKR (Yoneyama et al., 2004). It is unambiguous that the host cells developed complex sensory systems to ensure its survival by creating antiviral state, where IFNs, specifically IFN- α/β , are key players. Following viral assault, IFN- α/β are the first cytokines that are secreted from host cells. They bind to their receptors to activate highly regulated Janus tyrosine kinases (JAK)-Signal transducer and activator of transcription (STAT) pathway signaling cascades.

4.4. Interferon signaling pathway

During viral infection, cells respond by production of IFNs. Interferons act on cells to stimulate both early and late responses. Early response occurs as consequence of the interaction between cell-PRRs and viral-PAMPs which results in activation of IFN- α/β expression/secretion. IFN- α/β acts in a paracrine and autocrine manner to elicit the late response which is achieved by ISGs expression through the JAK-STAT signaling pathway. Type I IFN receptors consists of IFN- α R1 (IFNAR1) and IFN-

α R2c (IFNAR2) chains, while IFN- γ signal through receptor chains IFN- γ R1 (IFNGR1) and IFN- γ R2 (IFNGR2) (Pestka et al., 2004). Ligands binding to the receptor mediate heterodimerization of IFNAR1 and IFNAR2 subunits and IFNGR1 and IFNGR2 subunits (Samuel, 2001).

Interferons and IFN-like cytokines bind to their cognate receptors and JAK-STAT pathway (Stark et al., 1998). The signal transducers and activators of transcription (STAT) proteins are a group of cytoplasmic transcription factors that are activated through tyrosine phosphorylation by the Janus family of tyrosine kinases (JAK) in response to cytokines. There are seven identified members of the STAT transcription factors family; Stat-1, Stat-2, Stat-3, Stat-4, Stat-5a, Stat-5b and Stat-6 and four members of the JAK family; Jak-1, Jak-2, Jak-3, and Tyk-2 (Horvath, 2000). The JAKs are constitutively associated with receptors; hence, ligand binding to IFN transmembrane receptor brings receptor-associated JAKs into close proximity leading to transphosphorylation and resultant activation. Activated JAK phosphorylates specific tyrosine residues on the membrane cytoplasmic domain which acts as docking sites for STAT monomer binding. After binding to the tyrosine docking sites, STAT monomers become phosphorylated and released from the receptors. Phosphorylated STAT monomers form either homo- or hetero-dimers and move from the cytoplasm into the nucleus to bind their target gene promoter elements (Darnell, 1997; Horvath, 2000; Stark et al., 1998).

The JAK-STAT signaling pathway activation by Type I IFN to induce interferon stimulated genes (ISGs) is well-established (Haller et al., 2006; Samuel, 2001; Stewart et al., 2001; Stewart et al., 2002; Stewart et al., 2001). The JAK-STAT

signaling components include the Jak-1, Jak-2 and Tyk-2 kinases and the Stat-1 and Stat-2 transcription factors. IFN- γ signals through Jak-1 and Jak-2 kinases while IFN- α/β utilizes Jak-1 and Tyk-2 kinases. Tyrosine kinase-2 associates with the IFNAR-1 subunit, and Jak-1 associates with IFNAR-2 subunit, whereas, Jak-1 also interacts with IFNGR-1 subunit and Jak-2 interacts with IFNGR-2 subunit (Bach et al., 1997; Mogensen et al., 1999). Phosphorylation of the receptor-associated JAKs leads to the subsequent phosphorylation of STAT proteins. In the case of IFN- α/β , the phosphorylated Stat-1 (p91) and Stat-2 (p113) heterodimer, and non-STAT transcription factor, p48 (also known as IRF9) translocate to the nucleus and form interferon stimulated gene factor-3 (ISGF-3) complex (Samuel, 2001). The trimeric ISGF-3 complex binds to interferon stimulated response elements (ISRE) on the promoter regions of interferon stimulated genes (Haller et al., 2006; Harada et al., 1996; Hug et al., 1988; Ronni et al., 1998; Samuel, 2001; Stewart et al., 2001; Stewart et al., 2002). For IFN- γ , the activated stat-1 homodimerizes to form the gamma-activated factor (GAF) which translocates to the nucleus and subsequently binds the gamma-activated sequence (GAS) elements in IFN-gamma inducible genes (Pine et al., 1994; Samuel, 2001). The interferon signaling pathway is controlled by positive and negative regulatory factors. For example, interferon regulatory factor-1 (IRF-1) and IRF-2 act as positive and negative regulators of ISGs expression, respectively (Choi et al., 2001; Harada et al., 1989). The suppressors of cytokines signaling (SOCS) proteins and protein inhibitor of activated STAT (PIAS) prevent STAT phosphorylation and nuclear translocation and transcriptional actions of STAT,

respectively (Haller et al., 2006). Overexpression of SOCS-1 and SOCS-3 inhibits 2',5'-OAS and MxA stimulation by IFN- α (Vlotides et al., 2004).

4.5. Interferon stimulated genes (ISGs) in the ovine uterus

In addition to the antiluteolytic role of IFN- τ in blocking the pulsatile release of PGF-2 α from uterus (Bazer et al., 1986; Ott et al., 1993; Spencer et al., 2004), IFN- τ acts on the uterine tissues in a paracrine manner to stimulate expression of a large number of genes. Similar to other type I IFNs, the conceptus-secreted IFN- τ utilizes the JAK-STAT signaling pathway to stimulate uterine interferon stimulated genes (ISGs) (Stewart et al., 2001). Several known ISGs are up-regulated in response to IFN- τ in the sheep uterus such as Mx protein (Charleston and Stewart, 1993; Johnson et al., 2002; Ott et al., 1998), STAT-1 and -2 (Choi et al., 2001), IFN regulatory factor-1 (IRF-1) and IRF-9 (Choi et al., 2001), interferon stimulated gene-15 (ISG15) (Johnson et al., 2002; Johnson et al., 2000; Johnson et al., 1999), 2',5'-oligoadenylate synthetase (OAS) (Johnson et al., 2001; Mirando et al., 1991), major histocompatibility complex (MHC) class I (Choi et al., 2002), β -2-microglobulin (β 2MG) (Choi et al., 2002; Vallet et al., 1991) and cathepsins (CTS; (Song et al., 2005). The antiviral properties of several ISGs and their activation by type I IFN are well established; however, their roles in establishment of pregnancy have not been determined (Spencer and Bazer, 2002).

Interferon stimulated gene-15/17 (ISG-15), previously known as ubiquitin cross-reactive protein (UCRP), is synthesized and secreted by pregnant ewe endometrium in response to the conceptus IFN- τ (Johnson et al., 1999). In response

to pregnancy, ISG-15 mRNA and protein levels are increased in the ovine endometrium starting from d 13 through 19. Expression of ISG-15 mRNA and protein is confined to endometrial stroma (ST) and glandular epithelium (GE) during early pregnancy (Johnson et al., 2002; Johnson et al., 1999). Recently, ISG-15 was found to conjugate with cytosolic proteins and to be expressed in deep stroma throughout pregnancy (Joyce et al., 2005). Progesterone is required for ISG-15 stimulation by IFN- τ (Johnson et al., 2000) and along with IFN- τ increases both free and conjugated ISG15 protein (Joyce et al., 2005). Secretion of ISG-15 during early pregnancy and its expression in the ovine endometrium beyond the maternal recognition of pregnancy and at the maternal-fetal interface would suggest that it may play important roles in implantation and pregnancy maintenance (Joyce et al., 2005).

Beta-2-microglobulin (β 2MG) is ~12kD protein that was shown to be secreted from cyclic ewe endometrial explants in response to IFN- τ (Vallet et al., 1991). β 2MG associates with MHC class I molecule heavy α chain by non-covalent bonds (Choi et al., 2002). In the ewe endometrium, β 2MG mRNA and protein are localized in the luminal epithelium (LE) and GE on Days 10 and 12 of the estrous cycle and pregnancy (Choi et al., 2002). As pregnancy progresses (Day 12-20), β 2MG expression is reduced in the LE and superficial GE (sGE) and expressed in endometrial ST and GE only (Choi et al., 2002). The mechanism by which IFN- τ modulates the spatial expression of β 2MG in the ovine endometrium is not clear, however, it is thought to be important for maternal immune-tolerance toward the conceptus semi-allograph (Choi et al., 2002).

2',5'-oligoadenylate synthetase (OAS) is induced by viral dsRNA and mediates both cellular and viral RNA degradation by activating RNase L (Samuel, 2001). In the sheep endometrium, OAS enzymatic activity was higher in pregnant than cyclic ewes (Mirando et al., 1991). In sheep, endometrial OAS protein level was low in cyclic ewes, and increased in ST and GE during early pregnancy. In ovariectomized ewes, intrauterine infusion IFN- τ increased OAS expression in a progesterone-dependent manner (Johnson et al., 2001).

Mx proteins are large GTPases which belong to the dynamin superfamily of mechanochemical enzymes. Mx proteins are the only members of this superfamily with known antiviral actions (Haller and Kochs, 2002). In the ewe endometrium, Mx1 protein is up-regulated by conceptus IFN- τ during early pregnancy (Ott et al., 1998). Similar to other ISG in the uterus, Mx1 expression is co-regulated by steroids in the uterus (Ott et al., 1999). Expression and function of Mx proteins during early pregnancy in the uterus and peripheral blood immune cells is the focus of research in our laboratory.

5. Mx proteins

Mx proteins are antiviral proteins that play an important role in the innate immune system against viruses in different species (Lee and Vidal, 2002). Mx proteins were first discovered as antiviral proteins that conferred resistance to influenza A virus as a heritable trait in A2G mice (Lindenmann, 1964, 1962). In 1962, Lindenmann found that inbred A2G mice were resistance to influenza A virus infection which is lethal to other inbred mouse strains (Lindenmann, 1962). The

resistance of A2G mice was attributed to a single autosomal locus which was eventually named Mx because it confers resistance to myxovirus (influenza A) infection (Lindenmann, 1964). Since Lindenmann's discovery of Mx proteins, 1-3 Mx genes have been cloned from every species studied including; fish, birds, ruminants, rodents, and humans (Horisberger and Gunst, 1991; Lee and Vidal, 2002; Leong et al., 1998; MacMicking, 2004). Structurally, Mx proteins are classified as large GTPases with mechanochemical properties that belong to the dynamin superfamily (Haller and Kochs, 2002; Nakayama et al., 1993; Praefcke and McMahon, 2004). The dynamin superfamily of proteins are involved in many processes including budding of transport vesicles, division of organelles, cytokinesis and pathogen resistance (Praefcke and McMahon, 2004). All Mx protein sequences are characterized by the presence of a tripartite GTP-binding domain, a dynamin family signature domain, and a leucine zipper domain, which are strikingly conserved in all species (Lee and Vidal, 2002). In all investigated species, there are 2-3 Mx proteins; however, not all of them have antiviral actions (Lee and Vidal, 2002; Nakamura et al., 2005). Therefore, Mx proteins which lack antiviral activity may have other physiological roles in the host cells (King et al., 2004; Toyokawa, 2005).

5.1. Overview of known Mx proteins in humans, rodents and ruminants

Myxovirus-resistance (Mx) proteins were identified in humans (Aebi et al., 1989; Staeheli and Haller, 1985), mice (Horisberger et al., 1983; Jin et al., 1999; Lindenmann, 1964; Staeheli et al., 1983; Staeheli and Sutcliffe, 1988), rats (Horisberger, 1988; Meier et al., 1988), sheep (Charleston and Stewart, 1993; Ott et

al., 1998; Szuchet et al., 2002), and cattle (Ellinwood et al., 1998; Horisberger, 1988). Because of human health concerns, the roles of Mx proteins as antiviral agents are heavily studied in human and rodents (Haller and Kochs, 2002). In humans, there are two distinct Mx proteins with guanosine triphosphatase (GTPase) activity; human cytoplasmic MxA (MxA) (Staeheli and Haller, 1985) and MxB (MxB) protein which is found in the nucleus and cytoplasm (Melen et al., 1996). Human MxA and MxB proteins are products of distinct genes encoded on human chromosome 21 (Haller and Kochs, 2002; Horisberger, 1995; Horisberger et al., 1988) and share approximately 62% sequence homology (Aebi et al., 1989). To date no antiviral properties have been described for MxB (Pavlovic et al., 1990). Human MxA has molecular mass of 76 kDa, a cytoplasmic granular staining pattern, and is associated with actin, tubulin, the smooth endoplasmic reticulum (sER) (Accola et al., 2002; Horisberger, 1992). Human MxB has molecular mass of 78 and 76 kDa for nuclear and cytoplasmic forms, respectively. Human nuclear MxB has a nuclear localization signal on the amino terminus and is localized as granular structures in the heterochromatin region beneath the nuclear envelope. The cytoplasmic form of MxB has granular staining in the cytoplasm similar to MxA (Melen et al., 1996). However, both nuclear and cytoplasmic forms of MxB can bind to each other and form hetero-oligomers, thus, the cytoplasmic form can be carried to nucleus (Melen and Julkunen, 1997).

Structural and functional characteristics of Mx proteins have been studied extensively in rodent species. Since the first discovery of the Mx allele in the A2G mice strain (Lindenmann, 1964, 1962), two and three Mx proteins were identified in mice and rats, respectively (Lee and Vidal, 2002). In mice, there are Mx1 and Mx2

genes mapped on chromosome 16, a homologous chromosome to human chromosome 21 where human MxA and B are located (Reeves et al., 1988). Murine Mx1 is a nuclear protein of 72 kDa molecular weight whereas Mx2 is cytoplasmic protein with 74 kDa molecular weight (Horisberger et al., 1983; Jin et al., 1999; Jin et al., 2001; Staeheli and Sutcliffe, 1988). This represents the converse localization of that exhibited by their human counterparts, MxA and MxB. This makes the rodent, a less than ideal model for human MxA function. In contrast to the rodent Mx1 gene, Mx2 gene cloned from BALB/c and CBA strains has no antiviral activity as result of frame-shift mutation (Staeheli and Sutcliffe, 1988). However, feral mice strains NJL (*Mus musculus musculus*) and SPR (*Mus spretus*) carry a normal Mx2 gene that has antiviral action against vesicular stomatitis virus (VSV) (Jin et al., 1999). In rats, three Mx proteins (Mx1, Mx2 and Mx3) have been identified and Mx1 and Mx2 but not Mx3 has antiviral activity (Horisberger, 1988; Meier et al., 1988).

Similar to other species, sheep and cows expresses Mx genes (Charleston and Stewart, 1993; Ellinwood et al., 1998; Hicks et al., 2003; Ott et al., 1998; Phua and Wood, 1997; Szuchet et al., 2002; Yankey et al., 2001). In cattle, there are two distinct genes; Mx1 (Ellinwood et al., 1998) and Mx2 (GeneBank accession # NM_173941). Bovine Mx1 gene alleles (Ellinwood et al., 1998), splice variants (Kojima et al., 2003), and genetic polymorphism (Nakatsu et al., 2004) have been reported with no apparent affect on its antiviral activity against VSV (Nakatsu et al., 2004). The bovine Mx1 protein has a molecular weight of 75 kDa (Hicks et al., 2003) and mapped to chromosome number 1 (Ellinwood et al., 1999). The molecular weigh for bovine Mx2 has not been determined, but it was localized, along with Mx1, on the

bovine chromosome 1 (*Bos taurus* chromosome 1 genomic contig; NW_929716). In sheep, there is only one Mx1 gene (Mx1) and it was cloned from sheep endometrium (Charleston and Stewart, 1993) and brain (Szuchet et al., 2002), mapped to chromosome 1 (Phua and Wood, 1997). The antiviral activity of ovine Mx1 protein is not yet characterized. However, during early pregnancy, ovine Mx1 and bovine Mx1 are highly expressed in the endometrium (Hicks et al., 2003; Ott et al., 1998; Stewart et al., 1992).

5.2. Structure of Mx genes and proteins

The estimated size of the mouse Mx1 gene is approximately 55 Kb and made up of 14 exons (Hug et al., 1988). Although the size of mouse Mx2 was not determined but it was predicted to be similar to Mx1 in size and made up of 14 exons (Asano et al., 2003). Both genes encode for 631 and 655 amino acids (aa) for Mx1 and Mx2, respectively (Lee and Vidal, 2002). The human MxA gene is estimated to be approximately 33 Kb and comprised of 17 exons (Tazi-Ahnini et al., 2000). Human MxA and MxB genes encode for 661 and 715 aa, respectively (Lee and Vidal, 2002). Rat Mx genes encode for 651, 659 and 659 for Mx1, Mx2 and Mx3, respectively (Lee and Vidal, 2002). Ovine Mx1 genomic organization and size are not known, but the putative protein is ~653 aa (Charleston and Stewart, 1993; Szuchet et al., 2002). The genomic structure and approximate size of the bovine Mx1 gene has been reported to be over 50 Kb containing 15 exons that encodes a 654 aa protein (Ellinwood et al., 1998; Gerardin et al., 2004). Regardless of the gene organization,

the Mx proteins from various species share conserved domains that are important for their antiviral and physiological functions (Haller and Kochs, 2002).

Because of structural similarities, the Mx proteins are classified as members of the dynamin superfamily of large GTPases that include; the Mx proteins, the *Arabidopsis thaliana* GTP-binding proteins, and the yeast vacuolar sorting protein Vps1p (van der Blik, 1999). The Mx protein sequences contain a consensus tripartite GTP-binding domain (GBD) or GTPase domain, and the dynamin family signature domain on the amino terminus. In addition, they possess a central interactive domain (CID) and GTP effector domain (GED) which contains leucine zipper residues on the carboxy terminus region (Haller and Kochs, 2002). The mouse Mx1 nuclear localization signal (NLS) is located on the carboxy terminus (Noteborn et al., 1987), while human MxB NLS is located on the amino terminus of the protein (Melen et al., 1996). Mx proteins lack a pleckstrin-homology (PH), and proline-rich domains which are distinctive features of dynamins and are thought to mediate interactions with membranes (Haller and Kochs, 2002). Mx proteins possess a high intrinsic rate of GTP hydrolysis which is required for mouse Mx1 and human MxA antiviral activities (Pavlovic et al., 1993; Pitossi et al., 1993). The self-assembly motif is located on the amino terminus of human MxA and mouse Mx1 proteins, which enable, in a GTP-dependent mechanism, them to form large oligomeric-complexes (Kochs et al., 2002; Nakayama et al., 1993). Although, the carboxy-terminal leucine zipper motif of human MxA was found to mediate homo-oligomerization or interaction with other proteins (Melen et al., 1992), oligomerization between human MxA and MxB does not occur (Melen and Julkunen, 1997). Collectively, both amino-

and carboxy termini are important for Mx protein antiviral and physiological functions.

5.3. Antiviral properties of Mx proteins

The induction of Mx proteins by IFN α/β and by viruses make them unique markers to discriminate between viral and bacterial infections and to monitor the body's response to immunization or administration of type I IFNs (Chieux et al., 1999; Halminen et al., 1997; Roers et al., 1994; Spencer et al., 1999). The antiviral spectrum of Mx proteins has been described using experimental infections with RNA viruses, such as Influenza A viruses, Thogotovirus, Dhorivirus, VSV, Measles virus, Bunyavirus, Semliki Forest virus, Parainfluenza 3 viruses, and Hantavirus (Frese et al., 1996; Frese et al., 1995; Haller et al., 1998; Hefti et al., 1999; Thimme et al., 1995). The Mx proteins antiviral specificity and activity depend on several factors such as, Mx GTPase activity, type of virus, host/species, and Mx cellular localization (Lee and Vidal, 2002).

Cells stably transfected with nuclear mouse Mx1 cDNA acquire resistance against three orthomyxoviruses; Thogoto virus, Dhori virus and Batken virus (Garber et al., 1991; Haller et al., 1995; Thimme et al., 1995), as well as influenza (Arnheiter et al., 1990) . However, the same cells were susceptible to infection by other rhabdovirus; including VSV and Semliki Forest virus (SFV). The cytoplasmic mouse Mx2 protein was shown to confer resistance against VSV and Hantavirus, but was not effective against influenza virus (Jin et al., 2001; Zurcher et al., 1992). Single amino acid substitutions in the NLS of mouse Mx1 relocalized the protein to the cytoplasm

and abolished its antiviral activity. The antiviral activity was restored by targeting the NLS-mutated protein back into the nucleus (Zurcher et al., 1992). In rats, Mx1 protein (nuclear) is active against influenza virus but has weak activity against VSV. On the other hand, Mx2 (cytoplasmic) is effective in inhibiting VSV but had no activity against influenza virus. Interestingly, specificity against influenza virus was attained but lost against VSV, when experimentally-engineered Mx2 was shuttled into the nucleus (Johannes et al., 1993; Meier et al., 1990). Human MxA is active against wide range of viruses such as, Influenza A, VSV, Measles, Parainfluenza-3, SFV, Hanta, La Crosse and Thogoto orthomyxovirus (Frese et al., 1996; Frese et al., 1995; Kochs et al., 2002; Landis et al., 1998; Pavlovic et al., 1990; Schnorr et al., 1993; Staeheli and Pavlovic, 1991; Zhao et al., 1996). Recently, human MxA was shown to have antiviral activity against Crimean-Congo hemorrhagic fever virus (CCHFV) (Andersson et al., 2006). Despite known antiviral functions of Mx proteins, the exact mechanism by which they accomplish their antiviral tasks is still largely unknown; nonetheless there are antiviral differences between various Mx proteins.

Influenza virus adsorption, penetration, uncoating, and transport are not the targets of Mx1 protein (Broni et al., 1990; Horisberger et al., 1980; Meyer and Horisberger, 1984). Mouse Mx1 protein inhibits influenza virus replication by interfering with viral transcription (i.e. viral RNA synthesis) in the nucleus (Krug et al., 1985; Pavlovic et al., 1992). Mx1 protein expression caused an approximate 50-fold reduction in the primary transcription of viral genes that encode for virus polymerase proteins PB1, PB2, and PA (Pavlovic et al., 1992). In the same study, the authors reported that cells expressing human MxA showed normal nuclear

accumulation of viral mRNA transcripts, therefore, MxA blocked post-transcriptional processes of the influenza virus life-cycle (Pavlovic et al., 1992). Intriguingly, when MxA (MxA-R645) was expressed in the nucleus by the help of foreign NLS, it behaved in a similar fashion to the mouse Mx1 and interfered with primary transcription of influenza virus (Zurcher et al., 1992). Influenza virus-infected cells that express human MxA had apparently normal viral transcription in the nucleus and protein synthesis *in vitro*, but impaired cytoplasmic viral protein synthesis and genomic amplification *in vivo*. These results suggested that MxA might interfere with viral protein translocation or assembly of the newly synthesized viral proteins to the cell nucleus (Pavlovic et al., 1992; Zurcher et al., 1992). Recent studies show that MxA inhibit bunyaviruses such as La Crosse virus, and Bunyamwera virus and Rift Valley fever virus by sequestering the viral nucleocapsid proteins into perinuclear complexes (Kochs et al., 2002).

In sheep, the antiviral activity of Mx proteins has not been studied. However, bovine Mx1 protein has antiviral activity against VSV (Baise et al., 2004; Nakatsu et al., 2004), and paramyxoviruses (Leroy et al., 2005). Bovine Mx1 and human MxA proteins blocked VSV replication. Interestingly, bovine Mx1 was able to block 2 strains of another member of the Rhabdoviridae family, Rabies virus (SAG2 and SADB19 strains) in Vero cells. Human MxA was only able to block replication of the SAG2 strain (Leroy et al., 2005; Leroy et al., 2006). Genetic polymorphisms in the bovine Mx1 gene have been reported (Nakatsu et al., 2004). Nevertheless, bMx1 isoforms exhibit antiviral action against VSV (Nakatsu et al., 2004). Mx1 genetic polymorphism was reported in different chicken breeds as well (Ko et al., 2002).

However, antiviral activity of chicken Mx1 against VSV was abolished by single amino acid substitution at position 631(Ser to Asn) (Ko et al., 2002). Overall, different specie express several Mx proteins and some with several isoforms that posses varied antiviral properties. However, the existence of Mx proteins lacking antiviral activity fuel speculation that Mx proteins have normal cellular functions outside the response to viral infection.

5.4. Cellular functions of Mx proteins

Not all Mx proteins have known antiviral function including human MxB, rat Mx3, duck Mx, and dog Mx1 proteins (Bazzigher et al., 1993; Lee and Vidal, 2002; Meier et al., 1990; Nakamura et al., 2005). Based on their structural similarities to dynamins, it is plausible to speculate that Mx proteins have normal cellular function (Haller and Kochs, 2002). In addition to its antiviral characteristics and in a striking resemblance to dynamin, human MxA was shown to self-assemble into rings-like structures which tubulated lipids in vitro (Accola et al., 2002). Evidence that supports the ability of Mx proteins to have dynamin-like behavior (i.e. vesicle trafficking) came from experiments in which overexpressed human MxA was able to perturb transferrin endocytosis (Jatiani and Mittal, 2004). King et al., (2004) found that human MxB was localized at the cytoplasmic face of nuclear pores and appeared to be involved in nuclear import and cell cycle progression (King et al., 2004). Studying the temporal and spatial expression of Mx proteins under normal physiological circumstances may help in understanding their cellular functions.

5.5. Mx proteins in reproduction

Mx proteins are expressed in the endometrium of sheep, cows, gilts, mare, mice and women (Chang et al., 1990; Charleston and Stewart, 1993; Ellinwood et al., 1998; Hicks et al., 2003; Ott et al., 1998). Sheep Mx (now called; ovine Mx1) mRNA and protein are expressed in the endometrium during estrous cycle and highly up-regulated in response to pregnancy (Ott et al., 1998). In the ewe endometrium, oMx1 protein was detected on day 1, substantially increased on day 13 and declined on day 15 of the estrous cycle (Ott et al., 1998). In cyclic ewes, oMx1 was expressed in LE on day 1-3, increased in shallow GE (sGE) from day 5-13, and decreased on day 15. Interestingly, the temporal pattern of oMx1 expression correlated with peak levels of progesterone during the estrous cycle which suggests that P4 may regulate oMx1 expression as has been shown for other ISG (Spencer and Bazer, 2002). Indeed, intrauterine administration of IFN- τ failed to induce oMx1 expression in the absence of ovaries. Mx1 expression in response to IFN-tau was restored partially or completely by treatment of ovariectomized ewe with estrogen or progesterone, respectively (Ott et al., 1999). The effect of both steroids on oMx1 stimulation by IFN- τ was abolished with addition of progesterone receptor antagonist (Ott et al., 1999).

Ovine Mx1 expression was highly up-regulated (10-fold higher than cyclic ewes) in early pregnancy starting from day 13 through day 19 in LE, GE and stroma and myometrium (Ott et al., 1998). Expression continued to be strong out to day 25 after mating. By comparing the spatial expression of oMx1 and ISG-15 proteins in the

ewe endometrium it was determined that the pattern of expression of these two ISG differed in the endometrium with Mx1 expression being maintained in the LE and superficial GE, while ISG15 expression was confined to the stroma and deep GE (Johnson et al., 2002). This pattern described for ISG15 has been shown for a number of ISG and has led to the hypothesis the IRF-2 restricts ISG expression in the LE and sGE (Choi et al., 2001).

IFN- τ acts on the endometrium in a paracrine manner, however, oMx1 mRNA and protein are highly expressed in the peripheral leukocytes from pregnant sheep, a discovery that is being utilized to detect failed pregnancy in ruminants (Yankey et al., 2001).

The dynamin-like oMx1 protein has the potentials to have normal cellular function in the endometrium such as participating in the uterine gland secretions and/or endometrial cell remodeling during early pregnancy. In addition, the unique expression of oMx1 gene in the endometrial LE and co-regulation by steroids makes oMx1 protein an excellent candidate to study the cross-talk between the endocrine-immune systems during pregnancy and enhance our understanding of sexually transmitted diseases. One of the strategies to understand gene's function(s) is to study factors which regulated its expression.

5.6. Regulation of known Mx gene promoters

Studying IFN and viral regulation of Mx genes was originally initiated by cloning the mouse Mx1 gene promoter (Hug et al., 1988). Subsequent work has characterized Mx promoters from different species including; human MxA, mouse

Mx2, bovine Mx1, chicken Mx, and zebrafish, rainbow trout, and pufferfish Mx1 (Altmann et al., 2004; Asano et al., 2003; Chang et al., 1991; Collet and Secombes, 2001; Gerardin et al., 2004; Kojima et al., 2003; Ronni et al., 1998; Schumacher et al., 1994; Yap et al., 2003). All Mx promoters studied contain 1-3 interferon stimulated response elements (ISRE), cis-acting elements that are required for IFN responsiveness (Samuel, 2001). For example, the Chicken Mx1 promoter has a single ISRE, mouse Mx1 and Mx2 promoters have 2 ISRE and human MxA promoter has 3 ISRE. In fact, ISREs are common elements in all ISGs. Excluding the chicken Mx1 promoter which contains one ISRE, the two proximal ISRE sites apparently act synergistically and are required for IFN responsiveness in Mx1 promoters in human, mice, Zebrafish, and Fugu (Altmann et al., 2003; Hug et al., 1988; Ronni et al., 1998; Yap et al., 2003). Although it is not important for IFN induction, mouse Mx1 and Mx2 and rainbow trout promoter regions have a TATA box which is interestingly absent in the promoters of human MxA, bovine Mx1, chicken Mx, Zebrafish, and Fugu Mx (Altmann et al., 2003; Asano et al., 2003; Chang et al., 1991; Collet and Secombes, 2001; Gerardin et al., 2004; Hug et al., 1988; Schumacher et al., 1994). In most species, putative binding sites for Sp1, IL6-RE, and NF- κ B transcription factors are found in Mx promoter regions; however their roles in the regulating promoter activity are not clear. Because Mx proteins are best known as IFN-induced components of innate immunity, most of the focus on regulation of the Mx promoter has been focused on identifying ISRE sites and may have overlooked other transcription factor binding sites which might be involved in Mx gene regulation.

Transfection of appropriate cell lines with reporter plasmid driven by promoter of interest is a useful tool to determine the promoter transcriptional activity.

6. Cell lines

6.1. Rationale of using cell culture system

Cell culture can be divided into two systems; primary cell culture and cell line systems. Primary cell culture is defined as in vitro isolating and propagating cells of interest from tissues obtained from animals. Primary cells are considered physiologically more relevant to the in vivo system than cell lines. However, primary cell cultures have some disadvantages such as low propagation rate, and due to extensive culturing, deviation of cells from the original cell phenotype. Therefore, transforming cells to make them immortal can help to preserve cell characteristics and provide an unlimited cells supply by freezing. Cell immortalization can be achieved by transfecting cells with DNA viruses such as SV40 and adenoviruses. Using immortalized cell line to address certain clinical problems, novel scientific ideas and hypothesis testing is a more cost-effective, practical and humane approach than using whole animals.

Cell culture is a powerful tool to study gene regulation and functions in a less complex environment. In certain experimental applications, the cost, methodology and regulations make using animal systems is not feasible. In addition, studying the basic gene regulation in cell culture is more advantageous than whole animal where other variables are difficult to control. However, extrapolating scientific findings from cell culture experiment to whole animal physiological or pathological state

should be done with caution. Separating cells from their tissue-context where cell-to-cell contact or interaction is critical for gene regulation/function might lead to abnormal cell physiology and therefore, misleading response to different treatments. Therefore, in vitro experimental findings should be confirmed by in vivo studies.

6.2. Ovine immortalized endometrial cell lines

In most laboratories, using immortalized cell lines is a common scientific methodology to understand specific physiological or pathological condition. Ovine endometrial cell lines including luminal epithelium (LE), glandular epithelium (GE) and stroma (ST) were developed by Dr. Greg Johnson at Texas A&M University (Johnson et al., 1999). Ovine endometrial cells were prepared by enzymatic digestion from day 5 cyclic ewe endometrium and transformed by using a retroviral vector (Johnson et al., 1999). Transformed cells were allowed to propagate by using geneticin-selection medium. Examination of cell morphology using cell-specific markers confirmed each cell line identity. Cells were found to be responsive to IFN treatment (Johnson et al., 1999), thus, they are reasonable in vitro model to study ISGs regulation and functions.

Hypothesis:

We hypothesize that there are more than one Mx proteins in sheep which may have different regulations and conserved functions which are important in the establishments of pregnancy in sheep.

Objectives:

1. Clone Mx genes from the sheep endometrium and characterize their expression during late diestrus and early pregnancy in the sheep endometrium, peripheral blood leukocytes (PBL), and corpus luteum (CL). Characterize sheep Mx proteins cellular localization in the ovine glandular epithelium (oGE) cell line.
2. Clone and characterize the oMx1 gene promoter/enhancer region.

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CHAPTER TWO

Effects of estrogen, progesterone, oxytocin and pregnancy on prostaglandin $F_{2\alpha}$ secretion by porcine endometrial stromal and polarized luminal epithelial in vitro

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Abstract:

Endometrial Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) secretion causes luteolysis in pigs. During early pregnancy, luteolysis is by blocked a conceptus-induced switch in the direction of $PGF_{2\alpha}$ secretion favoring secretion into the uterine lumen. To examine the effect of estrogen (E_2) and progesterone (P_4) on the direction and magnitude of $PGF_{2\alpha}$ secretion in response to oxytocin (OT), luminal epithelial cells (LEC) from day 16 cyclic ($n=7$) and pregnant gilts ($n=7$) were isolated and grown on 12-mm Millicell-HA inserts. The LEC were co-cultured in steroid free medium (control) or in media containing 10 nM E_2 , 100 nM P_4 , or 10 nM E_2 + 100 nM P_4 . When cells reached confluence and were polarized (i.e., electrical resistance $>500 \Omega/cm^2$), they were treated on both the apical and basolateral surfaces with 0 or 100 nM oxytocin (OT) and the medium was collected 3 hr later for RIA of $PGF_{2\alpha}$. Stromal cells (SC), obtained from the same animals and grown directly on the plastic surface of 24-well culture plates, were subjected to the same steroid treatment and used as a positive control for response to OT treatment. $PGF_{2\alpha}$ secretion from the basolateral surface of LEC was greater than the apical surface secretion in both cyclic and pregnant gilts ($P < .01$). In cyclic gilts, $PGF_{2\alpha}$ secretion from the basolateral surface was greater than in pregnant gilts ($P = 0 .01$). In cyclic gilts only, E_2 increased $PGF_{2\alpha}$ secretion from the basolateral surface in response to OT treatment ($P < 0.01$). In the absence of OT, P_4 increased basolateral surface $PGF_{2\alpha}$ secretion ($P < 0.05$) in cyclic gilts. Cells incubated with P_4 secreted less $PGF_{2\alpha}$ from the basolateral surface than controls ($P = 0.07$) in response to OT. In LEC from pregnant gilts, P_4 stimulated $PGF_{2\alpha}$ secretion from the basolateral surface ($P < .05$) after OT treatment. In cyclic gilts, the

basolateral surface of LEC treated with $P_4 + E_2$, secreted more $PGF_{2\alpha}$ in the absence of OT ($P < 0.01$); however, treatment with OT decreased $PGF_{2\alpha}$ secretion ($P < .05$). In pregnant gilts, $E_2 + P_4$ did affect $PGF_{2\alpha}$ secretion in response to 0 or 100 nM OT. Results support the hypothesis that the status of the animal (pregnant vs. cyclic) and steroidal environment of the uterus affect the directional secretion of $PGF_{2\alpha}$. Not surprisingly, E_2 had a profound effect on secretion from the basolateral surface, but P_4 also has an important effect. In the directional $PGF_{2\alpha}$ secretion from polarized LEC, it appears that changes in secretion from the basolateral surface are more critical than the apical surface in the endocrine-exocrine shift of $PGF_{2\alpha}$ secretion in pigs.

Introduction:

In domestic animals, physiological control of the estrous cycle is a uterine-dependent event (McCracken et al., 1999). Release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the uterus in a pulsatile manner causes corpus luteum (CL) regression (i.e., luteolysis) in ungulates (McCracken et al., 1972; Miranda et al., 1996; Schramm et al., 1983). Oxytocin (OT), acting through cell surface OT receptors, causes pulsatile release of $PGF_{2\alpha}$ from the endometrium (McCracken et al., 1999; McCracken et al., 1995; Miranda et al., 1996). In the absence of the proper embryonic signal that conception has ensued, luteolysis occurs and is marked by sharp decline in plasma progesterone (Moeljono et al., 1977). The decline in progesterone allows for ovarian follicular growth and ovulation, which leads to another opportunity for female receptivity and pregnancy (Bazer, 1989).

Oxytocin stimulates $\text{PGF}_{2\alpha}$ secretion from endometrial tissue of pig in vivo (Carnahan et al., 1999; Carnahan et al., 1996) and in vitro (Carnahan et al., 1999; Uzumcu et al., 1998). In gilts, systemic administration of exogenous OT during late diestrus shortens the interestrus interval (Prince et al., 1995; Sample et al., 2000). Therefore, OT plays an important role in stimulating $\text{PGF}_{2\alpha}$ secretion leading to luteolysis during late diestrus in pigs (Mirando et al., 1996).

Progesterone during early to mid-diestrus inhibits OT receptor expression in the endometrium, an effect called the progesterone-block, and thus prevents early luteolysis (McCracken et al., 1984). Prior to luteolysis during late diestrus, down-regulation of progesterone receptors (PR) in the endometrium results in increase of OT receptors expression, an absolute requirement to initiate luteolytic $\text{PGF}_{2\alpha}$ production in sheep (Spencer and Bazer, 2004). The down-regulation of PR is thought to be caused by the continuous exposure of the endometrium to progesterone, which causes down-regulation of its own receptors (Geisert et al., 1994; McCracken et al., 1999). In addition to preventing premature luteolysis, progesterone has other roles which are important for endometrial release of $\text{PGF}_{2\alpha}$. Progesterone increases accumulation of phospholipids in the endometrial cells (Brinsfield and Hawk, 1973), which in turn, makes arachidonic acid available for $\text{PGF}_{2\alpha}$ synthesis and induces cyclooxygenase-2 (Cox-2) enzyme that is required for converting arachidonic acid to $\text{PGF}_{2\alpha}$ (Eggleston et al., 1990).

In the late 1970s, Bazer and Thatcher were first to introduce the endocrine-exocrine theory of maternal recognition of pregnancy in pigs (Bazer and Thatcher, 1977). This theory states that during early pregnancy, uterine $\text{PGF}_{2\alpha}$ secretion is

directed away from the uterine vasculature (endocrine) toward the uterine lumen (exocrine). This redirection of $\text{PGF}_{2\alpha}$ secretion was proposed to be mediated by conceptus-secreted estrogen (Bazer and Thatcher, 1977); however, the exact physiological mechanism and the contribution of the endometrial cell populations to redirection of $\text{PGF}_{2\alpha}$ secretion during early pregnancy are not well-understood. In cultured porcine endometrial cells, each cell type exhibits a different response to OT treatment (Uzumcu et al., 1998; Uzumcu et al., 2000). For example low, intermediate and high responses were observed in LE, GE and stroma, respectively (Uzumcu et al., 1998). However, the reproductive status of the animal also modulated $\text{PGF}_{2\alpha}$ secretion in response to OT in vitro (Uzumcu et al., 2000). In polarized LE, the basolateral surface of LE cells secreted more $\text{PGF}_{2\alpha}$ than did the apical surface in the absence of OT and the PKC stimulant, phorbol 12-myristate 13-acetate (PMA), and treatment only with PMA and not with OT increased $\text{PGF}_{2\alpha}$ secretion from both the apical and basolateral surfaces (Braileanu et al., 2000).

During early pregnancy, the endometrium is under the influence of maternal progesterone and conceptus estrogen; however, the effect of both steroid hormones on directional secretion of $\text{PGF}_{2\alpha}$ from stromal cells and polarized LE cells of pregnant gilts is not known. Therefore, the objectives of this study were to determine the effect of OT on: 1) directionality of $\text{PGF}_{2\alpha}$ secretion from LE and stromal cells obtained from Day 16 cyclic and pregnant gilts endometrium; 2) effects of estradiol- 17β (E_2), progesterone (P_4) and $\text{E}_2 + \text{P}_4$ on directional secretion of $\text{PGF}_{2\alpha}$ from LE 3) effects of E_2 , P_4 , and $\text{E}_2 + \text{P}_4$ on $\text{PGF}_{2\alpha}$ secretion from stromal cells.

Materials and Methods:

Animal procedures and tissue collection

All animal handling procedures were approved by the institutional animal care and use committee (IACUC) at Washington State University. Peripubertal crossbred gilts (Yorkshire, Landrace, Large White, Duroc and Hampshire) were observed daily at 08:00–10:00 h for standing estrous behavior in the presence of an intact boar. Onset of behavioral estrous was considered Day 0, and twelve gilts were assigned randomly and equally to either cyclic or pregnant statuses (6 gilts/status). Gilts in the pregnant group were mated to fertile boar at Day 0 and again 24 h later. On day 16 of the estrous cycle or after mating (pregnant group), gilts were hysterectomized, uteri were flushed with 20 ml sterile 0.9% NaCl, and pregnancy was confirmed in mated gilts by the presence of apparently normal conceptuses in the uterine flushings. Day 16 was chosen because previous studies showed that endometrium of cyclic gilts was maximally responsive to OT in vivo (Carnahan et al., 1996) and in vitro (Ludwig et al., 1998). Endometrium (20–25 g) was collected aseptically from one randomly-selected uterine horn and placed in incomplete Hanks balanced salt solution (IHBSS: 5.36 mM KCl, 0.44 mM KH₂PO₄, 4.17 mM NaHCO₃, 0.34 mM Na₂HPO₄, 136.9 mM NaCl, and 5.55 mM glucose) (Uzumcu et al., 1998).

Separation of endometrial cells for culture

Endometrial cells [luminal epithelial (LE) and stromal cells] were isolated by enzymatic digestion and sieve separation, as described previously (Zhang et al., 1991) and modified subsequently (Uzumcu et al., 1998; Zhang et al., 1991). The purity of

each cell type was 97% and 99% for LE and stromal cells, respectively. Luminal epithelial cells were grown under polarizing condition, as described previously (Braileanu et al., 2000). Briefly, cells were seeded onto 12-mm Millicell-HA inserts (0.45 μm pore size; Millipore, Bedford, MA) at density of 2.5×10^6 cells/insert in a volume of 400 μl RPMI-1640. Each insert was then placed in a 24-well plate in a volume of 600 μl RPMI-1640 per well per well such that the level of medium inside the insert was approximately equal to that on the exterior of the insert. After 3 days, medium was replaced with fresh medium. Beginning on Day 5, cellular polarity was assessed daily by measuring electrical resistance with a Millicell Electrical Resistance System (Millipore). Cell monolayers were shown to have reached confluence when electrical resistance increased sharply from one day to the next and was $>500 \Omega/\text{cm}^2$ (Braileanu et al., 2000). Stromal cells were plated at density of 0.5×10^6 cells per well in 24-well plate. Luminal epithelial and stromal cells were cultured at 37°C in humidified atmosphere of 95% air and 5% CO_2 as previously described (Braileanu et al., 2000; Uzumcu et al., 1998). All experiments were carried out using RPMI-1460 supplemented with 10% charcoal-stripped fetal bovine serum (FBS). Endogenous steroids were removed from 500 mL FBS by adding 1.25 g charcoal and 125 mg dextran and gently stirring the mixture overnight at 4°C (Carnahan et al., 2002).

Experiment 1: Effect of 10 nM estradiol (E_2) and 100 nM progesterone (P_4) treatment on directional secretion of $\text{PGF}_{2\alpha}$ by LE from Day 16 cyclic and pregnant gilts in the absence (0 nM) or presence (100 nM) of OT

Luminal epithelial cells obtained from Day 16 gilts were isolated and grown in steroid-free media or media containing 10 nM E₂, 100 nM P₄, or 10 nM E₂ + 100 nM P₄. Luminal epithelial cells were grown on 12-mm Millicell-HA inserts until confluent and polarized (*i.e.*, electrical resistance >500 Ω/cm²), then treated on the apical and basolateral surfaces with 0 or 100 nM OT for 3 hr in serum-free medium. Treatment media were collected from both apical and basolateral surfaces and stored at -20°C until PGF_{2α} concentrations were measured by radioimmunoassay (RIA).

Experiment 2: Effect of 10 nM estradiol (E₂) and 100 nM progesterone (P₄) treatment on PGF_{2α} secretion by stromal cells from Day 16 cyclic and pregnant gilts in the absence (0 nM) or presence (100 nM) of OT

Stromal cells were separated and grown in steroid-free media or media containing 10 nM estradiol (E₂), 100 nM progesterone (P₄), or 10 nM E₂ + 100 nM P₄. After cells reached confluency (*i.e.*, 72-96 hr), the medium was replaced with serum-free medium containing 0 or 100 nM OT and cells were incubated for 3 hr. Treatment media were collected and stored at -20°C for until PGF_{2α} concentrations were measured by RIA.

PGF_{2α} RIA

Concentration of PGF_{2α} in 10 µl culture media from both LE apical and basolateral compartments and stromal cells were assayed in duplicate by RIA as previously described (Uzumcu et al., 1998).

Statistical analysis:

Data for each cell type were subjected to least-squares analysis of variance

(ANOVA) for randomized block designs using the General Linear Models procedure of the Statistical Analysis System (SAS, 1995). For $\text{PGF}_{2\alpha}$ secretion from polarized LE cells in experiment 1, a split-plot ANOVA was performed. The main effects of E, P, and OT were cross-classified, surface of secretion (apical or basolateral) was the split-plot effect, and pig was the block effect. For $\text{PGF}_{2\alpha}$ secretion from polarized stromal cells in experiment 2, the main effects of E, P and OT were cross classified and pig was the block effect. All tests of hypotheses were performed using the appropriate error terms according to the expectation of the mean squares (Snedecor and Cochran, 1980). Statistical significance was set *a priori* at $P < 0.05$ and for trends or tendencies was set *a priori* at $0.05 < P < 0.10$. Least square means and the appropriate standard errors were generated from the ANOVA using the LSMEANS statement of the General Linear Models procedure.

Results:

Prostaglandin- $\text{F}_{2\alpha}$ secretion from the basolateral surface of LE cells obtained at Day 16 post-estrus was greater ($p < 0.01$) than from the apical surface in both cyclic and pregnant gilts (Fig. 1). There was no significant difference between cyclic and pregnant gilts in $\text{PGF}_{2\alpha}$ secretion from the apical surface of LE cells; however, $\text{PGF}_{2\alpha}$ secretion from the basolateral surface was greater ($p < 0.01$) for LE cells obtained from cyclic than pregnant gilts (Fig.1). For LE cells obtained from both cyclic (Fig. 2a) and pregnant gilts (Fig. 2b), $\text{PGF}_{2\alpha}$ secretion from apical surface did not change ($p = 0.5$) in response to treatment with 10nM E_2 , 100nM P_4 or 10 nM E_2 + 100nM P_4 treatment (Fig. 2a and 2b). In addition, treatment with 100 nM of OT, alone or in combination with E_2 , P_4 or E_2 + P_4 , did not affect ($p = 0.9$) $\text{PGF}_{2\alpha}$

secretion from the apical surface in both cyclic (Fig. 2a) and pregnant gilts (Fig. 2b). Treatment with E₂, P₄, and E₂ + P₄ increased ($p < 0.01$) PGF_{2α} secretion from the basolateral surface of LE cells from both cyclic (Fig.3a) and pregnant (Fig. 3b) gilts. Oxytocin treatment failed to stimulate PGF_{2α} secretion from the basolateral surface of LE cells from both cyclic and pregnant gilts. In cyclic gilts and in response to stimulation with 100 nM OT, E₂ increased ($p < 0.01$) PGF_{2α} secretion from the basolateral surface of LE cells; however, treatment with P₄ or E₂ + P₄ abolished ($p = 0.5$) the positive effect of E₂ (Fig. 3a). In pregnant gilts, treatment with P₄ only increased ($p < 0.08$) PGF_{2α} secretion from the basolateral surface after OT treatment, but treatment with E₂ inhibited ($p = 0.8$) OT stimulation of PGF_{2α} secretion (Fig. 3b). In the absence of OT, secretion of PGF_{2α} did not differ ($p = 0.8$) from stromal cells obtained from Day 16 cyclic and pregnant gilts (Fig. 4). However, treatment with 100 nM OT increased ($p < 0.01$) stromal cells PGF_{2α} secretion from both cyclic (Fig. 4a) and pregnant (Fig. 4b) gilts. The increase in PGF_{2α} secretion by OT was attenuated ($p < 0.01$) by P₄ and E₂+P₄ treatment in cyclic (Fig. 4a) gilts and only ($p < 0.01$) by E₂+P₄ treatment in pregnant (Fig. 4b) gilts.

Discussion:

Maternal recognition of pregnancy in pigs is mediated by conceptus estradiol, which redirects PGF_{2α} secretion from an endocrine to an exocrine direction, thus, rescuing the CL from the luteolytic action of PGF_{2α} (Bazer and Thatcher, 1977). The present studies support this hypothesis, because PGF_{2α} secretion from the basolateral surface (*i.e.*, endocrine direction) of luminal epithelial cells was greater in cyclic than

in pregnant gilts. In addition, our results showed that the apical surface secretes less $\text{PGF}_{2\alpha}$ than the basolateral surface in pregnant and cyclic gilts. These results for cyclic gilts confirm previous work from cells obtained on Day 16 from cyclic gilts (Braileanu et al., 2000; Hu et al., 2003). In the present study, oxytocin (OT) failed to stimulate $\text{PGF}_{2\alpha}$ secretion from the apical surface of LE cells obtained from gilts of both reproductive statuses, which agrees with results of (Braileanu et al., 2000), but disagrees with those of (Hu et al., 2003) for cyclic gilts. One possible explanation for this discrepancy is that in the study by (Hu et al., 2003), cells were serum-starved for 16-18 hr before applying OT treatment. In contrast, in our study and that of (Braileanu et al., 2000), cells were cultured in the presence of serum until immediately before OT treatment. Therefore, the presence of serum might have prevented LE cells from becoming responsive to OT. In addition, this study showed that E_2 , and P_4 alone or in combination did not affect $\text{PGF}_{2\alpha}$ output from the apical surface of LE cells in either the presence or absence of a stimulatory dose of OT. In previous work (Hu et al., 2003) and in disagreement with our results, E_2 increased $\text{PGF}_{2\alpha}$ secretion from the apical surface of LE cells from cyclic gilts in response to OT. As indicated above the effect of E_2 could be attributed to serum-deprivation in the study by Hu et al. (2003). The current study showed that LE cells obtained 16 days after estrus were not responsive to OT stimulation in either cyclic or pregnant gilts. These findings are consistent with earlier work that showed LE cells were unresponsive to OT when obtained on Day 16 from cyclic, pregnant or pseudopregnant gilts (Braileanu et al., 2000; Uzumcu et al., 1998; Uzumcu et al., 2000). Although luminal epithelial cells express the greatest number of OT receptors

(Boulton MI, 1995), they are the endometrial cell type that is least responsive to exogenous OT treatment (Uzumcu et al., 1998). This unresponsiveness to OT may be attributed to endogenous OT secretion by LE cells, which acts in an autocrine manner to make LE cells less sensitive to exogenous OT stimulation (Hu et al., 2001).

Steroid treatment (E_2 and P_4) modulated $PGF_{2\alpha}$ secretion from the basolateral surface of LE cells in cyclic and pregnant gilts. In cyclic and pregnant gilts, treatment with E_2 , P_4 and $E_2 + P_4$ increased $PGF_{2\alpha}$ secretion in absence of OT in the present study; however, in the presence of OT, E_2 increased the stimulatory effect of OT in cyclic gilts only. The E_2 -mediated increase in $PGF_{2\alpha}$ secretion in response to OT was reduced by P_4 . In contrast to LE cells from cyclic gilts, P_4 only increased the stimulatory effect of OT on $PGF_{2\alpha}$ secretion from the basolateral surface of LE cells from pregnant gilts. Treatment with E_2 inhibited the increase in OT-induced $PGF_{2\alpha}$ secretion by P_4 . These findings suggest that an antagonistic effect of P_4 and E_2 are required to keep OT-induced $PGF_{2\alpha}$ secretion from the basolateral surface of LE cells in balance during early pregnancy. In our study, P_4 did not enhance OT stimulation of $PGF_{2\alpha}$ production from LE cells, an effect that was demonstrated previously by (Carnahan et al., 2002). These contradictory results may be explained by the use of polarized LE cells and prolonged culture with P_4 (for up to 8 days) in the present study that may result in reduced sensitivity of LE cells to P_4 compared with only 24 hr of treatment with P_4 by (Carnahan et al., 2002).

In stromal cells obtained on Day 16 after estrus, OT treatment stimulated $PGF_{2\alpha}$ secretion from cells from both cyclic and pregnant gilts. However, the stimulatory action of OT on $PGF_{2\alpha}$ secretion was affected by steroid treatment. The

OT-induced secretion of $\text{PGF}_{2\alpha}$ was reduced by treatment with P_4 alone or $\text{P}_4 + \text{E}_2$ in cells from cyclic gilts and by $\text{P}_4 + \text{E}_2$ in pregnant gilts. For Day 16 cyclic gilts, reduction of stromal cell responsiveness to OT in the presence of $\text{P}_4 + \text{E}_2$ was also observed after 72 hr of steroid exposure (Hu et al., 2003). Treatment with P_4 enhanced the basal secretion of $\text{PGF}_{2\alpha}$ but failed to increase the stimulatory action of OT on stromal cells obtained from Day 16 cyclic gilts (Carnahan et al., 2002). The discrepancy in the effect of P_4 and OT interactions in the current study and the results of (Carnahan et al., 2002) might be due to different durations of exposure to progesterone [*i.e.*, >72 hr in this study compared with 24 hr by (Carnahan et al., 2002)]. In the pig endometrium, stromal cells comprise the greatest portion (47%) of the cellular composition (Blackwell et al., 2003). Stromal cells are the most responsive endometrial cell type (*i.e.*, compared with luminal and glandular epithelial cells) in cyclic gilts (Uzumcu et al., 1998), but are somewhat less responsive to OT stimulation in pregnant gilts (Uzumcu et al., 2000). In addition, results from this study showed that P_4 alone or with E_2 reduced the response of stromal cells (the largest cellular component) to OT, which could contribute to redirection of $\text{PGF}_{2\alpha}$ secretion toward the uterine lumen during early pregnancy.

In summary, secretion of $\text{PGF}_{2\alpha}$ from the basolateral surface of LE cells was greater for cyclic gilts than pregnant gilts. Progesterone decreased OT-induced $\text{PGF}_{2\alpha}$ secretion from stromal cells. These results indicate that the reduced secretion of $\text{PGF}_{2\alpha}$ from the basolateral surface of luminal epithelial cells in pregnant pigs along with P_4 -mediated reduction of stromal cell responsiveness to OT may be

involved in endocrine to exocrine reorientation of $\text{PGF}_{2\alpha}$ secretion during early pregnancy in pigs.

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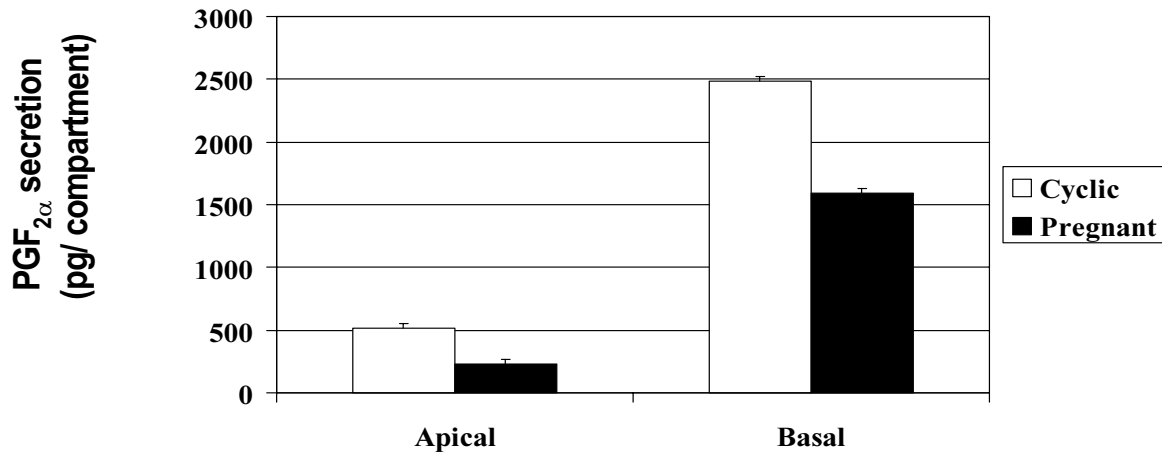


Fig.1. Secretion of $\text{PGF}_{2\alpha}$ from the apical and basolateral surface of LE cells from cyclic and pregnant gilts. Luminal epithelial cells secreted more ($P<0.01$) $\text{PGF}_{2\alpha}$ from basal than apical surface in both cyclic and pregnant gilts. The basolateral surface secretion of $\text{PGF}_{2\alpha}$ was higher ($P<0.01$) in cyclic than pregnant gilts.

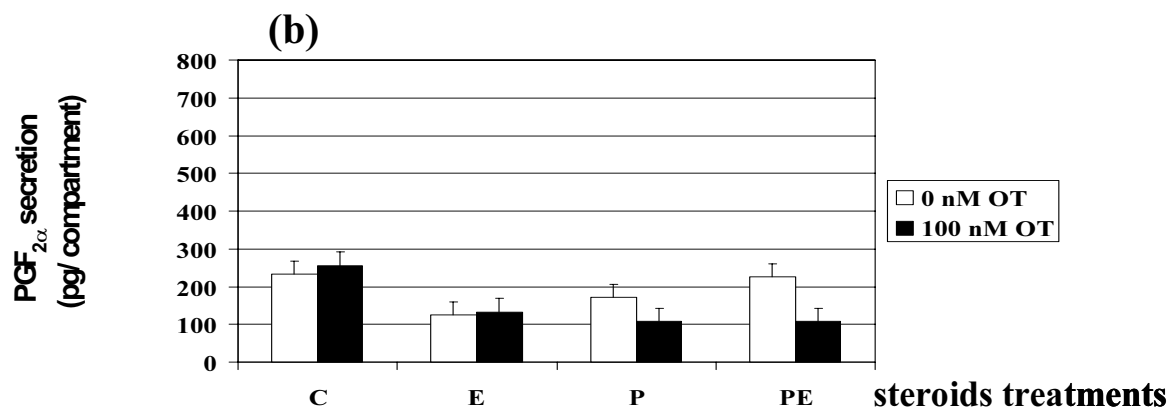
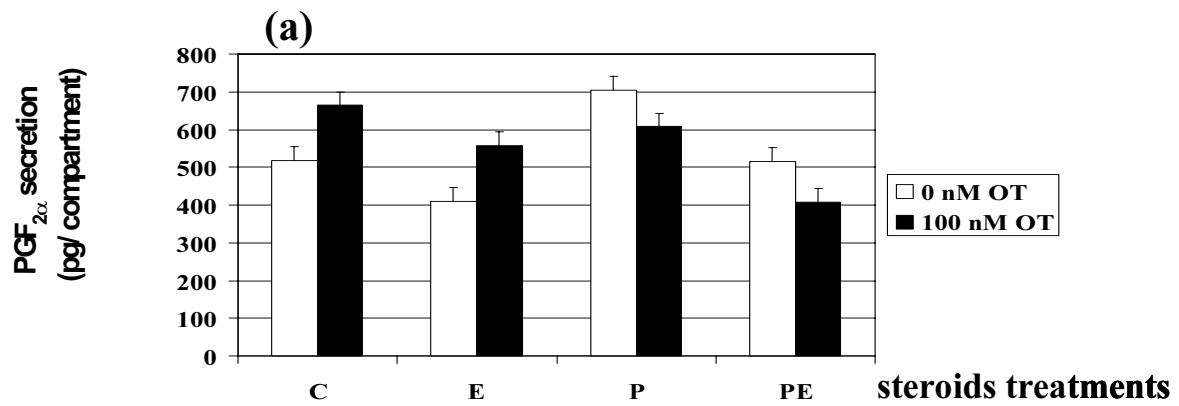


Fig.2. Apical surface secretion of PGF_{2α} from LE cells in cyclic (a) and pregnant (b) gilts. Treatment E2 (10nM), P4 (100nM) or E2 (10nM) + P4 (100nM) did not affect PGF_{2α} secretion ($p=0.5$). LE cells did not respond to OT treatment in the presence or absence of steroids ($P=0.9$)

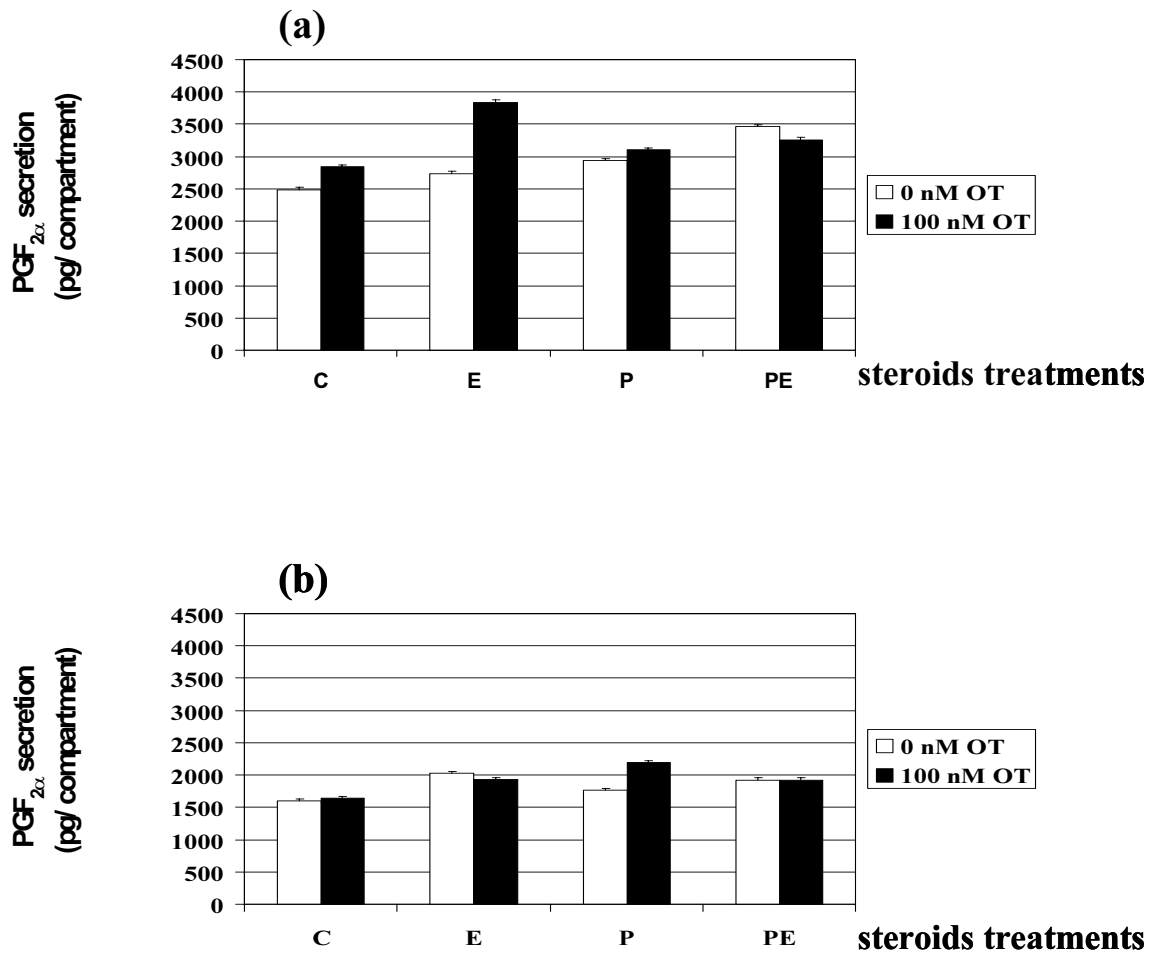


Fig.3. Secretion of $\text{PGF}_{2\alpha}$ from the basal surface of LE cells obtained 16 days after estrus in cyclic (a) and pregnant (b) gilts. Treatment with E2, P4, and E2 + P4 increased ($P < 0.01$) $\text{PGF}_{2\alpha}$ secretion from the basal surface of LE cells in both cyclic (a) and pregnant (b) gilts. Oxytocin treatment (100nM) increased $\text{PGF}_{2\alpha}$ in the presence of ($P < 0.01$) E and ($P < 0.08$) P in cyclic and pregnant, respectively.

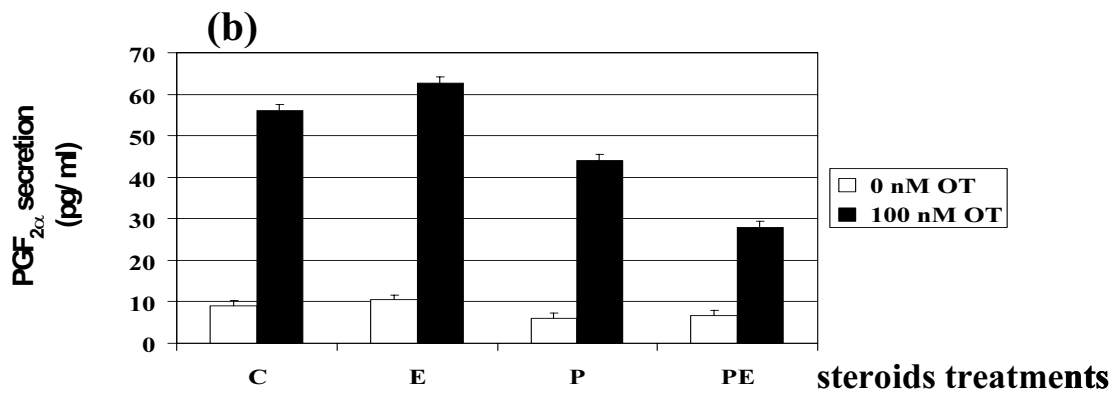
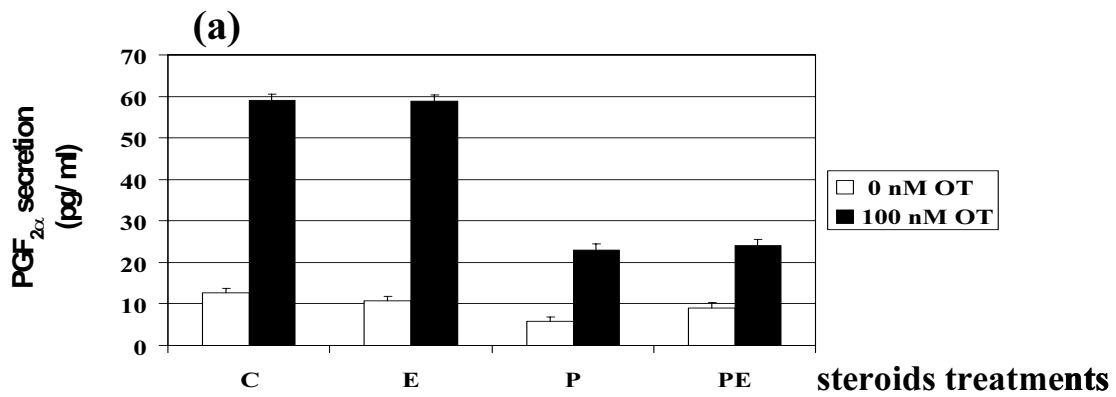


Fig.4. Effect of 0 or 100 nM OT on PGF_{2α} secretion from stromal cells from Day 16 cyclic and pregnant gilts. OT stimulated PGF_{2α} secretion ($P<0.01$) in both cyclic and pregnant gilts. Response to the stimulatory effect of OT was decreased by P 4 and E2+ P 4 in cyclic ($P<0.01$) and by E2+ P 4 in pregnant gilts ($P<0.01$).

CHAPTER THREE

Cloning and Characterization of the ovine Mx2 (oMx2) gene

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Abstract:

The Mx proteins are large GTPases that belong to the dynamin superfamily of mechanochemical enzymes. Mx proteins are induced by type I IFN and by viruses, and are found in most mammals, birds and fish as primary components of the innate response to viral infection. Mx protein is also expressed in the sheep endometrium in response to conceptus-produced IFN- τ . A role for Mx protein in establishment of pregnancy has not been described. Based on results in other species we hypothesized that sheep endometrium expresses more than one Mx protein. A second dynamin-like GTPase gene was cloned from the sheep endometrium and named ovine Mx2 (oMx2). Ovine Mx2 protein shared 93%, 57%, and 70% sequence homology with bovine Mx2, ovine Mx1 (oMx1) and human MxB proteins, respectively. PCR cloning and Northern blot analysis showed that oMx2 transcript size is approximately 2.9 Kb. Expressional profiles of mRNA for oMx2 and oMx1 in endometrium, peripheral blood leukocytes, and the corpus luteum were studied by qRT-PCR or slot blot analysis during late diestrus and early pregnancy in sheep. Endometrial oMx2 mRNA level was up-regulated by pregnancy ($P<0.01$) and increased by 10-fold on Day 13 and by 73-fold on Days 15 and 17, and by 40-fold on Day 19 of pregnancy. In peripheral blood leukocytes from pregnant ewes, oMx2 mRNA was up-regulated in response to pregnancy (Days 15, 17, and 19) ($P<0.07$) and increased on Day 15 12-fold, on Day 17 45-fold, and on Day 19 39-fold. The oMx1 mRNA level increased in response to pregnancy ($P=0.09$) by 7-fold, 22-fold, and 14-fold on Days 15, 17, and 19, respectively. Slot-blot analysis of oMx1 and oMx2 mRNA levels in corpus luteum showed that oMx1 mRNA was detectable but not increased ($P=0.5$) from Day

11 to 19 of pregnancy. In contrast, oMx2 mRNA levels increased ($P<0.07$) in response to pregnancy between Days 13 and 15 and remained elevated through Day 19. In situ hybridization for oMx2 mRNA showed distinct localization of oMx2 transcripts in cells resembling immune cells in the endometrium immediately beneath the luminal epithelium on Day 17 and 19 of pregnant ewe endometrium. The cellular localization of oMx2 protein was different from oMx1 and appeared to be both cytoplasmic and nuclear. The presence and differential expression of two Mx genes sharing moderate sequence homology suggests that they might have distinct functions in the establishment and maintenance of pregnancy in domestic ruminants.

Introduction:

In domestic animals, the estrous cycle is a uterine-dependent and the presence of a conceptus is vital to maintain a functional corpus luteum and therefore establishment of pregnancy (Ott, 1999; Spencer et al., 2004). Maternal recognition of pregnancy in ruminants is achieved by the conceptus-produced interferon tau (IFN- τ) (Bazer et al., 1997). Interferon tau is a novel type I IFN that is secreted by the conceptus mononuclear trophoctoderm between days 10 and 21 of pregnancy in sheep (Bazer et al., 1997). Similar to other type I IFN, IFN- τ activates its target genes using the JAK/STAT signaling pathway (Stewart et al., 2001; Stewart et al., 2001). Conceptus-derived IFN- τ acts locally on the endometrium to up-regulate a number of interferon-stimulated genes (ISGs) including the signal transducer and activators of transcription (STAT) 1 and 2 (Choi et al., 2001), IFN regulatory factor-1 (IRF-1) and IRF-9 (Choi et al., 2001), interferon stimulated gene-15 (ISG15) (Johnson et al., 2002; Johnson et al., 2000; Johnson et al., 1999), 2',5'-oligoadenylate synthetase

(OAS) (Johnson et al., 2001; Miranda et al., 1991), β -2-microglobulin (β 2MG) (Choi et al., 2002; Vallet et al., 1991) and the large GTPase; Mx protein (Charleston and Stewart, 1993; Hicks et al., 2003; Johnson et al., 2002; Ott et al., 1998).

Mx proteins are antiviral proteins that play important roles in the innate immune response to virus infection in different species (Lee and Vidal, 2002). Several distinct Mx genes have been cloned from different species including, fish, birds and mammals (Lee and Vidal, 2002; MacMicking, 2004). Not all Mx proteins have known antiviral actions. In addition, their antiviral properties depend on their sub-cellular localization, species of animal and virus type. For example, mouse Mx1 is a nuclear protein that is active against Influenza A virus (Pavlovic et al., 1992; Zurcher et al., 1992), while Mx2 is a cytoplasmic protein with antiviral action against VSV but not Influenza A virus (Jin et al., 1999; Zurcher et al., 1992). In addition, human MxA (Mx1 ortholog) is a cytoplasmic protein that protect against Influenza virus, and VSV (Pavlovic et al., 1992; Pavlovic et al., 1990) while human MxB protein has both cytoplasmic and nuclear forms and does not have any identified antiviral actions (Melen et al., 1996).

In sheep, only one Mx protein has been identified to date (Charleston and Stewart, 1993; Ott et al., 1998; Szuchet et al., 2002). Ovine Mx1 protein was shown to be up-regulated in response to conceptus IFN- τ in early pregnancy (Hicks et al., 2003; Johnson et al., 2002; Ott et al., 1998). Recently, Mx1 protein was also shown to increase in the peripheral blood leukocytes of early pregnant sheep (Yankey et al., 2001). The number of Mx genes in sheep is not known, however, based on other species, we hypothesize that there is more than one Mx proteins in the sheep.

Therefore, the objectives of the following studies are 1) clone additional Mx genes from pregnant sheep endometrium; 2) characterize Mx gene expression during early pregnancy in the ovine endometrium, peripheral blood leukocytes, and the corpus luteum (CL).

Materials and Methods:

Animal Procedures and Tissue Collections:

All animal procedures and handling were approved by the University of Idaho's IACUC. Thirty two white face ewes were observed daily for estrus using a vasectomized ram. Following 2 consecutive estrous cycles of normal length (18-24 days), ewes were assigned randomly to either cyclic or pregnant groups (3-4 ewes/day/group). Ewes in the pregnant group were mated with a fertile ram at estrus (Day 0) and 24 h later. Ewes were slaughtered and tissues were collected at Days 11, 13 and 15 of the estrous cycle and Days 11, 13, 15, 17, and 19 of pregnancy. Pregnancy was confirmed by the presence of corpus luteum and apparently normal conceptus tissues in uterine flushes. Endometrial tissue and CL were dissected and either immediately homogenized in TRIZOL[®] reagent (Invitrogen, Carlsbad, CA) or snap-frozen in liquid nitrogen and stored at -80C[°] for future RNA extraction. Peripheral blood leukocytes were obtained following centrifugation (300 x g) from jugular vein blood samples collected into EDTA containing Vacutainer tubes (BD Biosciences, San Jose, CA).

Screening of Day 15 pregnant sheep endometrial cDNA library:

Endometrial cDNA libraries were prepared from Day 15 pregnant sheep. Under stringent conditions, approximately 1×10^6 clones were hybridized with partial P³²-labeled cDNA probe that recognized ovine Mx1 gene (GenBank accession # X66093). Plasmid DNA from thirty two positive clones was isolated by using QIAGEN midi-prep kit (QIAGEN, Valencia, CA) and inserts were sequenced by using M13F and M13R universal sequencing primers. Sequencing of large insert was achieved by gene-specific primer walking.

Total and poly-A ribonucleic acid (RNA) isolation:

Endometrial tissues and CL were homogenized in TRIZOL[®] reagent (Invitrogen, Carlsbad, CA) and total RNA was extracted according to the manufacturer's recommendations. Isolation of PBL RNA was performed as previously described (Yankey et al., 2001). Poly-(A) RNA was prepared by using Oligotex mRNA Midi Kit according to the manufacturer's protocol (QIAGEN, Valencia, CA). RNA quality and quantity were examined by RNA-denaturing gel electrophoresis and spectrophotometry, respectively.

Rapid amplification of cDNA ends (5' and 3' RACE) and cloning of oMx2 full-length cDNA:

The SMART[™] RACE cDNA Amplification kit (BD Biosciences, San Jose, CA) was used to synthesize SMART[™] first-strand 5'-RACE-Ready cDNA and 3'-RACE-Ready cDNA from Day 15 pregnant ewe endometrial RNA. The SMART[™] first-

strand cDNA was used to amplify the oMx2 5'UTR and 3'UTR by using BD Advantage™ 2 PCR kit (BD Biosciences, San Jose, CA) .The PCR was carried out by using the Universal primer A mix (UPM) which is provided with SMART™ RACE kit and the oMx2 cDNA specific primer; (oMx2-5'RACE: GCC TCT GCC AAG CTC AGC TTG TT) and (oMx2-3'RACE: TCT GTT ACA TAA GCG AGA CTC TG) to amplify oMx2 5'UTR and 3'UTR. Another set of oMx2 gene-specific primers (oMx2FLfpr: AGC CAA GAA GAC CAG AGG CGC TGA G and oMx2FLrpr: GCT CCA CTT CAG GCA GGC GAG TT) were designed to amplify the full coding sequence of oMx2 from Day 15 pregnant sheep endometrial RNA. The PCR products were gel purified using GenElute™ Spin Columns from (Sigma-Aldrich, St. Louis, MO) and subsequently cloned into PCR® II-TOPO® TA vector (Invitrogen, Carlsbad, CA). The PCR reactions were performed at 94°C for 5 sec and 72°C for 3 min for 5 cycles, then 5 cycles at 94°C for 5 sec, 70°C for 10 sec and at 72°C for 3 min, then followed by 35 cycles at 94°C for 5 sec, 68°C (65°C for 3'RACE) for 10 sec and at 72°C for 3 min.

Generating oMx2 and oMx1 specific cRNA probes:

Northern blot and Slot blot cRNA probes: To distinguish transcript sizes for oMx2 and oMx1 mRNA, gene specific cRNA probes were made by PCR utilizing gene-specific primers coupled with Sp6 and T7 polymerase binding sites. The following primers; oMx2Sp6: *ATT TAG GTG ACA CTA TAG AATTG AGA CTG TCG TGA AGC ATT AC* and oMx2T7: *TAA TAC GAC TCA CTA TAG GGAAC CAG CTT CTC CAT CCT GAA CT* and oMx1Sp6: *ATT TAG GTG ACA CTA TAG AAC GCA CCG ACA CCA GAG*

ACA A, and oMx1T7: TAA TAC GAC TCA CTA TAG GGT GGC TGA AGG CTG ACC ACT A, were used to generate PCR-templates. Touchdown PCR was done using the following thermocycler settings; 5 cycles at 94°C for 25 sec and 76°C for 3 min (-1°C/cycle), then 30 cycles at 94°C for 25 sec, and 69°C for 3 min, then followed by 69°C for 7 min. A single amplified band of the expected size (approximately 250 bp) was generated and the PCR product was purified using QIAquick PCR purification kit (QIAGEN, Valencia, CA) and used for subsequent probe generation. Biotin-labeled cRNA probes for oMx2 and oMx1 were prepared using the MAXIscript Sp6/T7 kit according to the kit protocol (Ambion, Austin, TX).

Poly-A Northern and Slot Blot analysis:

Endometrial poly-A mRNA (1 ug) from Days 15 pregnant, and 11 and 15 cyclic ewes was separated on a 1% agarose, 0.62 M formaldehyde gel and transferred to a nylon membrane (Nitran SuPerCharge; Schleicher & Schull, Keene, NH) by overnight capillary diffusion. The blots were baked for 30 min at 80°C and cross-linked using ultraviolet illuminator (Stratagene, La Jolla, CA) set on auto cross-link. The blots were hybridized overnight with 5 ng/mL of the anti-sense probe using ULTRA-Hyb buffer (Ambion, Austin, TX). The anti-sense cRNA probes were denatured at 95°C for 10 min, cooled on ice for 3 min, added to hybridization buffer and then incubated at 65°C for oMx1 or at 55°C for oMx2 cRNA probe in a rotating hybridization oven (Fisher Scientific, Pittsburgh, PA). Following hybridization, blots were subjected twice to a low stringency wash (2X SCC, 0.1% SDS) at 68°C for 5 min, then to high stringency wash (0.1X SCC, 0.1% SDS) at 68°C for 20 min. Blots were then incubated with blocking solution containing a 1:300 dilution of streptavidin-horse

radish peroxidase conjugate and developed according to the kit's protocol (North2South Chemiluminescent hybridization and detection kit, PIERCE, Rockford, IL). The chemiluminescent signal was detected using the Bio-Rad Fluor-S Multi-imager system (40-mm zoom lens, f-stop = 2.7, exposure time = 600 sec; Bio-Rad Laboratories, Hercules, CA) and quantified using Quantity One software. To quantify oMx2 and oMx1 mRNA levels, 4µg of total RNA from the corpus luteum (CL) (3-4 ewes/day/status) were immobilized on nylon membrane by vacuum filtration, baking and cross-linking as described above (Schleicher & Schull, Keene, NH). After probing with oMx2 and oMx1 cRNA probes, blots were stripped using 0.1% SDS at 90C [should be 100 C] for 30 min, and then hybridized with biotin-labeled anti-sense ovine 18s ribosomal RNA (rRNA) cRNA probe and developed as described above. Data for oMx2 and oMx1 mRNA levels in the CL were adjusted for variation in amount of RNA loaded and transferred using 18s signal as a covariate in the ANOVA.

Endometrial cDNA synthesis:

For cDNA synthesis, 5 ug total RNA was incubated with 1 uL RQ1 DNase (Promega, Madison, WI) and 1 uL Strata Script RT buffer (Stratagene, La Jolla, CA) in 8 uL total volume at 37° C for 30 min. One microliter DNase stop solution (Promega, Madison, WI) was added, and samples were incubated at 65° C for 10 min. Samples were then frozen at -80° C for 1 hr to completely inactivate the DNase enzyme. Three microliters of random primers (Invitrogen, Carlsbad, CA) and 27 uL nuclease free water was added to each sample and incubated at 65° C for 5 min followed by 25° C for 10 min. Nine microliters of a master mix containing 5 uL Strata Script RT

buffer (Stratagene, La Jolla, CA), 1 uL RNase inhibitor (Invitrogen, Carlsbad, CA), 2 uL 100mM dNTPs (Invitrogen, Carlsbad, CA), and 1 uL Stratascript RT (Stratagene, La Jolla, CA) was added to each sample followed by incubation at 42° C for 2 hours and 90° C for 5 minutes. Samples were stored at -20° C.

Peripheral blood leukocytes (PBL) cDNA synthesis:

Because of limited total RNA amounts obtained from PBL, cDNA synthesis was carried out by using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) which is very efficient in synthesizing cDNA from low RNA concentration. One microgram of total RNA was used to make cDNA according to the manufacturer's recommendation. Generated cDNA was subsequently used for qRT-PCR.

Quantitative real time polymerase chain reaction (qRT-PCR):

A working solution of cDNA was prepared by diluting samples 1:10 with nuclease free water. Five microliters of cDNA working solution was added to 15 uL of master mix containing 10 uL SYBR Green (FINNZYMES DyNAmo™ SYBR® Green qPCR Kit, New England BioLabs, Ipswich, MA), 2.5 uL forward primer (2.4 uM for oMx2 and 18s; 0.8 uM for oMx1), and 2.5 uL reverse primer (2.4 uM for oMx2 and 18s; 0.8 uM for oMx1). Duplicate wells for each transcript and for 18s ribosomal RNA from the same animal were run using DNA Engine Opticon® 2 thermocycler (Bio-Rad Laboratories, Hercules, CA). The following primers oMx2 gene (oMx2RTfpr: TTG AGA CTG TCG TGA AGC ATT AC and oMx2RTTrpr: AAC CAG CTT CTC CAT CCT GAA CT), oMx1 gene (oMx1RTfpr: GAA GAT ATG GTG AGG GAA CAT GAA A and

oMx1RTrpr: GAG GGC GAC CCC AGA CAG), and 18s (18Sfpr: AAA CGG CTA CCA CAT CCA AG and 18Srpr: CGC TCC CAA GAT CCA ACT A) were used. Thermocycler settings; 3 min at 95°C, then 41 cycles at 95°C for 30 sec, annealing temp (58.1°C for oMx2 or 56.6°C for oMx1) for 30 sec, 72°C for 30 sec, and then plate read at 78°C for 1 sec, followed by incubating at 72°C for 5 min, then melting curve analysis from 60°C to 92°C at every 0.2°C for 1 sec.

In situ hybridization:

Localization of oMx2 and oMx1 transcripts was performed in serial sections of day 17 and 19 pregnant endometrium using in situ hybridization (ISH) as previously described (Johnson et al., 1999; Ott et al., 1998). Briefly, sections (0.5 µm) were deparaffinized, rehydrated, deproteinated and hybridized overnight with oMx2 and oMx1 anti-sense cRNA probes labeled-[α -³⁵S] uridine 5-triphosphate (PerkinElmer Life Sciences, Boston, MA). After hybridization, washes, and RNase A digestion, autoradiography was performed using NTB liquid photographic emulsion (Eastman Kodak, Rochester, NY). Sections were exposed at 4 °C for 5 days, developed in Kodak D-19 developer, counterstained, dehydrated, and mounted with Parmount (Fisher Scientific, NJ). Images were taken with a Nikon E1000 microscope using DXM 1200 digital camera and ACT-1 software (Nikon, Emeryville, NY). Photographic plates were assembled using Adobe Photoshop (Version 6.0, Adobe Systems Inc., San Jose, CA).

Cell culture, IFN- τ and polyinosinic-polycytidylic acid [poly(I-C)] treatment:

Ovine glandular epithelial (oGE) endometrial cells (Johnson et al., 1999) were cultured in 6-well plates (Corning Inc, Corning, NY), 5% CO₂ at 38.5°C for 24h in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, St. Lois, MO) supplemented with 10% FBS (Invitrogen, Carlsbad, CA). When cells achieved approximately 90-95% confluency, medium was replaced with DMEM medium (no treatment control-NT) or medium containing 10,000 antiviral units (AVU)/mL IFN-tau or 10 ug/mL of polyinosinic-polycytidylic acid [poly (I-C)] (Sigma Chemical Co, St. Louis), and incubated for 20h. Cells were then washed twice with Hanks buffered salt solution (HBSS) and 250 μ L of mammalian protein extraction reagent (M-PER, PIERCE, Rockford, IL) was added and then incubated at room temp for 10 min with gentle rocking. Cell lysates were collected and stored at -20°C until assayed.

Generating oMx2 amino and carboxyl terminus specific polyclonal antibodies:

The oMx2 amino (oMx2-53; ESN PDF LPN NFN QLN LD) and oMx2 carboxyl peptides (oMx2-541; CNL HQT VQN KIE DIK TKQ M) were synthesized and used to immunize rabbits (2 rabbits/peptide) (Alpha Diagnostics Intl. Inc., San Antonio, TX). These peptides were designed to be 100% homologous to ovine Mx2, and bovine Mx2 with no cross-reactivity with oMx1 or bMx1 proteins.

Protein quantification and Western blotting analysis:

Protein concentrations of cell lysates were measured by BCA protein assay using bovine serum albumin (BSA) standards (PIERCE, Rockford, IL). Twenty five micrograms of oGE cell lysate were dissolved in 1X sample buffer (4X: 7.5 mL dH₂O / 760 mg Tris base/ 2 g SDS/ 10 mL glycerol/ pH to 6.8/ 5 mL 2- β mercaptoethanol/

300 μ L bromphenol blue). Proteins were separated on a 12% SDS-PAGE gel with 6% stacking gel in 1X electrode buffer (10X: 30.3g Tris base/ 144.2 g glycine/ 10g SDS/ pH to 8.3/ add dH₂O to 1.0 L) at a constant current 70 mA for approximately 2h. The proteins in the gel were transferred to nitrocellulose membranes (Protran BA83; pore size = 0.2 μ m, Schleicher & Schuell, Keene, NH) in a Mini-Protean III Cell apparatus (Bio-Rad Laboratories, Hercules, CA) at a constant 70 V for 1h with an ice pack. Blots were rinsed with TBST (20 mL 1M Tris pH 7.5/ 137 mL 1M NaCl/ 500 μ L Tween 20/ dH₂O to 1.0L) and non-specific binding was blocked by incubating membranes in 5% nonfat dried milk in TBST for 2h. Membranes were incubated overnight in TBST including 2% nonfat milk and a 1:100 dilution of the amino terminal rabbit polyclonal oMx2 antiserum (9708 bleed #3, Alpha Diagnostics Intl. Inc., San Antonio, TX) or 1:1000 dilution of oMx1 antiserum (90618 bleed #2, Multiple Peptide Systems, San Diego, CA) at 4 °C. After incubation with primary antibodies membranes were washed 4 times with TBST for 5 min each, and then incubated with HRP-conjugated secondary antibodies (goat anti-rabbit IgGs, PIERCE, Rockford, IL) at 1:200,000 dilution for 1 h. Following incubation with secondary antibodies, membranes were washed 4 times with TBST, for 10 min each and signal was developed with the SuperSignal® West Femto kit according to the manufacturer's protocol (PIERCE, Rockford, IL). Images were collected using a Bio-Rad Fluor-S Multi-imager system and Quantity One software (40-mm zoom lens, f-stop = 2.7, exposure time = 600 sec; Bio-Rad Laboratories, Hercules, CA).

Cellular localization of oMx2 and oMx1 proteins by Immunofluorescence:

Ovine GE cells (3×10^5 cells/well) were cultured as described above. Cells were plated on 3 cover slips per well in 6 well plates for 24 h and then treated with IFN- τ (10,000 AVU/mL) for 12 h. Cover slips were placed in 24-well plates and rinsed at room temperature (RT) with 1 mL of 1X PBS (10X: 80 g NaCl/ 2 g KCl/ 14.4 g Na_2HPO_4 / 2.4 g KH_2PO_4 / dH_2O to 1.0L/ adjust pH to 7.2). Following washing, cells were fixed with 1 mL of freshly-prepared 3% formaldehyde (Polysciences, Inc, Warrington, PA) for 10 min at RT on a rocker and then washed with 1 mL of 1XPBS for 5 min twice, at RT, on a rocker. The following procedure was used for oMx1 and oMx2 localization with exception of that non-fat milk was used instead of BSA for blocking non-specific binding, primary and secondary antibodies incubation for oMx2. Non-specific binding was blocked with 1 mL of Base (1XPBS/0.05% Triton X-100/ 2% BSA) overnight at 4°C. Following overnight blocking with Base, cells were incubated overnight at 4°C in 1 mL of 1XPBS-2% BSA containing 1:2000 dilution of an amino terminal rabbit polyclonal oMx2 antisera (9708 bleed #3, Alpha Diagnostics Intl. Inc., San Antonio, TX) or 1:1000 dilution of an amino terminal rabbit polyclonal oMx1 antisera [90618 bleed #2, Multiple Peptide Systems, San Diego, CA]. Pre-immune rabbit serum at a dilution of 1:1000 for oMx1 or 1:2000 for oMx2 was used as a negative control. Cells were also incubated in 1XPBS-2% BSA without immune serum as a negative control. After incubation with primary antibodies, cells were washed with Base three times for 5 min on a rocker at RT. For localization of Lamin A/C, cells were incubated in 1 mL of 1XPBS-2% BSA containing 1:1000 dilution of conjugated monoclonal mouse anti-human Lamin A/C

for 3 h at room temp and then washed with 1XPBS. Following washing, cells were incubated in 1 mL of 1XPBS-2% BSA including a 1: 2000 dilution of goat anti-rabbit Alexa 488 (Molecular Probe, Inc, Eugene, OR) for 1 h at room temperature. Hoechst reagent (Sigma, St. Lois, MO) was used for nuclear staining for 5 min on a rocker. After washing cells with 1XPBS, each cover slip was whole-mounted in a mounting medium (Vector Laboratory, Burlingame, CA) on a glass slide sealed by nail polish. Immunofluorescence signals were detected using an Nikon Eclipse E-1000 fluorescence photomicroscope (Nikon, Japan) and MetaMorph version 6.1 (Universal Imaging Corp., Downingtown, PA).

Protein multi-alignments, DNA sequencing and analysis:

DNA sequencing was conducted by the Molecular Biology Core laboratory in the UI/WSU Center for Reproductive Biology. All DNA and protein sequences were blasted against the NCBI sequence databanks. For DNA alignments and generating consensus sequence, the contig alignment program (CAP) was used (Infobiogen, France, www.infobiogen.fr/services/analyseseq/cgi-bin/cap_in.pl). Protein alignments and phylogentic tree analysis were done using the CLUSTALW program from the San Diego Supercomputer, Biology Workbench website, (<http://workbench.sdsc.edu/>). The NCBI ORF Finder program used to predict oMx2 open reading frames (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>).

Statistical Analysis:

Data from slot-blot analysis of the CL total RNA and from the qRT-PCR for endometrial and PBL were analyzed using the Mixed procedures of Statistical Analysis System (version 9.1, SAS Inc., Gary, NC). The model included main effect of status (cyclic vs. pregnant) and Day (11, 13, 15 cyclic and 11, 13, 15, 17, 19 pregnant) and their interactions. Data are reported as least-squares means and standard errors. The 18s values were run as a covariate in the analysis.

Results:

Screening of Day 15 pregnant sheep endometrial cDNA library:

Thirty two positive clones were isolated and sequenced. Sequence multi-alignments generated 2 contigs (contig A and B) that have homology with published Mx sequences. Contig A shared approximately 99% nucleotide sequence homology with the only known sheep Mx sequence (GenBank Acc.# X66093); however, contig B sequence shared approximately 93% and less than 70% homology with bovine Mx2 (GenBank Acc.# AF355147) and sheep Mx1, respectively.

Rapid amplification of cDNA ends (5' and 3' RACE) and cloning of oMx2 full-length cDNA:

Based on the contig B nucleotide sequence, 5' and 3' RACE primers were designed to recognize contig B but not the published oMx1 sequence. The 5' RACE PCR reaction resulted in a single ~1.3 Kb band and the 3' RACE generated a major band of ~1.2 Kb that were cloned and sequenced. Utilizing the 5' and 3' RACE sequences,

oMx2 gene-specific primers were used to clone 2.4 Kb cDNA containing the full coding region. Sequence alignments of the oMx2 5' and 3' RACE and the full coding region generated 2.889 Kb consensus sequence (GenBank Acc. # AY859475) with a major open reading frame (ORF) of 714 amino acids sequence. The putative oMx2 protein share 93%, 70% and 57% sequence homology with bovine Mx2 (bMx2), human MxB, and ovine Mx1, respectively (table.1). Similar to other Mx proteins, the oMx2 protein had a conserved tripartite GTP binding domain (GDQSSGKS, DLPG and TKPD) and a dynamin-signature motif (LPRGSGIVT) in the amino terminus and leucine zipper motif in the carboxy terminus (fig.1). Phylogenetic tree analysis of Mx proteins from sheep (oMx1 and oMx2), cow (bMx1 and bMx2), human (MxA and MxB), mouse (Mx1 and Mx2), and rat (Mx1, Mx2, and Mx3) revealed that oMx2 is related to bMx2 and human MxB, while oMx1 is related to bMx1 and human MxA (fig.2).

Poly-A Northern analysis:

To determine ovine Mx transcript sizes, specific cRNA probes were designed to recognize either oMx1 or Mx2 and used to hybridize poly-A Northern blots prepared from Day 15 pregnant and 11 and 15 cyclic endometrium. In RNA collected from Day 15 pregnant ewe endometrium, anti-sense oMx1 and oMx2 cRNA probes recognized a single mRNA transcript of approximately 2.5 Kb and 2.9 Kb, respectively (fig.3). Consistent with previous reports (Charleston and Stewart, 1993; Ott et al., 1998), the transcript size of oMx1 was similar to the oMx1 while oMx2

transcript was similar in size to the bovine Mx2 (GenBank Acc.# NM_173941) which is approximately 2.9 Kb.

Steady-state mRNA level of Mx2 and oMx1 during late diestrus and early pregnancy:

Endometrium: Ovine Mx2 mRNA levels in the endometrium were quantified by qRT-PCR. Analysis showed that oMx2 mRNA was detectable in cyclic animals (Days 11, 13, and 15) and up-regulated by pregnancy ($P < 0.01$; fig.4A). During early pregnancy compared to day 11 of the cycle, oMx2 mRNA levels increased 10-fold on day 13, 73-fold on Days 15 and 17 and by approximately 40-fold on Day 19. Statistical analysis of oMx2 transcript levels showed that there was a Day*Status interaction ($P < 0.01$). Analysis of oMx1 mRNA levels by qRT-PCR agrees with previous work (Ott et al., 1998) and showed that oMx1 is up-regulated in pregnancy ($P < 0.01$; fig.4B). For oMx1 levels, a Day*Status interaction ($P < 0.07$) was detected. In comparison to Day 11 of the cycle, oMx1 mRNA increased on Day 13 by 14-fold, Day 15 by 45-fold, Day 17 by 73-fold and by 21-fold on Day 19 of pregnancy, but did not change from days 11 to 15 in cyclic ewes.

Peripheral blood leukocytes (PBL): Steady-state levels of mRNA for oMx2 and oMx1 in the PBL were quantified by qRT-PCR. The oMx2 mRNA was detectable on Days 11, 13, and 15 of the estrous cycle and up-regulated in response to pregnancy (Days 15, 17, and 19) ($P < 0.07$; fig.5A) in pregnant ewes. Levels increased by 12-fold on day 15, 45-fold on day 17 and 39-fold on day 19 of pregnancy compared to oMx2 mRNA levels on Day 11 of the estrous cycle. Statistical analysis of Mx2 levels

revealed that there was Day*status interactions ($P<0.07$). These results are in general agreement with previous work from our lab (Yankey et al., 2001). The oMx1 mRNA levels in PBL from pregnant ewes were higher ($P=0.09$) than cyclic ewes (fig.5B) and increased by 7.5, 12.6, and 14-fold on day 15, 17 and 19 of pregnancy, respectively. There was an effect of day ($P<0.01$) but no effect of status or day*status interactions.

Mx levels in the corpus luteum: oMx2 and oMx1 specific cRNA probes were used in slot-blot analysis of oMx1 and oMx2 mRNA levels in the CL. Slot-blot analysis showed that oMx1 was detectable but did not increase ($P=0.5$) during early pregnancy (fig6B). However, oMx2 increased in response to pregnancy ($P<0.07$) on Day 15 by 2-fold and remained elevated through day 19 (4-fold) of pregnancy (fig.6A).

Localization oMx2 and oMx1 mRNA transcripts in the ewe endometrium by ISH:

Serial cross-sections from Day 17 and 19 pregnant ewe endometrium were hybridized with oMx2 or oMx1 specific cRNA probes (fig.7). The oMx1 cRNA probe hybridization was abundant throughout the luminal epithelium (LE), glandular epithelium (GE) and stroma (ST). However, the pattern for oMx2 localization differed. Hybridization signal for oMx2 was observed predominantly in cells beneath the luminal epithelium that appeared to be immune cells; however, this has not yet been confirmed. The overall intensity of the hybridization signal for oMx1 transcripts was greater than oMx2 which suggests that oMx1 is more abundant than oMx2.

Determination of oMx2 protein molecular mass, and induction by IFN- τ or polyinosinic-polycytidylic acid [poly (I-C)]: Serum from rabbits immunized against oMx2 peptides detected a protein of approximately 75 kDa. The level of the immune-reactive protein increased in response to IFN- τ but did not change in the poly (I-C)-treated or control (NT) samples (fig.8). Incubation of the same samples on a different blot with pre-immune serum did not show any binding of similar size which suggests that the immune-reactive band detected in the IFN-treated sample is specific band for oMx2. Incubation of the same cell lysate with oMx1 antiserum showed the expected size of oMx1 protein (~75 kDa) which was induced by IFN and did not change in other treatments (i.e. NT and poly (I-C)).

Ovine Mx2 cellular localization: oGE cells were treated with IFN- τ for 12 hr and then subjected to immunofluorescence analysis using oMx2 pre-immune and immune sera. Immunofluorescence analysis of oMx2 protein revealed that, in the absence of IFN stimulation, oMx2 protein expression was very low (fig. 9 A). In response to IFN treatment, oMx2 protein staining was observed in the cytoplasm and formed a ring-like structure around the nucleus (fig. 9 D and G) and was co-localized with Lamin (fig9. F and I). Cells incubated with pre-immune sera showed weak and non-specific staining (fig. 9 J). Comparing the staining between pre-immune and immune sera strongly support that pattern of staining in the immune sera is oMx2 protein specific. Incubating cells with oMx1 antisera showed that oMx1 protein staining was predominantly cytoplasmic after induction by IFN (fig. 9 P and S) and there was no co-localization with Lamin (fig.9 R and U). Figure 10 show higher magnification of

the cellular localization of oMx1 (fig.10 A, C, and E) and oMx2 (fig.10 B, D, and F) proteins in oGE cells following IFN treatment.

Discussion:

This is the first report to show the existence of a second Mx protein in the sheep and characterizes its expression during early pregnancy. Sequencing data and Northern blot analysis show the presence of two different ovine Mx proteins; oMx1 and oMx2. Both genes produce different size transcripts (fig.3); Mx2 is approximately 2.9 Kb and Mx1 the expected 2.5 Kb. The similarity of the predicted amino acid sequences between oMx1 and oMx2 is only 57% (fig.2 and table.1). Nonetheless, both proteins possess the conserved tripartite GTP-binding domains, the dynamin signature motif and a leucine zipper domain characteristic of members of this superfamily (Haller and Kochs, 2002). Interestingly, the sequence alignments (table.1) and phylogenetic analysis (fig.2) of ruminant, human and rodent Mx proteins show: 1) ruminant (ovine and bovine Mx1 and 2) are more related to human Mx proteins (human MxA and B) than their rodent counterparts; 2) oMx2 is more related to human MxB (70% homology) than oMx1 (57% homology); and 3) Mx proteins from different species (sheep Mx2 vs. human MxB) have higher sequence homology than similar protein of the same family (oMx1 vs. oMx2, human MxA vs. MxB) within species. These observations strongly suggest that ovine Mx proteins are products of distinct genes that probably arose from a gene duplication event that predated the divergence of these species.

In response to pregnancy, oMx2 and oMx1 proteins were up-regulated in the ewe endometrium (fig.4). Previously we showed that oMx1 was expressed in the ewe endometrium during the estrous cycle (Ott et al., 1998) and increased in response to pregnancy (Charleston and Stewart, 1993; Ott et al., 1998). Here we show that oMx2 mRNA levels were also increased in response to pregnancy.

In situ hybridization of the ewe endometrium at day 17 and 19 of the pregnancy with oMx2 cRNA showed a distinctive localization of the oMx2 mRNA transcripts in cells beneath the luminal epithelium which resemble immune cells (fig.7). The identity of these cells was not established in this study; however, the hybridization pattern of oMx2 mRNA would suggest that these cells are either eosinophils or macrophages. The pattern of oMx2 hybridization resembled that shown for uterine eosinophils expressing monocyte chemoattractant protein-1 and -2 (MCP-1 & -2) (Asselin et al., 2001) or macrophages expressing IFN- γ -inducible protein 10 kDa (IP-10) (Nagaoka et al., 2003) in the early pregnant ewe endometrium. The precise functions of these immune cells in establishment of pregnancy in the sheep uterus are not clear. However, they produce certain chemokines that attract other immune cells into the endometrium such as Monocytes, Natural Killer (NK) cells and T lymphocytes (Farber, 1997; Taub et al., 1993). The physiological role of oMx2 in immune cells in the endometrium is unknown. However, due to the structural similarities between Mx proteins and dynamins which are involved in cellular vesicle trafficking, we postulate that oMx2 may be involved in secretion by the immune cells.

Work from our lab (Ott et al., 1998) showed that the Mx protein was expressed during the estrous cycle and strongly up-regulated by conceptus IFN during early pregnancy. That study demonstrated that sheep Mx mRNA and protein were expressed in the luminal, glandular epithelium, stroma and the myometrium during early pregnancy. In this study and by using oMx1 specific cRNA probe and RT-PCR primers, the temporal and spatial pattern of endometrial oMx1 expression was consistent with our previous work (Ott et al., 1998). Hybridization of the immune cells observed for oMx2 was not seen with oMx1 probe and that could be attributed to either lack of Mx1 expression in that type of immune cells (i.e. oMx2 specific) or possibly oMx1 is expressed by the same population of the immune cell but the signal was overshadowed by the intense hybridization in the adjacent tissues (luminal and glandular epithelium, stroma). Indeed, we previously showed that oMx1 protein immunoreactivity was detectable in immune cells at the interface of the endometrium and myometrium (Ott et al., 1998). In addition, recent reports showed that while oMx1 protein was expressed in the luminal and glandular epithelium and the stroma, ISG15 protein was not expressed in the luminal epithelium, but was expressed in the glandular and stromal tissue of pregnant ewes' endometrium (Johnson et al., 2002). These findings along with the present study are interesting because they show cell-specific expression of interferon-stimulated genes (oMx1, Mx2 and ISG15) in the endometrium which suggest that these genes are regulated differently.

Our comparative expressional profiling of oMx1 and oMx2 in tissues other than the uterus (PBL and CL) during early pregnancy are novel for both genes and

consistent with our previous results examining oMx1 expression in the PBL (fig. 5) (Yankey et al., 2001) but not in the CL (Spencer et al., 1999) (fig. 6). In early pregnant ewes, both genes were up-regulated by pregnancy in the PBL (oMx1; $P<0.09$, oMx2; $P<0.07$). However, it is noteworthy that the fold induction of both genes in the PBL differed on different days of pregnancy which may suggest that the oMx2 response to pregnancy is more robust than oMx1. In the CL obtained from early pregnant ewes, oMx2 expression increased ($P<0.07$) in response to pregnancy on day 15 by 2-fold and remained elevated through day 19 (4-fold) of pregnancy (fig.6A). Conversely, there was no difference in oMx1 mRNA levels in CL obtained from cyclic and pregnant ewes ($P<0.5$; fig6B). These findings may suggest that oMx1 is constitutively expressed in the CL to serve normal physiological functions while oMx2 has pregnancy specific functions. However, lack of oMx1 up-regulation in early pregnancy in this study is inconsistent with early work that demonstrated the Mx (oMx1) and the UCRP (ISG15) are expressed in the ewe CL in response to intrauterine or subcutaneous administration of roIFN- τ (Spencer et al., 1999). This discrepancy could be attributed to the differences in the experimental procedures between the 2 studies. In this study, CL were collected from cyclic/non-mated and from mated/pregnant ewes, whereas in the other study CL were collected from ewes that were exposed to intrauterine administration of IFN- τ at 5×10^6 AVU/horn/day for 4 days. Although, the used dose of IFN- τ was reported to mimic the of IFN produced by conceptuses during early pregnancy (Spencer et al., 1995), the maternal system during normal pregnancy in sheep is exposed to a gradual increase in conceptus-produced IFN- τ starting from day 10 of pregnancy (Bazer et al., 1996).

Infusing IFN- τ at high doses used in that study for 4 days may over-stimulate oMx1 expression in the CL; therefore the data from the present study are more representative of the maternal response to IFN- τ during early pregnancy. Nonetheless, the expression of these antiviral genes in CL in response to pregnancy is rather intriguing. In early pregnancy, the ovine conceptus secretes IFN- τ which acts locally on the endometrium to prevent uterine production of luteolytic pulses PGF2alpha (Spencer and Bazer, 2004). In addition, IFN-tau up-regulates a large number of ISGs including Mx (Ott et al., 1999). However, no antiviral activity could be detected in the uterine vein or lymphatics suggesting that IFN-tau does not escape the uterus (Bazer, 1998). Therefore the activation of Mx proteins and other ISGs in the PBL and the CL could occur via stimulation of uterine-factors that are secreted into the circulation and hence stimulate Mx expression. Another explanation of the observed stimulation of Mx proteins outside the uterine environment is an *in utero* stimulation of Mx genes in the immune cells which then migrate out of the uterus into the peripheral blood. Further studies are needed to elucidate if the oMx2 expression in the CL is confined to the luteal cells and/or to the immune cells.

To determine the protein molecular mass, induction by IFN- τ and by dsRNA and oMx2 cellular localization, oMx2 specific antisera were generated and used for immunoblotting and immunofluorescence of oGE cells. Western blot analysis revealed that oMx2 had a molecular mass of approximately 75 kDa and was induced by IFN- τ but not by poly-IC (fig.8). It was unexpected to find that both oMx1 and oMx2 were un-responsive to poly-IC treatment. Poly-IC is known to stimulate ISGs through the toll-like receptor 3 (TLR3) (O'Neill, 2006; Schaefer et al., 2005). The

lack of response to poly-IC by oGE cells may be due to either lack of TLR3 in these cells or the dose used was not enough to activate TLR3 and hence induce Mx expression. Although the poly-IC dose used in this experiment is consistent with that used by others to elicit IFN-mediated cellular response (Schaefer et al., 2005; Scherbik et al., 2006; Tissari et al., 2005), a dose-response experiment is needed to further investigate the effect of poly-IC on Mx gene expression in the oGE cell line.

Cellular localization of oMx2 and oMx1 proteins in oGE cells was different. Ovine Mx1 was predominantly cytoplasmic (fig. 10 A, C and E) and oMx2 had both cytoplasmic and nuclear staining (fig. 10 B, D, and F). In the absence of IFN stimulation, oMx2 protein levels were low (fig.9 A). However following IFN treatment, oMx2 protein staining increased and was localized throughout the cytoplasm and appeared to be concentrated in a ring-like structure around the nucleus (fig. 10 B, D and F). In addition, oMx2 but not oMx1 was co-localized with Lamin A/C (fig. 10 B, D and F), a component of the nuclear lamina. Human MxB was reported to be expressed in the cytoplasm (76 kDa) and in the nucleus (78 kDa) with both protein products being produced from the same transcript (Melen et al., 1996). The authors suggested that due to the lack of Kozak consensus for the first ATG, the cytoplasmic form is a result of translation of the second ATG (Melen et al., 1996). The nuclear form of MxB was localized in the nuclear matrix and associated to chromatin (Melen et al., 1996). Human MxB protein has a non-classical nuclear localization signal (NLS) within the first 24 N-terminal residues which is rich in basic residues (9/24 either arginines or lysines) (Melen et al., 1996). The human MxB cytoplasmic and nuclear proteins were shown to form hetero-oligomers via their C-

terminal leucine zipper motifs, thus the cytoplasmic form could be transported into the nucleus utilizing the NLS of the nuclear form (Melen and Julkunen, 1997). In the present report, Northern blot analysis of oMx2 showed a single mRNA transcript that migrated at 2.9 kb (fig. 3) and the 5'RACE generated a single PCR product (results not shown). The Northern blot and the 5'RACE strongly suggest that the putative oMx2 nuclear and cytoplasmic staining might represent 2 forms of the same protein that were translated from a single mRNA transcript. However, this has not been tested. The oMx2 protein does not possess a classical NLS, but comparison of the oMx2 N-terminal region to its human MxB-NLS region showed that it had some basic residues between the first and third methionine (5/24 are either arginine or lysine). Analysis of the oMx2 sequence revealed that the first methionine residues (first and third, but not second ATG) could be translated based on the Kozak consensus sequence present (Kozak, 1987). More experiments need to be done to confirm if oMx2 possess a NLS and test the contribution of the first 3 ATG codons in oMx2 protein translation. Although the Western blot analysis of oMx2 (fig. 8) showed a single immunoreactive band, it is not clear whether the SDS-PAGE and Western blot conditions were sufficient to separate and detect multiple oMx2 isoforms.

Human MxB has no known antiviral actions, but a recent report showed that MxB may have a role in nuclear import and cell cycle progression (King et al., 2004). The high sequence similarities between oMx2 and human MxB, and cellular localization would indicate that these proteins have a conserved cellular function.

In summary, this study showed the existence of a second dynamin-like GTPase in sheep, oMx2. Ovine Mx2 expression was up-regulated by pregnancy sheep endometrium, CL and PBL. In the endometrium, Mx2 expression appeared to be particularly abundant in cells resembling immune cells. These observations would suggest that oMx2 expression may be regulated differently than Mx1. Although oMx1 and oMx2 belong to the same family of proteins, they have unique cellular localization and they appear to have different regulation. These findings suggest that these proteins may have distinctive and non-redundant cellular functions.

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1 CGG GGG ACT TGC CCG CCT CCG TCC TAC ACG CGC ACA GGT GAC GAG CGC GGC ACC
58 AAA AGT GAC GGC AAA CCC TTG AAA CCC TCC CTT GGA AGC GTG GAG TCC CTT TGT CCC
115 GAG GGC CCA GGG GGC CAC CAA CAG CCC TTT TGT TTT CTG TGC CTG TTT CTT GGC ATC
172 AGC CCC TCC TCG AAA CAG GGC TGA GGT GGG CTG CTG TCC CCG GCA TCG GTG AGC
229 GGC AGA GGT ACC CAA GAA CAG CAG AGC CGC TGA GGC CCA GAG ACC CCA CCC ACC
286 TCT CCA ACC TAG ACA GTG ATG TCG ATG TCC TAT AGC GGT TTT AGG TTT AGC AGC CAT
343 CTT CCA ACC TCT ACT CAG CAG CAC CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG CAG
400 A P T S T Q H H P K E D M N F H Q Q P
CGA GGG CTA CCG GGC CCA CCA CTG GGC CAG ACA ATG AGT CCC CCA CCC TGG CAG ATG
457 P G L P G P A L G Q T M S P P W Q M
GGA GAG AGC AAC CCG GAC TTT CTT CCC AAC AAC TTT AAT CAG CTG AAT TTG GAC CTT
G E S N P D F L P N N F N Q L N L D P
514 CAG CAG CCA GAA CCA CAG GGC GGC CCA CAG AGC TCA AAC GGC TCC GAA AAC AAC CTG
Q Q P E A D G G Q Q R S K G S E N N L
571 TAC AGA AAG TAC CAG CAG AAG GTG GGC CCG TCC ATT GAC CTG ATC GAC TCC CTG CCG
Y R K Y E E K V R P C I D L I D S L R
638 GGC CTG GGC CTG CAG CAG CAG CTG GGC TTTG CCC GGC ATT GGC CTG ATT GGC GAG CAG
A L G V E Q D L A L P A I A V I E D Q
685 AGC TCA GGC AAG AGC TCC GTG CTG GAG CTT CTG TCG GGG GTT GGC CTC CCG AGC GGC
S S G K S S V L E A L S G V A L P R G
742 AGC GAA ATC ATC ACC CCG TGT CCA TTG GTG CTG AAA CTG ACA AAG CCG GAG TGT GAA
S G I I T R C P L V L K L T K R E C E
799 TGG ACG GGG AAG ATC ACC TAC CCG AAC GTA AAC CAG CAG CTC CAC AAC CCC TCC GAG
W T G K I T Y R N V T Q Q L H N P S E
886 GTG GAG CCG GAG ATT CCG AGA CCG CAG AAC ATC ATA GGT GGG AAT GGA GTT GGC ATC
V E R E I R R A Q N I I A G N G V G I
913 AGC CAT GAG CTC AAT CTG CAG CTC ACC TCC CTT GAG CTC CCA GAT CTG ACC CTC
S H E L I N L E V T S P E V P D L T L
970 AAT GAC CTT CCC GGC ATC ACC AGG GTG CTT GTG GAA AAC CAG CCA CAA GAC ATC GGA
I D L P G I T R V A V E N Q P Q D I G
1027 CTG CAG ATC AAG CCA CTC ATC AAA AAG TAC ATC CAG AGC CAG GAG ACC ATC AAC TTG
L Q I K A L I K T Y I Q R Q E T I N L
1084 GTG GTA CAC CCC TCC AAT CTG CAG ATC GGC ACC AGC GAG CCG CTG AGC ATG GGC CAG
V V V P C N V D I A T T E A L S M A Q
1141 GAG GTG GAG CCC GAT GGA CAG AAG ACC ATA GGG ATC CTG ACC AAA CCA CAG CTG CTG
E V D P D G D R T I G I L K P D L V
1198 GAG AAG GGC ACC CAG AAA GGT GTG CTG AAG CTC ATG CAG AAC CTC ACC TAT CAT CTC
D K G T E K G V L K V M Q N L T Y H L
1285 AAG AAG GGG TAT ATG ATC GTG AAG TGC CCG GGC CAG CAG CAT ATC ACC AAC AAG CTG
K K G Y M I V K C R G Q D I T N K L
1312 CCG TTG GCA CAG ACC AGG AAA GAA AAG ATG TTT TTT CAG ACC CAT CCG TAT TTT
R L A E A T R K E V M F E Q T H P Y F
1389 AAG GTT CTT CTG GAT GAA GAG AAG CCG AGC CCG CTT CTT CCG GCA GAA AAA CTT ACC
R V L L D E G K A T V V P L L A E R L T

1426 ACC GAG CTC AAT TCG CAC ATC AAT AAA TCG CTC CCC TTG TTA GAA AAC CAA ATA AAA
T E L I W H I N K S L P L L E N Q I K
1483 GAG AAA CAG CAG AGC GCA ACA CAG CAG CTG CAG CAG TAC GGG GAG GAC AAT CCC AGC
E K H Q R A T E E L Q Q Y G D D I P S
1540 AAT GAA GGG GAT AAA ATG TTT CTC ATC AAT GAG AAA AAT AAG TTA TTT AAT GAG GAG
N E G D K M F E L I E K I K L F N E D
1597 AAT GAA TTA ATA GGA GAA GAA AAT GAA AAT GAA AAT GAA AAT GAA AAT GAA AAT GAA
I E K L I E G E I V I E T E S R L C
1684 AAC AAT ATC ACA GAG CAG TTT ACA AAT TCG GTA CTC ATC ATC ACC AAC ATC GAA
N R I R E E F T R W V L I L T T N I E
1711 AAA GTT AAA AGT ATC CTT AAC GAA GAA GAA GAA TAT GAA AAG AAG TAT CCA GGC
K V K S I L N E E V S K Y E T K Y R G
1788 AAA GAA CTT CTC GGA TTT GAT AAT TAC AAG AGC TTT GAG ACT GTG CTG AAG CAT TAC
K E L L G F V N Y K T F E T V V K H Y
1825 CTG GCA CAA CTG ATA GAC CCA CCA CTC ATC AAG ATG CTC CAG AAG CCC ATG GAA AAT ATC
L G Q L I D P A L K M L Q K A M E I I
1882 TCG CAA ACT TTC AAG GAG ACA GGC AAA AAG CAA TTT GGT GAA TTT TCC AAT CTG CAA
W Q T F K D T A K K H F A E F C N L H
1939 CAA ACA CTG CAG AAC AAG ATC GAA GAC ATA AAA ACA AAG CAA ATG CCA GAA CCA GAA
Q T V Q N K I E D I K T K Q M A E A A
1996 AAT CTG ATC CAA CTT CAG TTC AGC ATG ATG AAG CTG GTT TTT TGT CAA GAG CAG AAT
N L I Q L Q F R M E K L V F C Q D Q I
2083 TAC GCG GTG GTT CTC AAC AAA CTC CCA GAA GAG AAT TTT AAT TCC GCG GAA AAG CCA
Y G V V L N K V R E E I F N S V G K A
2110 TCA GAG AAT CTT CAG TCG AAG CAT CCC TTT TTA AAT AAT CAG TCT TCA GTT TCC TCC
S E N P Q S K H P F L N N Q S S V S S
2187 AAT GTT GAG AAT GGG GTG CAG CTC GAT GAA TAT TTT ACC GAA ACC AAC AAA CCG CTC
I V E I G V H L N A Y F T E T S K R L
2224 CCG AAT CAG ATC CCG TTC ATC AAT CAG TAT TTT ATG CTC CAA GAG AAT GGT GAT AAG
A N Q I P F I I Q Y F M L Q E N G D K
2281 GTG CAG AAA GGC ATG ATG CAG CTT CTG CAG GAG ACG CAG CAC TAT TCC TCG CTG CTT
V Q K A M M Q Q E T Q H Y S W Q
2338 CAG GAG CAG ACC GAT ACA GCT ACC AAG AAG AAA TTT CTT AAG CAG AAG AAT TTT AGS
Q E Q S D T A T K R K F K E K I F R
2395 CTG AAT CAG GCA CAG CAA GCT CTC TAC GAA TTT CCC CAT TTT AAA GGC ATA TGA GGA
T T Q A Q Q A Y E F P H F K G I
2482 TGA GAT GGC TGG ATG GCA TCA CCG ACT CCA TGG ACG TGA CTC TGA CTG AAC TCC GGG
AGT TCG TGA TGG ACA GGG AGG CTT GGC CTG CCA TTT ATG GGG TCG CAA AGA GTG
2566 GGA CAT GAG TGA GGC ACT GAA CTG AAC TGA ACC ACC TTT TTG CTG GTT TGT GTT GTA
2633 CAT GAT AGT CCA GGC TTG ACC ATG TAG CCA CAA GAA AAC TCG GCT GCC TGA AGT GGA
2880 CTT GAT GAT GGA AGT GCG ACT TCT GTT CAA GAA GGA TGA AAG CCG TCG TGT TTT GGC
2737 CTT CTC TAT TTT TCC TTT GAT TAT GAA AAT GCA GGC CAG TAA TTT TAG GTG CAG
2794 CAC CTT CTT ACC TCC CTG ATG ATA AGT CAT ATA AAC TTT ATA TTT TAA TAA ATC ACT
2881 TCA TAT CTG AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA A

Fig.1: Ovine Mx2 nucleotide and the predicted amino acids sequence. The tripartite GTP-binding motifs and the dynamin signature are shaded in dark-gray and underlined, respectively. The leucine zipper residues are boxed and shaded in gray.

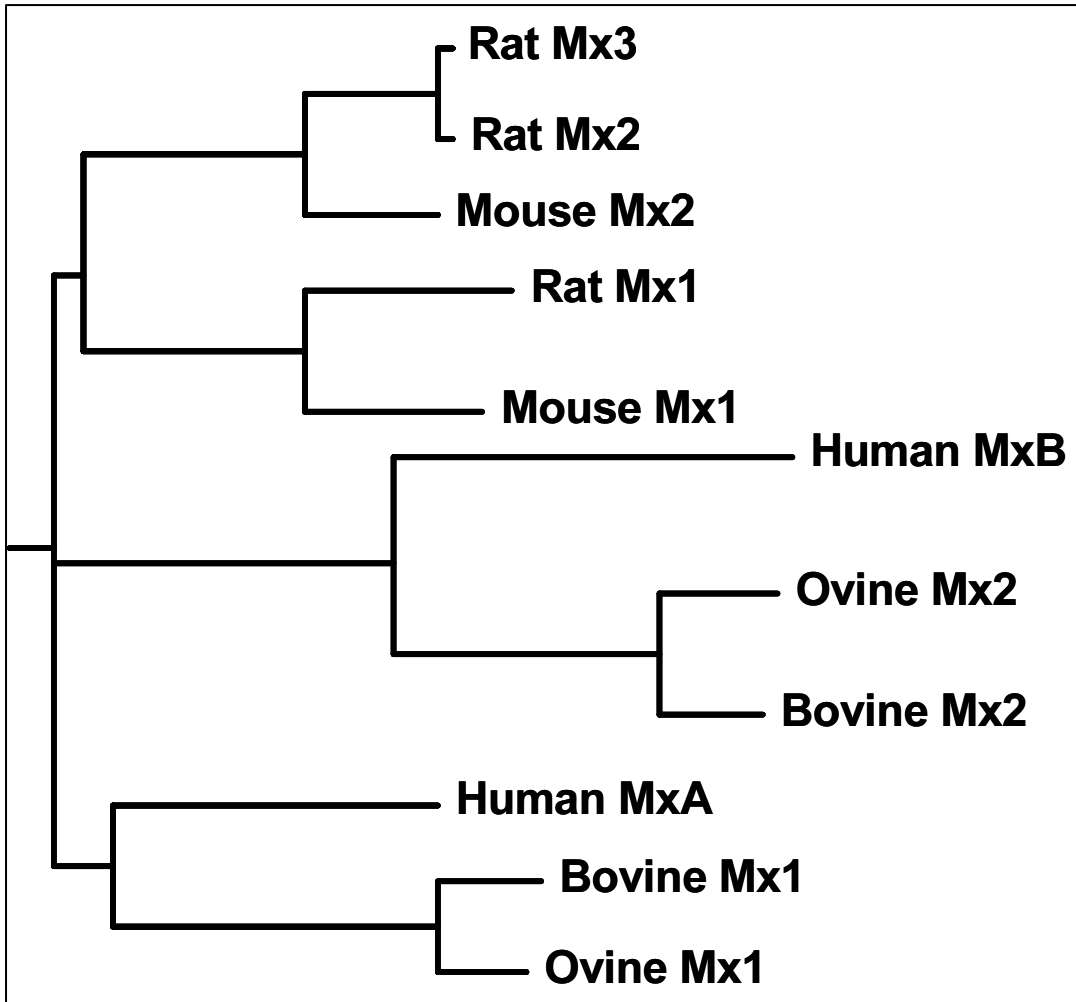


Fig.2: Phylogenetic rooted tree analysis of Human, Rodent, Ovine, and bovine Mx proteins utilizing CLUSTALW software. Analysis revealed that human MxA and MxB are closely related to ovine Mx1 and ovine Mx2, respectively. Ovine and human Mx proteins are more related to each other than to rodents Mx.

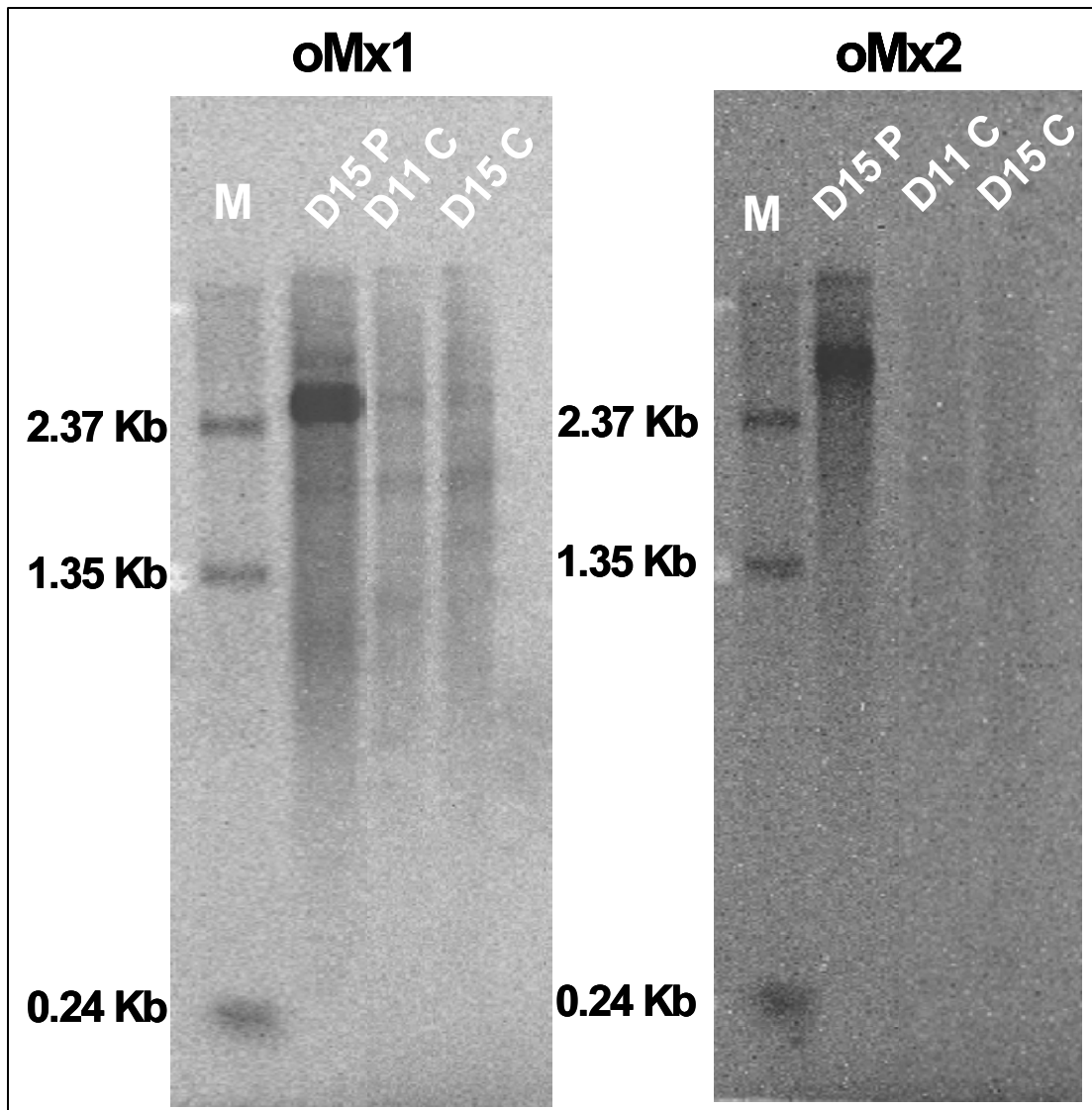


Fig.3: Poly-A Northern blot analysis of ovine Mx mRNA transcripts obtained from day 15 pregnant (D15P), 11 and 15 cyclic (D11C and D15C) endometrial mRNA using oMx2 and oMx1 specific cRNA probes. Sample from day 15 pregnant showed that oMx1 and oMx2 cRNA probes hybridized to a major single mRNA transcript. The oMx1 mRNA transcript migrated at approximately 2.5 kb (left image), while the oMx2 transcript migrated at approximately 2.9 kb (right image). M= RNA ladder.

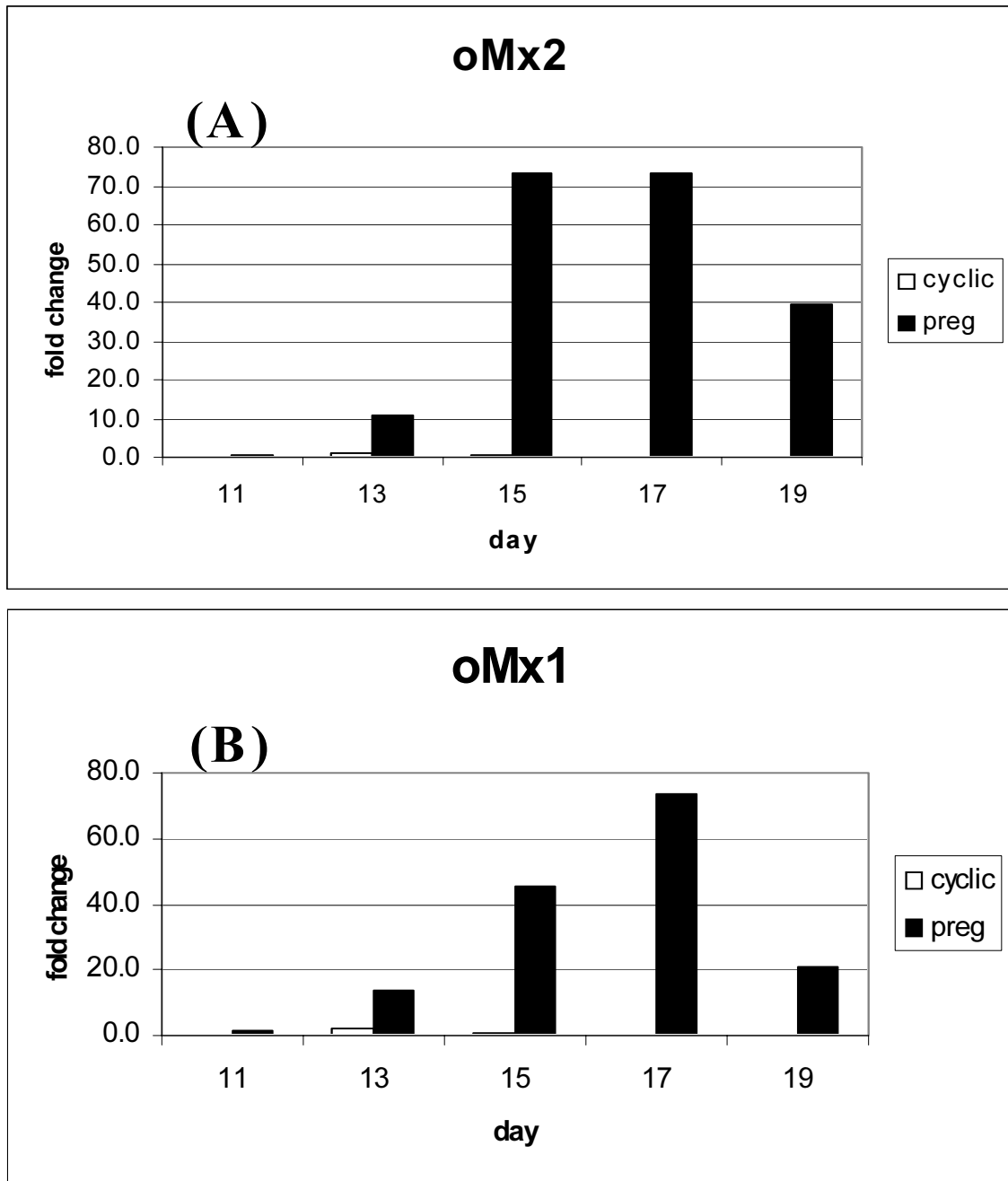


Fig.4: Quantitative RT-PCR analysis of oMx2 (A) and oMx1 (B) mRNA levels in the endometrium of early pregnant (Days 11,13,15,17, & 19) and late cyclic (Days 11, 13, & 15) ewes. The oMx1 and oMx2 mRNA levels were detected in cyclic ewes and up-regulated by pregnancy ($P<0.01$). Ovine Mx2 mRNA increased by 10-fold on day 13, 73-fold on days 15 and 17 and by approximately 40-fold on day 19. The oMx1 mRNA increased on day 13 by 14-fold, day 15 by 45-fold, day 17 by 73-fold and by 21-fold on day 19 of pregnancy.

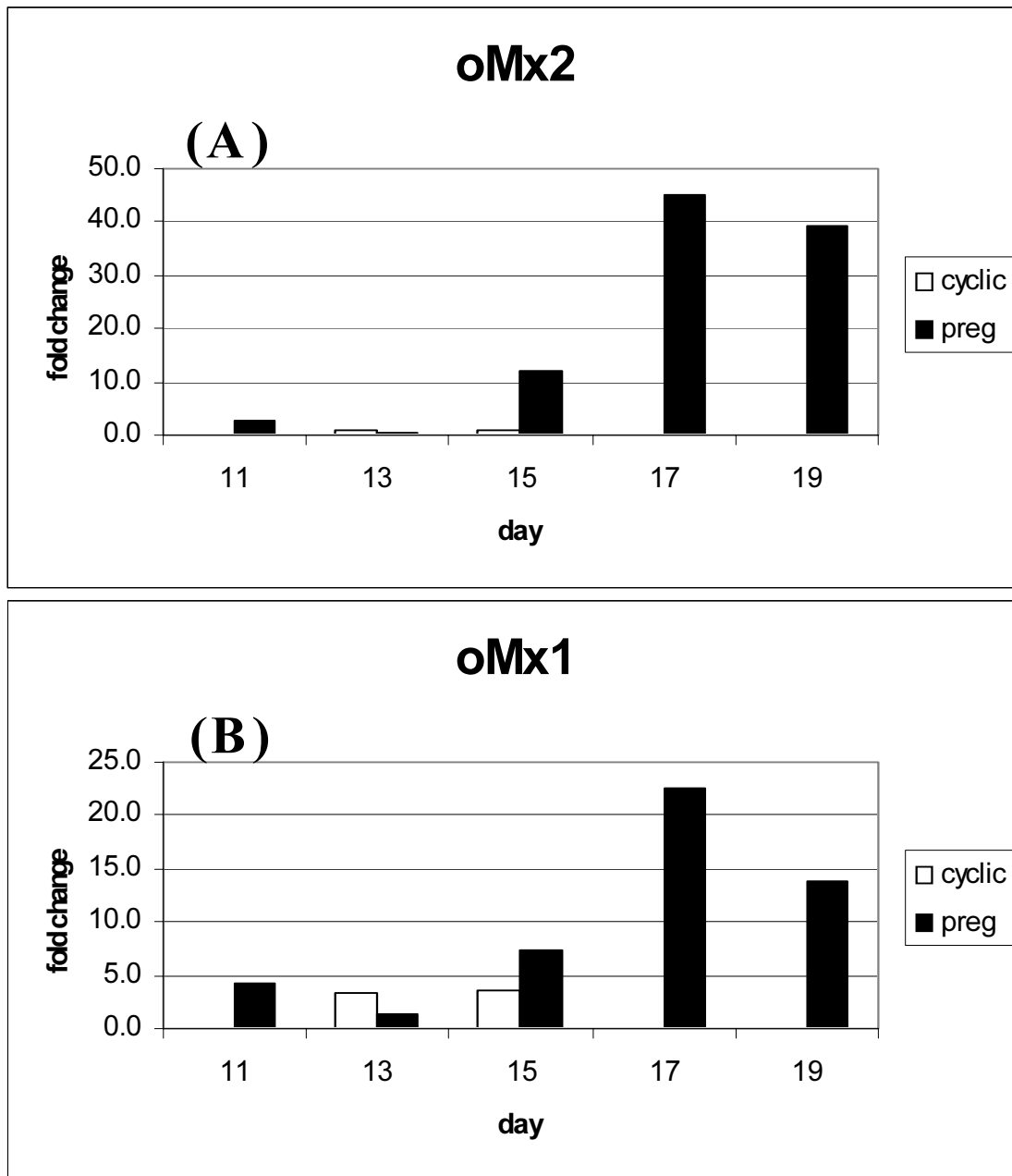


Fig.5: Peripheral blood leukocytes were obtained from late cyclic and early pregnant ewes and levels of oMx2 (A) and oMx1 (B) mRNA were quantified by qRT-PCR. The oMx2 mRNA levels were detectable on all days examined of the cycle and up-regulated in response to pregnancy (days 15, 17, and 19) ($P<0.07$). In pregnant ewes, oMx2 mRNA increased by 12-fold on day 15, 45-fold on day 17 and 39-fold on day 19 of pregnancy. The oMx1 mRNA levels in PBL from pregnant ewes were higher than cyclic ewes ($P=0.09$) and increased by 7.5, 12.6, and 14-fold on day 15, 17 and 19 of pregnancy, respectively.

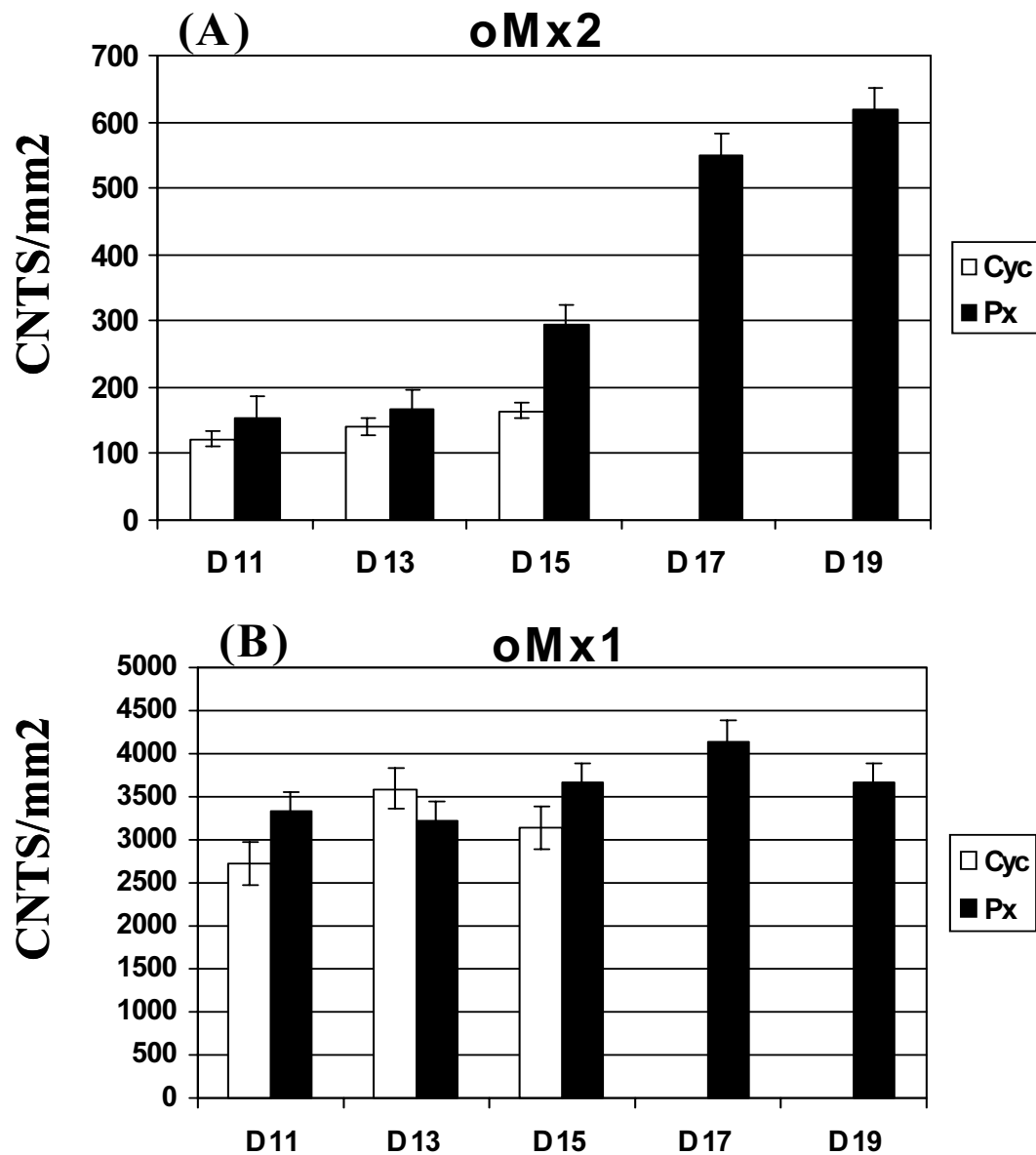


Fig.6: Slot-blot analysis of oMx2 (A) and oMx1 (B) mRNA levels in CL from cyclic and pregnant ewes. Both transcripts were detected in cyclic and pregnant animals, however only oMx2 was up-regulated by pregnancy ($P<0.07$) on day 15 by 2-fold and remained elevated through day 19 (4-fold) of pregnancy. Although oMx1 did not increase by pregnancy ($P=0.5$), the level of oMx1 appeared to be higher than oMx2.

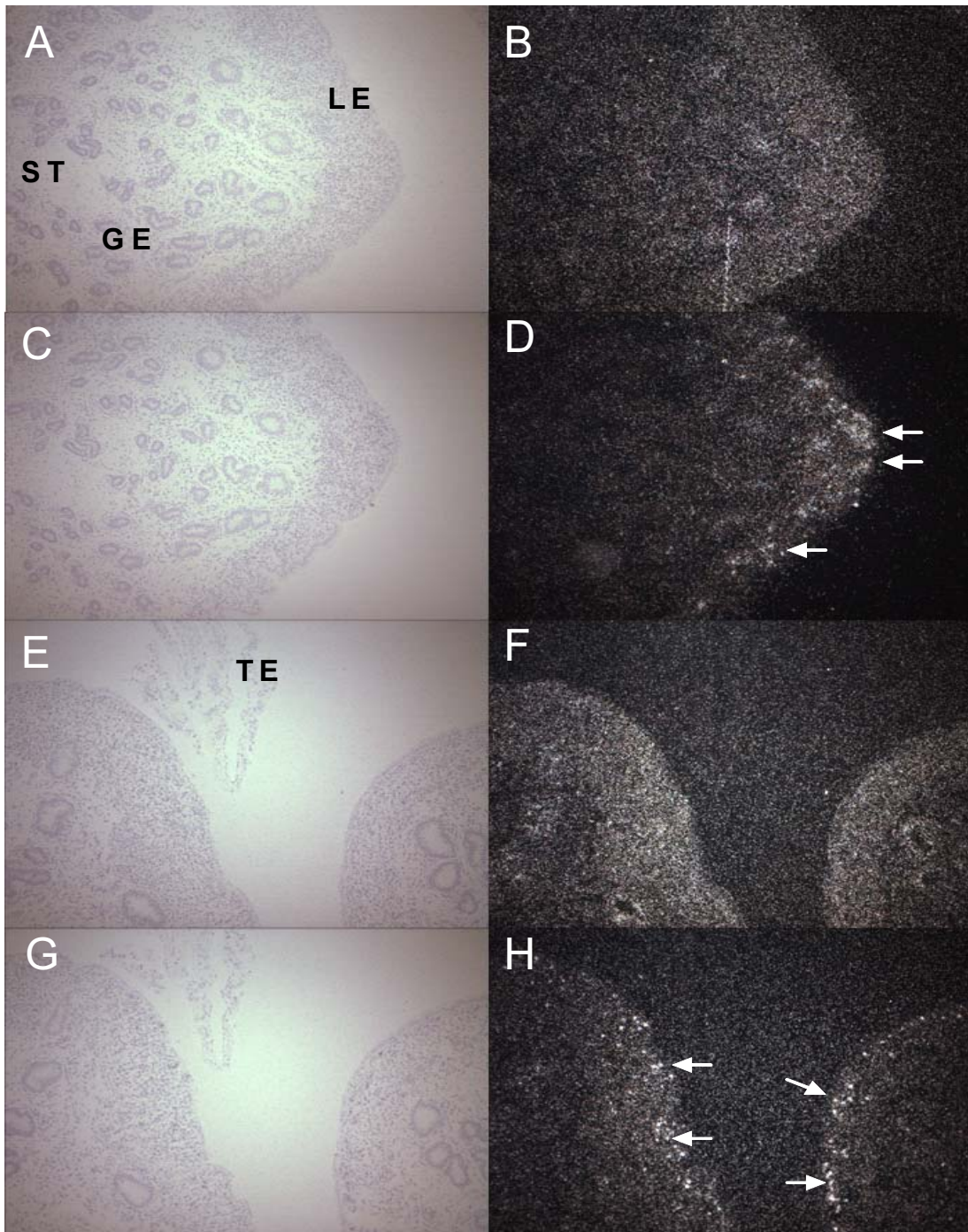


Fig.7: In situ hybridization of serial cross-sections of sheep endometrium at Days 17 (A, B, C,D) and 19 (E, F, G,H) of pregnancy under brightfield (A,C,E,G) and darkfield (B,D,F,H) illumination. The oMx1 anti-sense cRNA probe hybridization signal was observed in luminal epithelium (LE), glandular epithelium (GE), and stroma (ST) in both Day 17 and 19 (B, F). The oMx2 anti-sense probe hybridized strongly to distinct group of cells beneath the luminal epithelium (arrows; D, H). There was no hybridization of either probe to the trophoblast (TE).

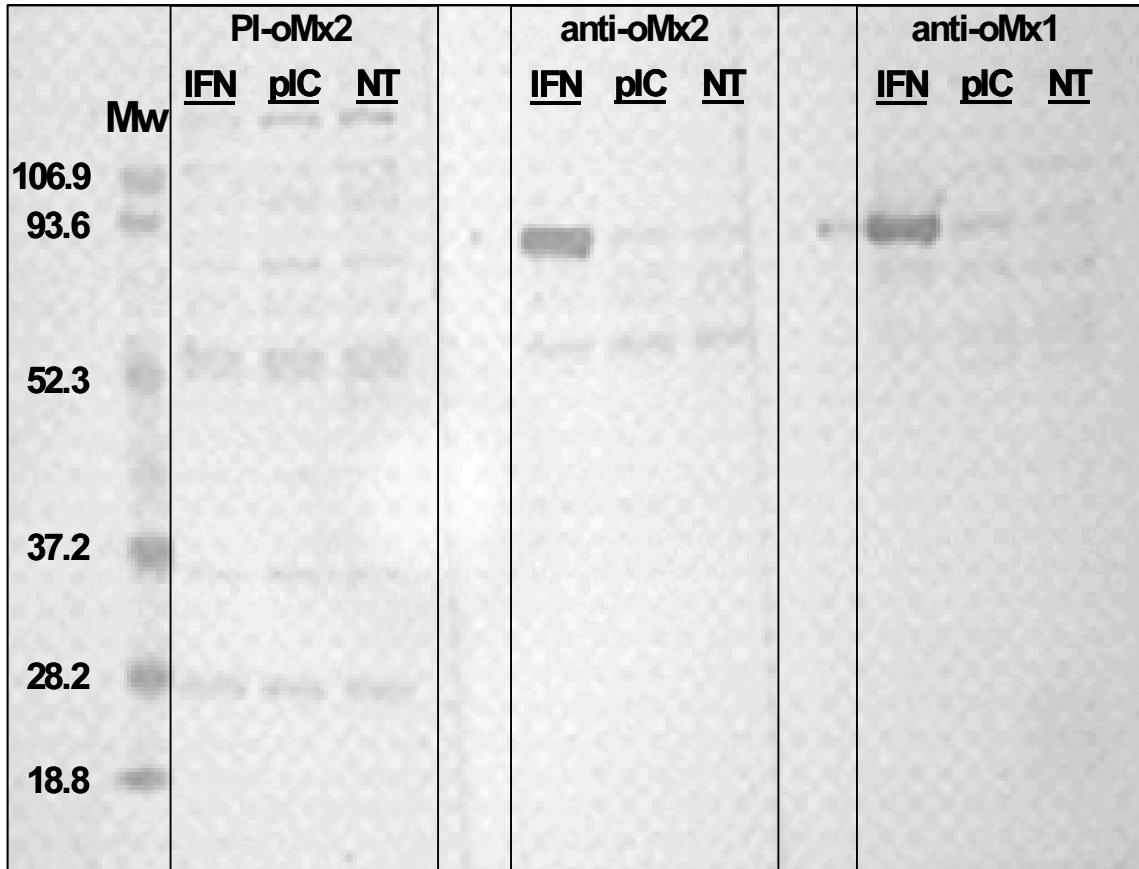


Fig.8: Western blot analysis of oMx1 and oMx2 proteins. Cell lysates were separated on SDS-PAGE gels and immunoblotting was performed using oMx2 pre-immune (left box), oMx2-specific (middle box) or oMx1-specific antisera (right box). Ovine Mx2 antiserum detected an immunoreactive protein of approximately 75 kDa which was very abundant in the IFN-treated and faint in untreated (NT) and poly-IC samples. Blotting with pre-immune sera did show bands of the size expected for Mx proteins, however, there were other minor cross-reacting bands. Anti-serum for oMx1 detected the expected protein of approximately 75 kDa.

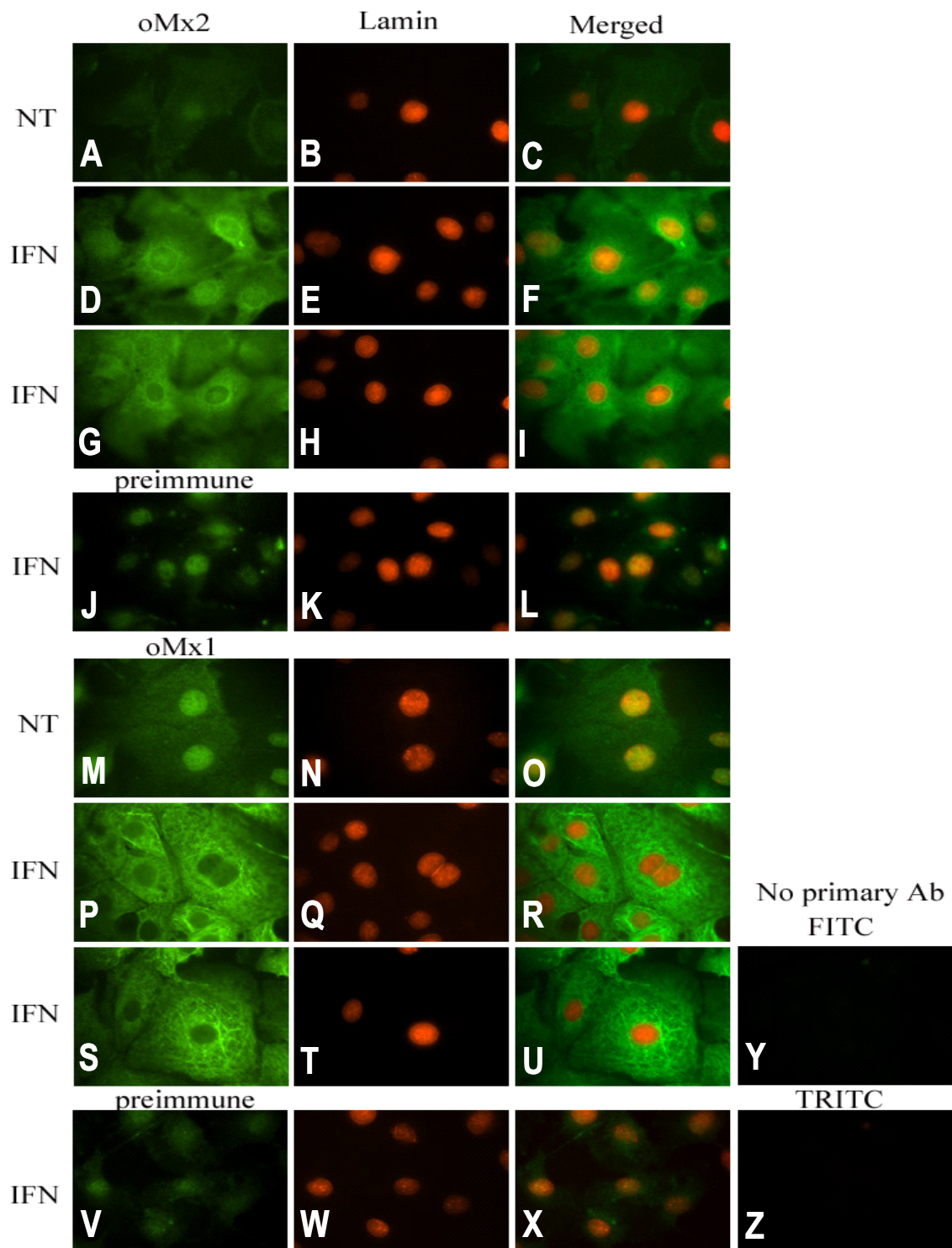


Fig.9: Comparative cellular localization of oMx2 (A, D and G) and oMx1 (M, P, and S) by immunofluorescence microscopy. Images show TRITC staining of nuclear Lamin A/C (middle panel), oMx1 and oMx2 FITC staining (right panel), and combination of TRITC and FITC staining (left panel). In absence of IFN stimulation, oMx2 protein showed low staining in the cytoplasm (A). Treatment with IFN increased oMx2 protein levels which was localized in the cytoplasm and formed a nuclear ring-like structure (D and G). Staining of oMx1 was predominantly cytoplasmic following IFN treatment (P and S). Lamin A/C was co-localized with nuclear oMx2 (F and I) but not with oMx1 (R and U). Preimmune sera for oMx2 (J) and oMx1 (V) showed weak and non-specific staining. No staining was seen in the absence of primary antisera (Y and Z).

oMx1

oMx2

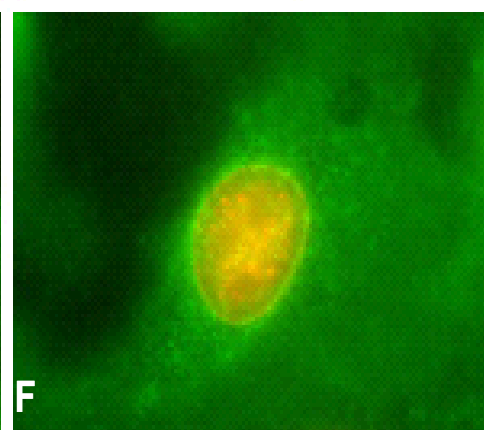
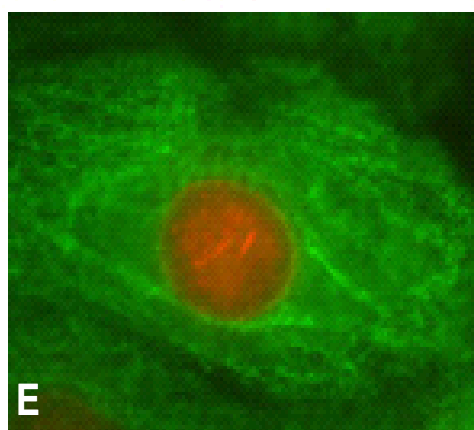
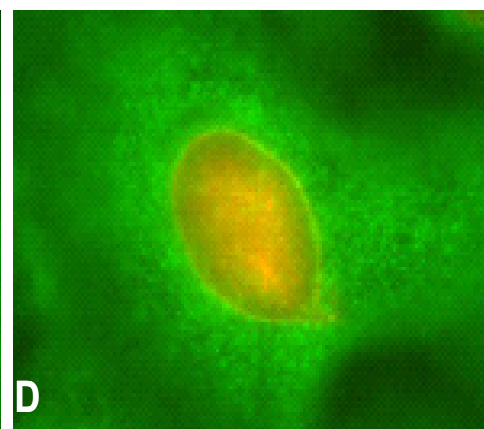
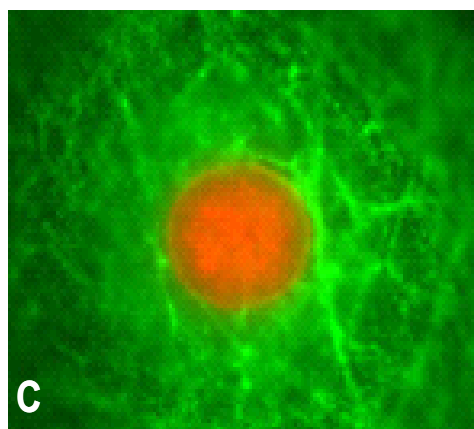
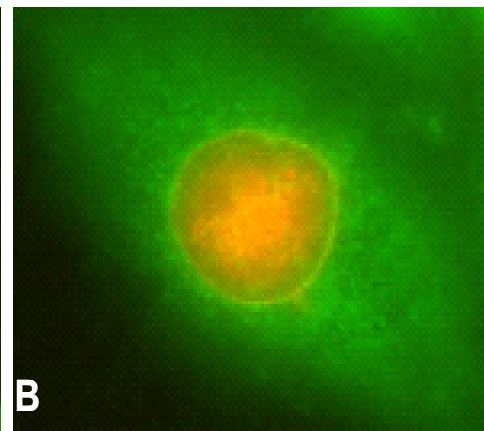
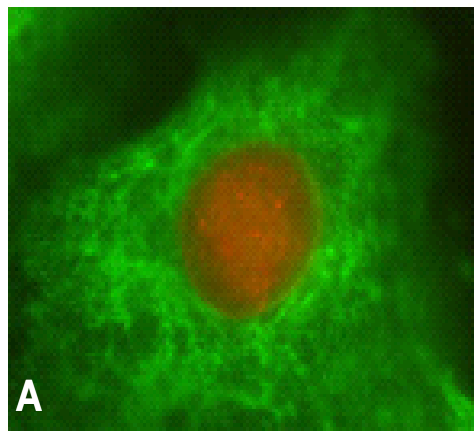


Fig.10: Immunofluorescence staining for oMx1 (A, C, and E) and oMx2 (B, D, and F) protein in oGE cells after IFN treatment. Lamin A/C was co-localized (yellow staining) with oMx2 but not with oMx1.

	oMx1	oMx2	bMx1	bMx2	hMxA	hMxB	mMx1	mMx2
oMx1	-	57%	93%	58%	75%	57%	63%	69%
oMx2	57%	-	59%	93%	59%	70%	57%	59%
bMx1	93%	59%	-	59%	74%	58%	63%	69%
bMx2	58%	93%	59%	-	60%	70%	58%	60%
hMxA	75%	59%	74%	60%	-	59%	66%	74%
hMxB	57%	70%	58%	70%	59%	-	56%	60%
mMx1	63%	57%	63%	58%	66%	56%	-	73%
mMx2	59%	59%	69%	60%	74%	60%	73%	-

Table .1: Amino acid sequence homology analysis using the CLUSTALW program of the newly cloned sheep Mx2 (oMx2) and ruminants (oMx1, bMx1, and bMx2), human (hMxA and hMxB), and mouse (mMx1 and mMx2) proteins. The oMx2 protein shares the highest homology with bovine Mx2 and human hMxB. Sequence homology between oMx2 and other Mx proteins including oMx1 is approximately 57-59%. It is interesting to note that homology between Mx proteins from different species (oMx2 vs. hMxB) is greater than the homology of Mx proteins within the same species (oMx1 vs. oMx2).

CHAPTER FOUR

Cloning and Characterizing of the Ovine Mx1 Gene Promoter/Enhancer Region

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Abstract:

Ovine Mx1 (oMx1) is an interferon stimulated gene that is expressed in the sheep endometrium during the estrous cycle and is strongly up-regulated during early pregnancy in both the endometrium and peripheral blood leukocytes. Mx proteins are large GTPases, many of which have antiviral activity and are important elements in innate immunity. Our results showed that estrogen and progesterone are required for oMx1 protein induction by IFN in the ovine uterus. To address the role of IFN and steroids in regulating oMx1 expression, 2.1 kb of the 5' end of the gene containing 1.7 kb of the promoter/enhancer, exon1 (25 bp) and 400 bp of intron A was cloned. Serial deletion mutants (-1666 bp to -99 bp) were prepared along with a clone that contained a full-length promoter including the 2 promoter-ISREs (-101 & -145) but lacking the 1 intronic-ISRE (+224). Luciferase reporter plasmids driven by the various oMx1 promoter deletions were transfected into an ovine endometrial glandular epithelial cell line. The full-length promoter was induced by interferon tau (IFN- τ) in a dose- and time-dependent manner. Treatment with 10,000 AVU/ml of IFN- τ increased luciferase activity 5- & 9-fold at 3 and 12 hours, respectively. The oMx1 promoter responded to doses of IFN- τ as low as 1 AVU/ml. Promoter deletions, showed the 2 proximal ISRE sites (-101 and -145), but not the intronic-ISRE site (+224), were required for maximal response to IFN. Deletions of a distal region (-920 to -715) resulted in a decreased luciferase activity (~3-fold) to IFN- τ treatment. However, subsequent deletion of the -715 to -437 region restored

maximal promoter response (~9-fold) to IFN- τ . Results may suggest that the regions -920 to -715 and -715 to -437 have positive and negative regulatory element binding sites, respectively. The importance of the 2 proximal ISRE sites for oMx1 promoter activation by IFN is consistent with results for the human MxA and mouse Mx1 promoters. Although, the intronic ISRE is present in the human MxA promoter, this is the first report to show that this site may not be required for oMx1 promoter activation by IFN. Identifying positive and negative regulatory regions in oMx1 promoter may help elucidate the apparent unique regulation of Mx1 during early pregnancy in ruminants.

Introduction:

In ruminant species, female cyclicity is a uterine-dependent event and rescue of the corpus luteum (CL) requires the presence of the conceptus (Ott, 1999; Spencer et al., 2004). Maternal recognition of pregnancy in ruminants is achieved by conceptus-produced interferon tau (IFN- τ) (Bazer et al., 1997). Interferon tau is a novel type I IFN that is secreted by the conceptus mononuclear trophoderm between days 10 and 21 of pregnancy in sheep (Bazer et al., 1997). During early pregnancy in sheep, the IFN- τ blocks uterine pulsatile release of the luteolysin, prostaglandin F₂Alpha (PGF₂ α), (Bazer et al., 1994). The antiluteolytic action of IFN- τ is achieved by suppression of endometrial estrogen (ER) and oxytocin receptors (OTR) expression (Fleming et al., 2001; Spencer et al., 1995) which in turn prevents endometrial luteolytic PGF₂ α production. Interferon tau does not suppress OTR expression directly, but suppresses ER gene transcription and therefore

precludes OTR expression by estrogen (Fleming et al., 2006). In addition to its antiluteolytic property, IFN- τ has antiviral, antiproliferative and immunomodulatory activities (Bazer et al., 1996).

Similar to other type I IFN, IFN- τ activates its target genes by the JAK/STAT signaling pathway (Stewart et al., 2001; Stewart et al., 2001). Binding of type I IFN causes phosphorylation of the receptor-associated Jak-1 and Tyk-2 kinases which leads to the subsequent phosphorylation of Stat-1 and Stat-2 proteins. The phosphorylated Stat-1 (p91) and Stat-2 (p113) form a heterodimer, and along with non-STAT transcription factor, p48 (also known as IRF9) translocate to the nucleus to form interferon stimulated gene factor-3 (ISGF-3) complex (Samuel, 2001). The trimeric ISGF-3 complex binds to interferon stimulated response elements (ISRE) on the promoter regions of interferon stimulated genes (ISGs) (Haller et al., 2006; Harada et al., 1996; Hug et al., 1988; Ronni et al., 1998; Samuel, 2001; Stewart et al., 2001; Stewart et al., 2002). The conceptus-derived IFN- τ acts locally on the endometrium to up-regulate several interferon stimulated genes (ISGs) including the signal transducers and activators of transcription (STAT) 1 and 2 (Choi et al., 2001), IFN regulatory factor-1 (IRF-1) and IRF-9 (Choi et al., 2001), interferon stimulated gene-15 (ISG15) (Johnson et al., 2002; Johnson et al., 2000; Johnson et al., 1999), and the large GTPase; Mx protein (Charleston and Stewart, 1993; Hicks et al., 2003; Johnson et al., 2002; Ott et al., 1998).

The Mx proteins are members of dynamin superfamily of large GTPases and are important components of the innate immunity against wide range of viruses (Haller and Kochs, 2002). The mouse Mx1 protein was the first to be discovered to

confer resistance in influenza A viral challenge (Lindenmann, 1962). In most species including fish, birds, and mammals, two to three Mx proteins were identified and their sequences are characterized by a conserved tripartite GTP binding, dynamin signature and leucine zipper motifs (Haller and Kochs, 2002; Lee and Vidal, 2002).

Mx proteins are expressed in the endometrium of sheep, cows, gilts, mare, mice and women (Chang et al., 1990; Charleston and Stewart, 1993; Ellinwood et al., 1998; Hicks et al., 2003; Ott et al., 1998). Ovine Mx1 mRNA and protein are expressed in the endometrium during estrous cycle; being low but detectable on day 1, substantially increased on day 13 and low on day 15 of the estrous cycle (Ott et al., 1998). In response to pregnancy, oMx1 expression was increased (10-fold) higher than cyclic animals and was expressed in endometrium starting from day 13 through day 19 in luminal epithelium (LE), glandular epithelium (GE), and stroma and myometrium (Ott et al., 1998). Temporal oMx1 expression correlates with peak levels of progesterone (P4) during the estrous cycle which suggests that steroids (P4) may regulate oMx1 expression (Ott et al., 1998). In ovariectomized ewes, intrauterine administration of IFN- τ failed to induce oMx1 expression in the absence of steroids. Estrogen, progesterone, and estrogen and progesterone treatment increased oMx1 expression in response to IFN- τ . The effect of both steroids on oMx1 stimulation by IFN- τ was abolished by the progesterone antagonist (Ott et al., 1999). Although oMx1 and ISG-15 are both stimulated by IFN, their temporal expression is different in the ewe endometrium (Johnson et al., 2002). Both proteins are expressed in GE and stroma during early pregnancy, however oMx1 is only expressed in LE (Johnson et al., 2002). These findings are rather intriguing, because both proteins are interferon

stimulate genes (ISG) and presumably, like other ISGs, activated through the JAK-STAT signaling pathway. Expression of oMx1 in LE would suggest that in addition to JAK-STAT, other signaling mechanisms may be involved in oMx1 regulation.

The Mx gene promoters from different species were cloned and the effects of IFN on promoter activity was studied (Altmann et al., 2004; Asano et al., 2003; Chang et al., 1991; Collet and Secombes, 2001; Gerardin et al., 2004; Kojima et al., 2003; Ronni et al., 1998; Schumacher et al., 1994; Yap et al., 2003). Interferon activates the Mx promoter via the JAK-STAT pathway, leading to formation of the ISGF3 complex which binds to the interferon stimulated response elements (ISRE) (Samuel, 2001). Although the chicken Mx promoter has one ISRE which is sufficient for stimulation by IFN (Schumacher et al., 1994), the two proximal ISRE sites act synergistically and are required for Mx promoter responsiveness to IFN in human, mouse, zebrafish, and Fugu fish (Altmann et al., 2003; Hug et al., 1988; Ronni et al., 1998; Yap et al., 2003).

Due to the unique production of IFN tau by ruminant conceptuses and the temporal and spatial expression of oMx1 during the estrous cycle and early pregnancy in sheep, this study was conducted to clone oMx1 gene 5' promoter/enhancer region and to characterize factors which affect its activity.

Materials and Methods:

Animal procedures, peripheral blood cells collection, genomic DNA isolation:

All animal procedures were approved by the University of Idaho IACUC. Jugular vein blood was collected from commercial white faced ewes in EDTA-containing

Vacutainer tubes (BD Biosciences, San Jose, CA) and kept on ice until further manipulations. The genomic DNA was isolated from whole blood using QIAGEN anion-exchange resin-based kit (QIAGEN Blood & Cell Culture DNA mini kit; QIAGEN, Valencia, CA). The quality and purity of the isolated genomic DNA was checked by spectrophotometer and Dra I digestion and gel electrophoresis.

5' Rapid amplification of cDNA ends (5'RACE) and oMx1 exon-exon structure analysis:

Sequence analysis of the available ovine Mx1 cDNA sequences, ovine Mx1 endometrial [GI: X66093] and oligodendrocyte GTP-binding protein [GI: AF399856] revealed some discrepancies in the 5' UTR (Fig. 1A). To obtain an accurate 5'UTR sequence and to map exon-exon junctions, we performed 5' RACE for the ovine Mx1 cDNA. The SMART™ RACE cDNA Amplification kit from BD Biosciences (BD Biosciences, San Jose, CA) was used to synthesize SMART™ first-strand cDNA from day 15 pregnant ewe endometrial total RNA. The SMART™ cDNA was used to amplify the oMx1 5'UTR by using BD Advantage™ 2 PCR kit from BD Biosciences (BD Biosciences, San Jose, CA). The PCR was carried out by using the universal primer A mix (UPM) which is provided with SMART™ RACE kit and the oMx1 cDNA specific primer (oMx1 5'RACE; GGA TGT CCT TGC CAT ACT TC). PCR reactions were performed at 94°C for 5 sec and 72°C for 3 min for 5 cycles, then 5 cycles at 94°C for 5 sec, 70°C for 10 sec and at 72°C for 3 min, then followed by 35 cycles at 94°C for 5 sec, 63°C for 10 sec and at 72°C for 3 min. A single PCR product (~ 1.1 kb) was gel purified by using GenElute™ Spin Columns from (Sigma-Aldrich, St. Louis, MO) and subsequently cloned into PCR® II-TOPO® TA vector (Invitrogen,

Carlsbad, CA). Following restriction enzyme screening, clones with expected size insert were sequenced by using M13F and M13R universal sequencing primers. The oMx1 5'UTR sequence (this study) and the published oMx1 sequences (GenBank accession No. X66093 and AF399856) were used to generate an oMx1 cDNA consensus sequence. The NCBI, Spidey program [<http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/>] was utilized to map the exon-exon junctions of the oMx1 cDNA consensus sequence against the bovine Mx1-a genomic sequence (GenBank accession No. 33111869).

Construction of sheep genomic libraries:

The Universal GenomeWalker™ kit from BD Biosciences (BD Biosciences, San Jose, CA) was used according to the manufacturer's recommendations. Briefly, 4 genomic libraries were constructed by digesting the sheep genomic DNA with 4 different restriction enzymes (*Dra I*, *EcoR V*, *Pvu II*, and *Stu I*). The blunt-ends of the digested genomic DNA were ligated to GenomeWalker adapters and used for the subsequent genomic polymerase chain reactions (PCR).

Cloning of the ovine Mx1 gene intron A, exon1 and partial promoter:

Following sequence analysis of the oMx1 5' UTR and exon-exon mapping of the oMx1cDNA, oMx1 exon 2 specific primers were designed by using Primer Designer program. Sheep genomic libraries were used as template, the primary PCR was carried out using Adapter primer1 (AP1; GTA ATA CGA CTC ACT ATA GGG C) which recognize the GenomeWalker adapter and oMx1 exon 2 specific primer (oMx1-ex2; TAG TGC TGA TTA GTT GGC AGA AAT GCA GAG). The BD

Advantage™ 2 PCR kit from BD Biosciences (BD Biosciences, San Jose, CA) was used for amplification with following thermocycler settings; 5 cycles at 94°C for 25 sec, 72°C for 5 min with progressive decrease of 1°C/cycle, then followed by 32 cycles at 94°C for 25 sec, 67°C for 5 min and then final extension at 67°C for 10 minutes. The secondary PCR was performed on 1:50 dilution of the primary PCR reaction by using Adapter primer 2 (AP2; ACT ATA GGG CAC GCG TGG T) and oMx1 exon 2 nested primer (oMx2-exnst; CAG AAA TGC AGA GCT CTC CTC CAA C). The secondary PCR was done using the following thermocycler settings; 5 cycles at 94°C for 25 sec, then at 72°C for 3 min with a decrease of 1°C/cycle, 20 cycles at 94°C for 25 sec, then 67°C for 3 min and followed by final extension at 67°C for 7 min. The secondary PCR products were run on 1% agarose gel and major bands (~0.25kb, 0.35kb, and 3kb) were gel purified and cloned into PCR® II-TOPO® TA vector (Invitrogen, Carlsbad, CA). The 3 kb clone contained partial exon2, intron A, exon1 and partial promoter sequence (~0.2kb). Our cloning and sequencing analysis revealed the size of oMx1 exon1 (25bp) and intron A (~2.8kb). Nucleotide sequence and size of exon1 confirmed our oMx1 5'RACE data and the Spidy program exon-exon junction mapping.

Cloning of oMx1 gene promoter/enhancer region:

The oMx1 intron A 5' nucleotide sequence was utilized to design primers to walk upstream of intron A. The following primer sets (AP1; GTA ATA CGA CTC ACT ATA GGG C, and oMx1INT121; TCC TAG TCT GCG CAA TAC CGG CAT C) and (AP2; ACT ATA GGG CAC GCG TGG T, and Mx1DG2; NNN CCG TCC CCA GCG CNN N, where N=A, C, G or T) were used for primary and secondary PCR

respectively. The nested degenerative primer MxDG2 was designed to contain the complementary sequence for the conserved 13 nucleotides between oMx1 and b Mx1-a allele exon1. The primary and secondary PCR conditions were carried out using similar conditions used for amplification of the oMx1 intron A region, with exception of that the annealing/final extension temp were increased from 72°C to 74°C and from 67°C to 69°C. The secondary PCR reaction was run on 1% agarose gel and 2 major bands (~0.7kb and ~1.7kb) were gel purified and cloned into PCR[®] II-TOPO[®] TA vector (Invitrogen, Carlsbad, CA) .

Generating oMx1 serial deletion constructs:

The oMx1 promoter specific primers linked with *Mlu* I and *Xho* I restriction sites (Table.1) were designed to clone a full-length, ~2.1 kb, (-1666 bp; Luc1) genomic fragment containing ~1.7 kb promoter/enhancer, exon 1 (25 bp), and ~0.4 kb partial intron A. The serial deletion mutants which ranged from -1425 bp to -99 bp (Luc2-9), relative to the transcription start site (TSS), and oMx1 promoter/enhancer lacking putative intronic-ISRE site (Luc10) were cloned as well (fig. 3). The thermocycler settings were similar to the previously described in intron A amplification. The PCR reactions generated the expected size products which were cloned into PCR[®] II-TOPO[®] TA vector (Invitrogen, Carlsbad, CA) and sequenced. The linked restriction sites were used to release oMx1 promoter mutants from the TOPO plasmid by double digestion with *Mlu* I and *Xho* I enzymes (Promega, Madison, WI). Subsequently, inserts were gel-purified using NucleoTrap[®] Gel Extract Kit (BD Biosciences, San Jose, CA) and subcloned into *Mlu* I and *Xho* I linearized pGL3Basic luciferase plasmid (Promega, Madison, WI) to generate oMx1p-Luciferase (Luc1-10)

constructs. To determine the orientation of inserts, the pGL3Basic flanking sequence and the 5' and 3' ends of inserts were sequenced. The oMx1p-Luciferase (Luc1-10) constructs were purified using QIAGEN HiSpeed Midi plasmid DNA purification kit (QIAGEN, Valencia, CA), and the purity and concentration of DNA preparation were determined by spectrophotometry and gel electrophoresis.

Cell culture and transient transfection:

All cell line experiments were repeated 2 times, with 3 wells /treatment. All transfections included the β -galactosidase plasmid to monitor transfection efficiency and cells were harvested using 1X passive lysis buffer (PLB) according to manufacturer's protocol (Promega, Madison, WI).

Ovine glandular epithelial (oGE) endometrial cells (Johnson et al., 1999) were cultured in 12-well plates (Corning Inc, Corning, NY), 5% CO₂ at 38.5°C for 24h in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, St. Lois, MO) supplied with 10% FBS (GIBCO; Invitrogen, Carlsbad, CA). At approximately 90-95% confluency, cells were either mock transfected or transfected with 500ng/mL of pGL3Basic (negative control), pGL3Control (positive control; Promega, Madison, WI) or oMx1p-Luciferase constructs and 50ng/mL β -galactosidase expression pEF1/Myc-His/LacZ plasmid (Invitrogen) using LipoFectamine2000™ reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, culture medium containing serum was aspirated and cells were washed twice with Opti-MEM Reduced Serum media (Opti-MEM RS; Invitrogen, Carlsbad, CA) Invitrogen) and after final wash, 800 uL of Opti-MEM RS media was added to each. Lipofectamine2000 reagent was mixed with Opti-MEM RS (1.5 uL/100 uL) and

incubated at room temperature for 5 min and then combined with DNA (500ng/100 uL of Opti-MEM RS) and incubated for 20-40 min at room temperature. Total of 200 uL of DNA-Lipofectamine2000 reagent was added to each well and cells were incubated for 4-6hr then, 500 ul of DMEM-10% FBS without antibiotic was added to each well. Cells were incubated for 24 hours and observed for cytotoxicity by microscope.

Human breast cancer (T47D) cell line was obtained from the American Type Culture Collection (ATCC No. HTB-133) and grown according the ATCC protocol. The T47D cells were cultured in 12-well plates (Corning Inc, Corning, NY) for 24 hours using phenol red RPMI-1640 supplemented with 10% FBS, 10 mM HEPES (GIBCO; Invitrogen, Carlsbad, CA), and 0.2 Units/ml bovine insulin (Sigma-Aldrich, St. Louis, MO) at 37°C and 5% CO₂. The T47D cells were either mock transfected or transfected with luciferase (500ng/mL) and β -galactosidase plasmids (100ng/mL) using FuGENE®6 transfection reagent (Roche, Indianapolis, IN) according to the product protocol. Briefly, 3 uL of FuGENE®6 were diluted in 47 uL phenol- and serum-free RPMI-1640 containing 10 mM HEPES, and 0.2 Units/ml of bovine insulin and incubated at room temperature for 5 min and then plasmids (4 uL) were added and mixed with diluted transfection reagent and then incubated at room temperature for 20-40 min. During incubation, cells were washed once with Opti-MEM RS medium (Invitrogen, Carlsbad, CA) and approximately 500 uL of phenol- and serum-free RPMI-1640 containing 10 mM HEPES, and 0.2 Units/ml bovine insulin was added to each well and then received 54 uL of FuGENE® 6-plasmid DNA mixture. Cells were incubated with transfection mixture for 5-6 hours and then

1 mL of serum containing RPMI-1640 medium was added and incubated for 48 hours before starting treatments.

Effect of time and dose of IFN- τ on oMx1 full-length promoter luciferase activity:

To determine effect of 10,000 AVU/mL of IFN- τ treatment on the oMx1 promoter luciferase activity, oGE cell were transfected with the full-length oMx1 promoter (Luc1), and then harvested at 0 (control), 3, 6, 12, 24, 36, and 48 hours after IFN- τ treatment. To establish effect of various IFN- τ doses, oGE cells were transfected with the full-length oMx1 promoter (Luc1) and untreated or treated with different doses of IFN- τ (1-10,000 AVU/mL) for 12 hours and then harvested.

Effect of IFN- τ on oMx1 full-length promoter and oMx1 promoter deletion mutants' luciferase activity in oGE cell line:

Ovine GE cells were transfected with oMx1 promoter constructs (6-wells/construct) and 24 hours after transfection; cells were incubated (3-wells/treatment) with medium only or medium containing 10,000 AVU/mL of IFN- τ . Cells were harvested after 12 hours of IFN treatment.

Effect of IFN- τ on oMx1 full-length promoter and oMx1 promoter deletion mutants' luciferase activity in T47D cell line:

The human breast cancer cell line, T47D cells were transfected with plasmid DNA (6-wells/construct) using FuGENE® reagent as described above. Forty eight hours after transfection, cells were untreated or treated with 10,000 AVU/mL of IFN- τ (3-wells/treatment) for 12 hours and then harvested using PLB.

Luciferase and β -Galactosidase Assays:

Cell lysates from each well were assayed for luciferase and beta-galactosidase activity using recommended protocols of Luciferase Assay System (Promega, Madison, WI) and Galactolight Plus (Applied Biosystems, Foster City, CA), respectively. Luciferase and beta-galactosidase assays were run using VICTOR2 Wallac1420 luminometer (Perkin Elmer, Boston, MA).

Statistical analysis:

Luciferase units from untreated and IFN- τ treated cells were analyzed using general linear models (GLM) procedures of the Statistical Analysis System (version 9.1, SAS Inc., Gary, NC). The model included main effect of treatment and construct and their interaction. Results are reported as fold increase or decrease.

Results:

5' Rapid amplification of cDNA ends (5'RACE) and oMx1 exon-exon structure analysis:

Sequence analysis revealed that oMx1 5'UTR had about 99% homology (fig. 1B) with known sheep Mx sequences. Our RACE clone had 50 and 18 base unique nucleotide sequences compared to the Mx1 published sequences; X66093 and AF399856, respectively. Interestingly, this sequence had 13 nucleotides which were

identical to the published full-size bovine Mx1a exon 1, (GenBank accession No. AY251193). The NCBI Spidy program was utilized to map the exon-exon structure of the ovine Mx1 cDNA consensus sequence against the bovine Mx1-a genomic sequence (GenBank accession No. AY251193). It was predicted that the oMx1 gene had 16 exons and 15 introns (Fig. 2).

Analysis of oMx1 gene promoter/enhancer sequence:

The oMx1 full-length promoter/enhancer region was cloned in 2 steps. In the first step, intron A, exon1 and partial promoter sequence was cloned and in the second step and based on the sequence information from the first step and 5'RACE, approximately 2.1 kb genomic fragment was cloned which represented the 5' region of the oMx1 gene (Fig. 3). The oMx1 promoter/enhancer sequence was searched for putative transcription factor (TF) binding sites using the default settings of the web-based program; the Transcription Elements Search System (TESS) [<http://www.cbil.upenn.edu/tess>]. Computer assisted analysis revealed the presence of the following putative TF binding sites (Fig. 3 and 4); two ISRE sites (-101 and -145) and one intronic-ISRE site at +244 that had high sequence similarity to the ISRE consensus sequence; [(G/A/T)GAAAN(1-2)GAAA(G/C)(A/T/C)] (Collet and Secombes, 2001). In addition there were several ISRE core sequences GAAAN and GC-rich boxes that were found in the oMx1 promoter/enhancer region. The oMx1 promoter lacked a TATA box and IFN- γ activated site (GAS). Putative half-sites for progesterone receptor (PR; -1650, -1263, -530), and estrogen receptor (ER; -1437, -1188, -849) were also predicted. The oMx1 promoter contained 2 putative serum

response factors (SRF) (-1688, and -1567), 3 putative activator protein-1 (AP-1; -1183, -1001, -800) and several stimulating protein 1 (Sp1; -1576, -1016, -657, -590, -262, -72) binding sites. There were 3 predicted NF-kappaB (NF-κB) binding sites at -920, -776, and -573, and 4 IL6 response elements (IL6-RE) at -1144, -536, -237, and -104.

Effect of IFN-τ treatment on oMx1 promoter/enhancer constructs:

To determine the effect of different doses of IFN-τ on the full-length oMx1 promoter (Luc1) luciferase activity, transfected oGE cells were treated with different doses of IFN-τ and the luciferase activities were measured. There was approximately 2-fold and 14-fold increase in luciferase counts in response to 1 and 1000 AVU/mL of IFN-τ (fig. 5A). The effect of different treatment times with 10,000 AVU/mL of IFN-τ was also tested (fig. 5B). The luciferase activity driven by the oMx1 promoter increased (5-fold) after 3 hours, peaked (9-fold) at 12 hours and remained elevated (4-fold) after 48 hours of treatment with IFN-τ.

To establish the transcriptional activity of oMx1 promoter and the minimal sequence that was required for IFN-τ responsiveness, serial deletion mutants were generated by PCR and transfected into oGE and human T47D cells and then treated with IFN-τ. In oGE cells (fig. 6), IFN-τ increased the luciferase activity ($P<0.01$) of the oMx1 full-length and truncated promoter constructs (Luc1-8 and 10). There were approximately 8 to 10-fold increases in the transcriptional activity of Luc1-4, 6, 7, and 10, only 3-fold in Luc5, and 2-fold in Luc8. Treatment with IFN-τ appeared to decrease (P

<0.01) luciferase levels by 1.5 fold in the Luc9 promoter constructs. In the T47D cell line (fig. 7), treatment with IFN- τ did not change ($P > 0.5$) the luciferase levels in cells transfected with full-length or truncated forms of oMx1 promoter.

Discussion:

This study reports the cloning and characterization of the 5' flanking region and predicts the genomic organization of the oMx1 gene. At the time when this study was conducted, only 2 sheep Mx1 cDNA sequences were available (GenBank accession No. X66093 and AF399856). The X66093 sequence was derived from pregnant ewe endometrium (Charleston and Stewart, 1993) while the AF399856 sequence was cloned from sheep brain (Szuchet et al., 2002). Although they share approximately 99% sequence homology, the reported 5' UTRs had some sequence discrepancies shown in figure 1A. To have a better understanding of the exon-exon junction structure of the oMx1 gene, we cloned the 5' UTR of the oMx1 cDNA. Utilizing day 15 pregnant ewe endometrial cDNA, oMx1 5' RACE generated a single product (~1.1 kb) which suggested that oMx1 mRNA transcript originated from a single transcription start site (TSS). Sequencing analysis of the 5' RACE product revealed a unique nucleotide sequence which is not found in the sheep Mx1 published sequences (fig. 1B). The oMx1 5' RACE contained an extra 50 and 18 nucleotides compared to Mx1 published sequences; X66093 and AF399856, respectively. The oMx1 cDNA consensus sequence was aligned with the bovine Mx1-a genomic sequence (GenBank accession No. 33111869) to predict exon-exon boundaries of oMx1 gene using the NCBI Spidy program. It was predicted that the oMx1 gene consists of 16 exons and

15 introns (fig. 2). The number of exons of oMx1 is close to the bovine Mx1-a and human MxA genes which possess 15 and 17 exons, respectively (Gerardin et al., 2004; Tazi-Ahnini et al., 2000). Exon 1 of oMx1 is 25 bp in size and contains 13 bp which represents the entire exon 1 sequence in bovine Mx1-a gene (GenBank accession No. AY251193) (Ellinwood et al., 1998; Gerardin et al., 2004). In addition, several rounds of PCR amplifications of the 5' region of the oMx1 gene confirmed exon 1 sequence and size. Therefore, the sequence disagreement between this report and the other reports is probably due to inaccurate sequence in the previous studies or unknown splice variance in the sheep Mx1 gene downstream of exon 1.

Unlike mice Mx1 and 2 promoters, the oMx1 promoter does not contain a TATA box. This is similar to the promoter regions in human MxA, bovine Mx1, chicken Mx, zebrafish Mx1, and Fugu fish Mx1 (Altmann et al., 2003; Asano et al., 2003; Chang et al., 1991; Collet and Secombes, 2001; Gerardin et al., 2004; Hug et al., 1988; Schumacher et al., 1994). The oMx1 5' region had two ISRE sites which were located upstream of the TSS and one site was located in intron A. In other species, Mx promoters contained at least 2 functional ISRE, with exception of chicken Mx promoter that had a single ISRE (Altmann et al., 2003; Hug et al., 1988; Ronni et al., 1998; Schumacher et al., 1994; Yap et al., 2003). In addition, the oMx1 promoter has several GAAAN type sequences that represent the ISRE core sequence [(G/A/T)GAAAN(1-2)GAAA(G/C)(A/T/C)] (Collet and Secombes, 2001), which are found in most Mx promoter regions (Gerardin et al., 2004; Hug et al., 1988). Analysis of the 5' flanking region of the oMx1 gene revealed the presence of several putative

binding sites for cytokine and steroid transcription factors such as IL6-RE, NF- κ B, PR and ER half sites, AP-1, SRF and Sp1. These transcription factors are found in human MxA, mouse Mx1, and bovine Mx1-a promoter sequences as well (Assiri AM, 2004, unpublished observations) which may suggest common regulation of these genes in different species. The presence of putative steroid receptor (PR and ER) binding sites in the oMx1 gene promoter was not entirely unexpected because previous studies showed that oMx1 expression was regulated by steroids in the ovine uterus (Ott et al., 1999; Ott et al., 1998). However, further studies are needed to verify if these sites are functional.

The full-length oMx1 promoter responded to the lowest dose (1 Unit) of IFN- τ that was used and to higher dosages as well which suggests that the promoter is very sensitive to IFN- τ induction. In addition, the full-length oMx1 promoter was induced within 3 hours, and showed maximal response at 12 hours of incubation with IFN- τ . Although still higher than the control, the decline of the transcriptional activity 12 hours after IFN- τ induction, could be attributed to either binding of negative regulatory factors such as interferon regulatory factor 2 (IRF2), a known suppressor of ISGs (Choi et al., 2001), or post-transcriptional/translational modifications which may affect the luciferase mRNA/protein stability.

Serial deletions and oGE transient transfection experiments showed the transcriptional behavior of truncated oMx1 promoter/enhancer in response to IFN- τ treatment. Both ISRE positioned at -101 and -145 are required for maximal oMx1 promoter response to IFN- τ . Deletion of the distal ISRE site (-145) decreased luciferase activity from approximately 8-fold (Luc7) to 2-fold (Luc8). Interestingly,

the presence of intronic ISRE at +244 (Luc8) did not compensate for the loss of the ISRE site (-145). In addition, deleting both proximal ISRE sites (-101 and -145) and maintaining the intronic ISRE site (Luc9) resulted in a decrease in promoter activity in response to IFN- τ treatment by 1.5 fold compared to non-treated construct. However, deleting the intronic ISRE site while maintaining the 2 proximal ISRE sites in Luc 10, did not affect the luciferase activity, and the promoter show the maximal response to IFN. Deletion of a distal region (-920 to -715) resulted in a 6-fold decrease in luciferase activity in response to IFN- τ treatment. However, subsequent deletion of the -715 to -437 region restored maximal promoter response (~9-fold) to IFN- τ . The simplest interpretation of these results is that the regions -920 to -715 and -715 to -437 have positive and negative regulatory element binding sites, respectively. However, this remains to be determined.

To study the role of steroids in oMx1 promoter stimulation by IFN- τ , the human T47D cell line was used. This cell line expresses high levels of estrogen receptor (ER) and progesterone receptor (PR) (Keydar I, 1979). T47D cells were transfected with the full-length and all truncated constructs of the oMx1 promoter/enhancer region. In transient transfection experiments the oMx1 promoter constructs, including the full-length promoter, failed to respond to IFN- τ treatment and it appeared that the oMx1 promoter was constitutively activated in this cell line. For example, the full-length oMx1 promoter showed ~ 100-fold and 4-fold luciferase activity in non IFN- τ -treated T47D and oGE cells, respectively. In each construct, the transcriptional activity in the absence IFN was very high and therefore, the effect of treatment could not be established. These findings were not expected because it

was reported that T47D cells respond to type I IFN treatment with reduced cell proliferation (Coradini et al., 1995) and activation of the Jak/Stat signaling pathway (Schaber et al., 1998). Interestingly, the patterns of luciferase activities by various oMx1 promoter constructs in T47D cells resembled that in oGE cells following IFN- τ treatment (fig. 6 and 7). Whether high expression of steroid receptors by the T47D cell line caused the apparent oMx1 promoter activation is unclear, however treatment with RU-486, a PR-antagonist, did not affect the full-length promoter response to IFN- τ in this cell line (data not shown). Additional studies will be required to examine signaling pathways that might activate the IFN-induced oMx1 promoter in the absence of IFN in T47D cells.

In this study, the oMx1 unique '5 UTR sequence, TSS, exon-exon structure were determined. In addition, the 5' flanking region of the oMx1 gene was cloned and its ability to drive reporter plasmid expression was studied. Results from this study suggest that IFN- τ activates the oMx1 promoter through the proximal ISRE sites. The importance of the 2 proximal ISRE sites for oMx1 promoter activation by IFN is consistent with results for the human MxA and mouse Mx1 promoters. Although, a predicted intronic-ISRE is present in the human MxA promoter, this is the first report to show that this site may not be required for oMx1 promoter activation by IFN. In addition, the oMx1 promoter transcriptional activity was affected by the presence of upstream positive and negative regulatory regions in the oMx1 promoter that was evident in both cell line examined. Finally, constitutive expression of the oMx1 promoter/enhancer in T47D cells may provide a novel model to examine sites which drive Mx1 expression.

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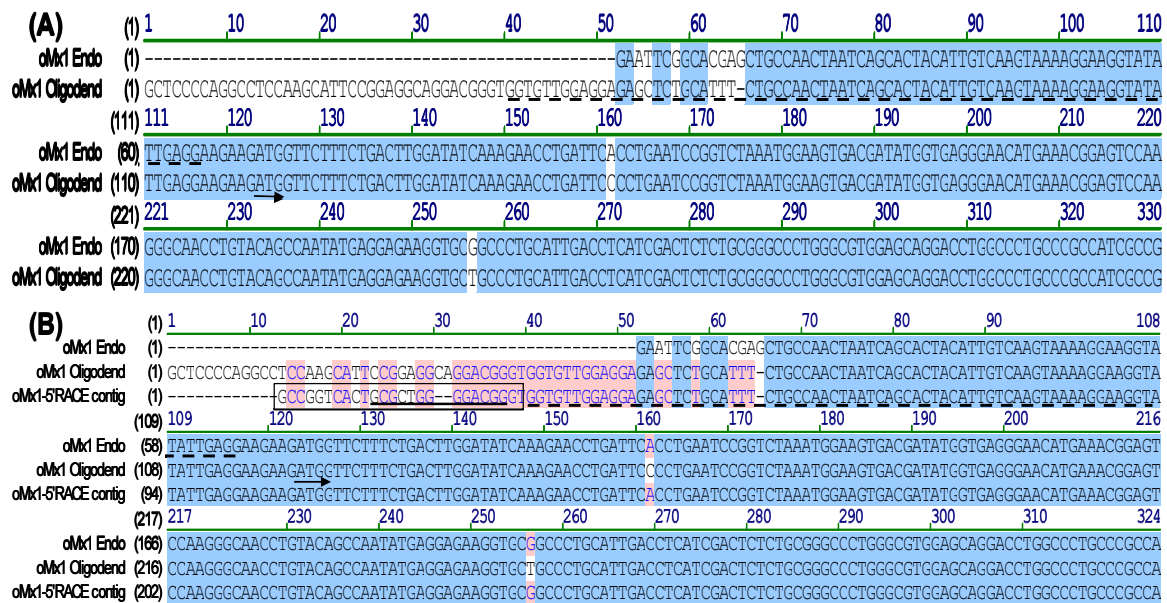


Fig1. Multiple sequence alignment (MSA) of the oMx1 endometrial cDNA (GeneBank# X66093) and oligodendrocyte GTP-binding protein cDNA (GeneBank # AF399856). The MSA analysis show sequence discrepancy in the 5'UTR region between the published Mx1 sequences (A). Alignments between known oMx1 sequences and the sequence of oMx1 5'RACE (this study) is shown (B). The translation start site (ORF) is indicated by the arrow, oMx1 exon1 sequence is boxed, the 13 bp conserved sequence of oMx1 the bovine Mx1-a exon 1 (AY251193) is underlined, and predicted exon 2 sequence is marked with dashed line. The white and blue (A&B)/ pink (B) background colors indicate sequence discrepancy and identical sequence, respectively.

Exon-exon structure of the oMx1 gene

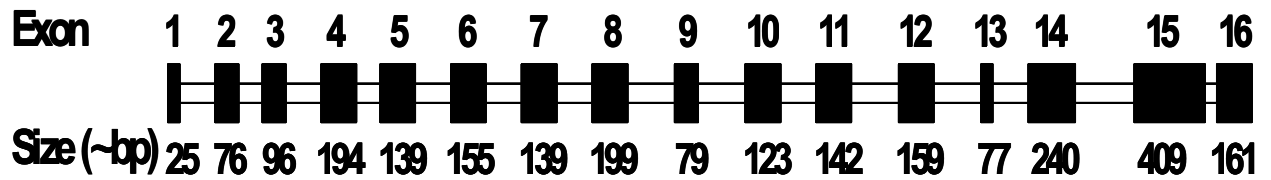


Fig2. The bovine Mx1-a genomic sequence (GeneBank # 33111869) was utilized to map ovine the Mx1 gene structure by using NCBI Spidy program. It was predicted that the oMx1 gene consists of 16 exons and 15 introns.

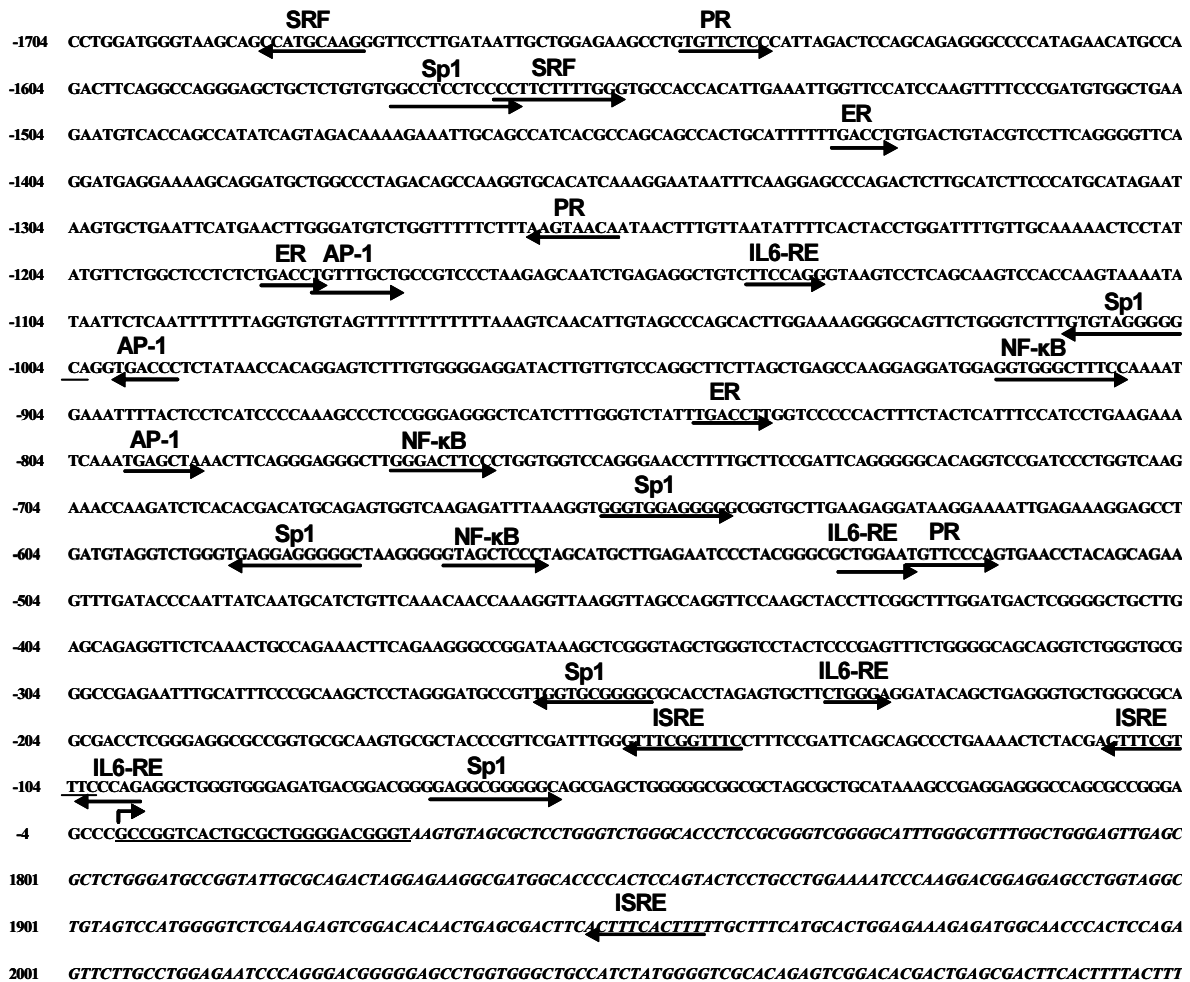


Fig3.The 5' nucleotide sequence of the oMx1 gene including 1.7 kb of promoter/enhancer, 25 bp exon 1(underlined), and partial intron A (in italic). The transcription start site is indicated with bend arrow. The putative transcription factor binding sites (TFBS) are marked with arrows pointed left to right (sense strand) and right to left (reverse strand) to indicate orientation of TFBS. AP-1; activator protein-1, ER; estrogen receptor, ISRE; interferon stimulatory response element, IL-6RE; interleukin-6 response element, NF-κB; nuclear factor-kappa B, PR; progesterone receptor, Sp1; stimulating protein-1, SRF; serum response factor.

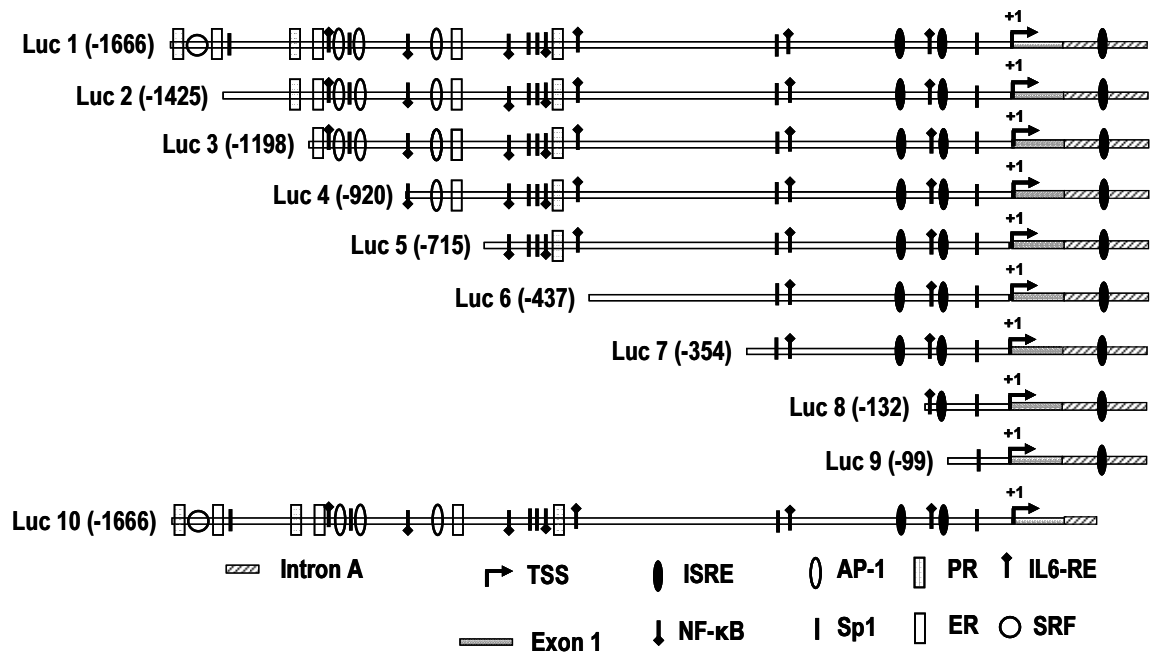


Fig4. Schematic diagram showing 5' region of oMx1 gene deletion mutants and putative transcription factor binding sites. Luc1 construct represents the full-length promoter/enhancer region including all 3 predicted ISRE sites while Luc 2-7 constructs represent truncated forms of the full-length maintaining the 2 proximal ISRE sites. Luc8 and 9 represents construct that lack 1 and 2 proximal ISRE sites, respectively. Luc10 construct represent the full-length (Luc1) but missing an intronic ISRE site.

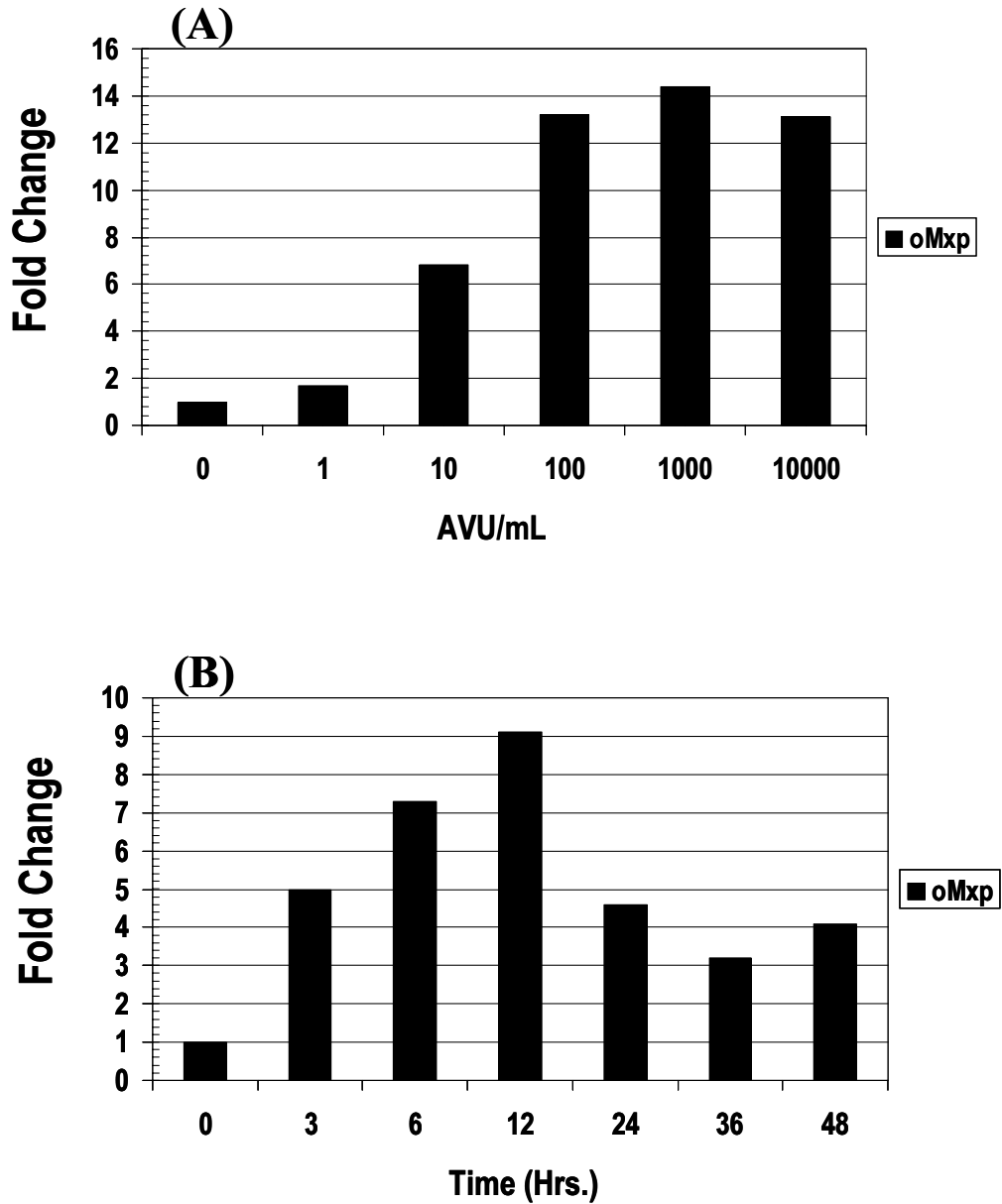


Fig5. Full-length oMx1 promoter/enhancer luciferase activity increased with increasing concentrations of IFN-tau (A) and treatment with 10,000 AVU/mL IFN increased luciferase activity 5- and 9-fold at 3 and 12 h, respectively (B).

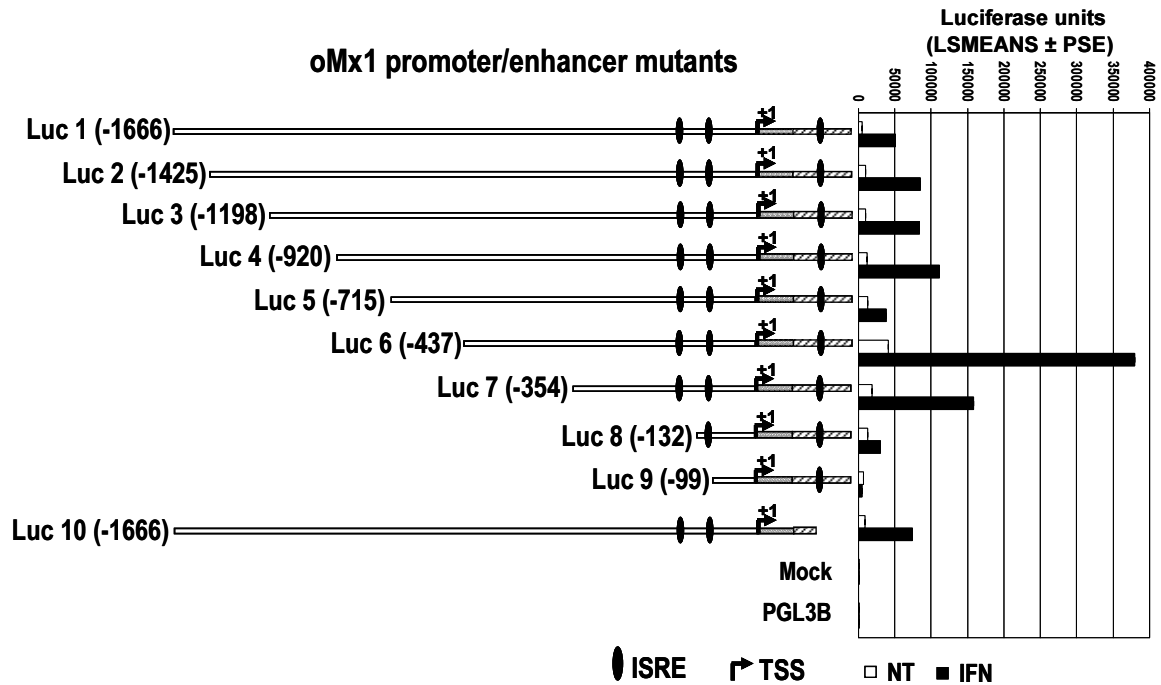


Fig6. Transcriptional activity of various oMx1 promoter/enhancer deletion constructs in oGE cells in the absence and presence of IFN τ . Treatment with IFN- τ increased the luciferase activity ($P < 0.01$) of the oMx1 full-length and truncated promoter constructs (Luc1-8 and 10). There were approximately 8 to 10-fold increase in the transcriptional activity of Luc1-4, 6, 7, and 10, only 3-fold in Luc5, and 2-fold in Luc8. Treatment with IFN- τ decreased ($P < 0.01$) luciferase levels in oMx1 promoter constructs; Luc9 by 1.5-fold.

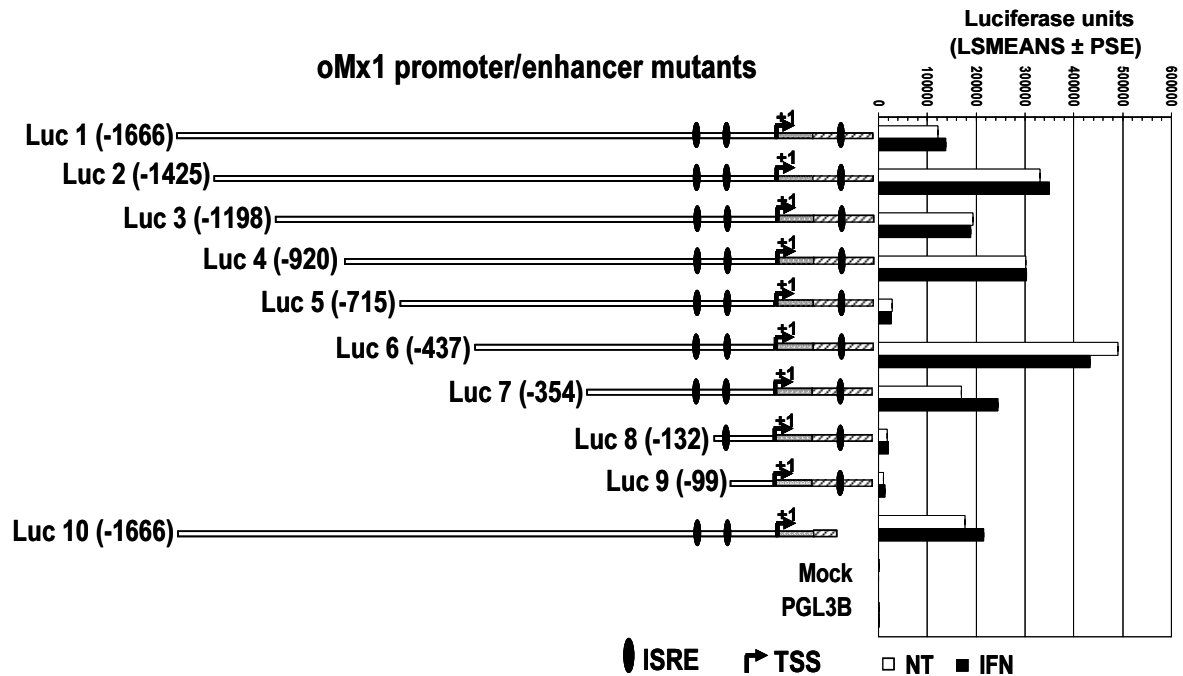


Fig7. Transcriptional activity of various oMx1 promoter/enhancer deletion constructs in T47D cells in the absence and presence of IFN τ . Treatment with IFN- τ did not change ($P > 0.5$) the luciferase levels in cells transfected with full-length or truncated forms of oMx1 promoter. Regardless of treatment, oMx1 deletion mutant luciferase levels in T47D, show similar pattern to the luciferase activity following IFN treatment in oGE (fig. 6).

oMx1p-Luciferase	Regions: TSS	Forward primer- <i>Mlu</i> I (located upstream of TSS)	Reverse primer- <i>Xho</i> I (located downstream of TSS)
Luc1	-1666bp	(oMx1PF-39) <i>ATACGCGTATTGCTGGAGAAAGCCCTGTGT</i>	(oMx1PR-2080) <i>ATCTCGAGCAGTCGTCGTGCCGACTCTGTG</i>
Luc2	-1425bp	(oMx1PF-280) <i>ATACGCGTTGTACGTCCTTCAGGGGTTTC</i>	(oMx1PR-2080) <i>ATCTCGAGCAGTCGTCGTGCCGACTCTGTG</i>
Luc3	-1198bp	(oMx1PF-507) <i>ATACGCGTTGGCTCCTCTCTGACCTGTT</i>	(oMx1PR-2080) <i>ATCTCGAGCAGTCGTCGTGCCGACTCTGTG</i>
Luc4	-920bp	(oMx1PF-785) <i>ATACGCGTGTGGGCTTCCAAAATGAAA</i>	(oMx1PR-2080) <i>ATCTCGAGCAGTCGTCGTGCCGACTCTGTG</i>
Luc5	-715bp	(oMx1PF-990) <i>ATACGCGTCCCTGGTCAAGAAAACCAAGA</i>	(oMx1PR-2080) <i>ATCTCGAGCAGTCGTCGTGCCGACTCTGTG</i>
Luc6	-437bp	(oMx1PF-1268) <i>ATACGCGTTACCTTCGGCTTTGGATGAC</i>	(oMx1PR-2080) <i>ATCTCGAGCAGTCGTCGTGCCGACTCTGTG</i>
Luc7	-354bp	(oMx1PF-1351) <i>ATACGCGTGGGTAGCTGGGTCTACTCC</i>	(oMx1PR-2080) <i>ATCTCGAGCAGTCGTCGTGCCGACTCTGTG</i>
Luc8	-132bp	(oMx1PF-1573) <i>ATACGCGTGACAGCCCTGAAAACTCTACG</i>	(oMx1PR-2080) <i>ATCTCGAGCAGTCGTCGTGCCGACTCTGTG</i>
Luc9	-99bp	(oMx1PF-1606) <i>ATACGCGTAGAGGCTGGGTGGGAGAT</i>	(oMx1PR-2080) <i>ATCTCGAGCAGTCGTCGTGCCGACTCTGTG</i>
Luc10	-1666bp	(oMx1PF-39) <i>ATACGCGTATTGCTGGAGAAAGCCCTGTGT</i>	(oMx1PR-1872) <i>ATCTCGAGTTCAGGCAGGAGTACTGG</i>

Table1. List of primers that were used to clone oMx1 promoter deletion constructs Luc 1-10. The Mlu I and Xho I restriction sites are in italic font.

CHAPTER FIVE:

Summary and general discussion

Infertility is a major problem in humans and in animal reproduction. One factor contributing to infertility is early embryonic loss (within first 30 days of pregnancy) which constitutes ~ 75% of total embryonic losses in humans (Shahani et al., 1992). In farm animals, fertilization is a quite efficient process. For example, the fertilization rate is estimated to be 95% in pigs, but early embryonic loss is relatively high (~25-30%) (Pope, 1988). In dairy cattle, estimates of fertilization rates range from 75-90% and by day 35-40 after insemination, roughly 35% of lactating cows will be pregnant (Santos et al., 2004). Miscommunication between embryo and maternal system is thought to be one of the major components contributes to early embryonic loss and infertility. Coordinated communication between the developing conceptus and the maternal system is a prerequisite for extending the CL lifespan and establishing pregnancy (Spencer and Bazer, 2004; Spencer et al., 2004). In sheep, the embryonic signal is the conceptus-secreted IFN- τ that acts locally on the endometrium to suppress the release of luteolytic pulses of PGF $_{2\alpha}$ and up-regulates interferon stimulated genes (ISGs) including the large GTPase; Mx proteins (Ott et al., 1998). In pigs, maternal recognition of pregnancy is characterized by redirection of PGF $_{2\alpha}$ secretion away from the uterine vasculature toward the uterine lumen (Bazer and Thatcher, 1977) and this response is mediated by conceptus-produced estrogen.

In this dissertation we report the results from a series of experiments designed to understand the hormonal regulation and endometrium cellular contribution to PGF $_{2\alpha}$ secretion in cyclic and pregnant gilts. In addition, we report results from a series of experiments designed to study the antiviral Mx gene

regulation in the endometrium, peripheral blood leukocytes, and corpus luteum during early pregnancy in sheep.

The first set of experiments studied the effects of estradiol (E_2) and progesterone (P_4), and oxytocin (OT) on $PGF_{2\alpha}$ secretion from the basolateral and apical surfaces of luminal epithelial (LE) and stromal (ST) cells obtained from day 16 pregnant and cyclic gilts endometrium. The basolateral surface of LE cell secretes more ($P < 0.01$) $PGF_{2\alpha}$ than the apical surface in both cyclic and pregnant gilts. In addition, $PGF_{2\alpha}$ secretion from the basolateral surface of LE cells obtained at Day 16 after estrus was greater ($P < 0.01$) in cyclic than pregnant gilts. These results for cyclic gilts confirm previous work from cells obtained on Day 16 from cyclic gilts (Braileanu et al., 2000; Hu et al., 2003). Regardless of steroids treatments, OT failed to stimulate $PGF_{2\alpha}$ secretion from the apical surface of LE cells obtained from both cyclic and pregnant gilts. Hu et al. 2003 showed that estradiol enhanced the OT stimulation of $PGF_{2\alpha}$ secretion from the apical surface of LE cell obtained from cyclic animals. The discrepancy between our study and that of Hu et al. 2003 could be attributed the serum starvation that was used in the previous study which might cause the cells to be more responsive to OT treatment. Failure of OT to stimulate $PGF_{2\alpha}$ secretion from the apical surface of LE cell is consistent with earlier work that showed LE cells were unresponsive to OT when obtained on Day 16 from cyclic, pregnant or pseudopregnant gilts (Braileanu et al., 2000; Uzumcu et al., 1998; Uzumcu et al., 2000). Although luminal epithelial cells express the greatest number of OT receptors (Boulton MI, 1995), they are the endometrial cell type that is least responsive to exogenous OT

treatment (Uzumcu et al., 1998). This unresponsiveness to OT may be attributed to endogenous OT secretion by LE cells, which acts in an autocrine manner to make LE cells less sensitive to exogenous OT stimulation (Hu et al., 2001). In stromal cells obtained on Day 16 after estrus, OT treatment stimulated PGF_{2α} secretion from cells from both cyclic and pregnant gilts. However, the stimulatory action of OT on PGF_{2α} secretion was affected by steroid treatment. The OT-induced secretion of PGF_{2α} was reduced by treatment with P₄ alone or P₄ + E₂ in cells from cyclic gilts and by P₄ + E₂ in pregnant gilts. For Day 16 cyclic gilts, reduction of stromal cell responsiveness to OT in the presence of P₄ + E₂ was also observed after 72 hr of steroid exposure (Hu et al., 2003). Results from this study showed that P₄ alone or with E₂ reduced the response of stromal cells (the largest cellular component, (Blackwell et al., 2003)) to OT, which could contribute to redirection of PGF_{2α} secretion toward the uterine lumen during early pregnancy.

In summary, the basolateral surface secretion of PGF_{2α} is higher from LE cells in cyclic than pregnant animals and treatment with progesterone decreased the stromal cells response to OT. Collectively, these findings would suggest that the reorientation of PGF_{2α} secretion from endocrine to exocrine direction is probably mediated by the decreased in the basolateral PGF_{2α} output from LE cells in pregnant gilts and by P₄-mediated reduction of stromal cell responsiveness to OT. Future experiments are needed to study whether the prolonged exposure of LE cells to the conceptus estrogen caused the observed reduction in the PGF_{2α} secretion from the LE cells' basolateral surface at Day 16 of pregnancy. This can

be achieved by comparing PGF_{2α} secretion from polarized LE cells obtained from Day 12 cyclic and pregnant gilts.

In the second two experiments the goal was to identify different Mx genes expressed in sheep endometrium and study their regulation during pregnancy in different reproductive tissues and to clone and characterize oMx1 gene promoter. The first set of experiments was aimed to clone sheep Mx genes and characterize their expression in the sheep endometrium, peripheral blood leukocytes (PBL), and CL during late diestrus and early pregnancy. We are the first group to report the existence of a second Mx gene in sheep; ovine Mx2 gene (oMx2). The oMx2 protein shares 93%, 57%, and 70% sequence homology with bovine Mx2, ovine Mx1 (oMx1) and human MxB proteins, respectively. However, the existence of a second Mx gene in sheep was not totally unexpected; results presented here are the first to examine Mx expression in the endometrium, PBL and CL of any species. During early pregnancy, the expressional profile of oMx2 and oMx1 were similar in the endometrium and PBL but dissimilar in the CL. Pregnancy up-regulates oMx1 and oMx2 mRNA starting at Day 13 and Day 15 in endometrium and PBL, respectively. In the ewe CL, oMx2 mRNA was up-regulated by pregnancy while oMx1 mRNA appeared to be constitutively expressed and was not affected by pregnancy. In addition, oMx2 and oMx1 the spatial expression and intracellular localization were different *in vivo* and *in vitro*, respectively. *In situ* hybridization results utilizing serial cross-sections from Day 17 and 19 pregnant ewes endometrium showed that the oMx2 mRNA was highly expressed

in immune cells in the endometrium immediately beneath the luminal epithelium (LE), while oMx1 was highly expressed (LE), glandular epithelium (GE) and stromal cells. In addition, localization of both proteins in ovine glandular epithelium (oGE) following IFN treatment by immunofluorescence revealed that oMx1 was predominantly cytoplasmic while oMx2 was cytoplasmic and nuclear protein.

In this study we showed that sheep has 2 dynamin-like large GTPases, oMx1 and oMx2 which had unique cellular expression and regulation during early pregnancy. The sequence homology and cellular localization between oMx2 and human MxB proteins are similar which suggest that these proteins may serve normal physiological functions that are conserved between species. In addition, we demonstrated that closely related and IFN-induced oMx1 and oMx2 genes had apparent regulation in the reproductive tissues during early pregnancy.

Different experimental approaches can be done to understand function and differential regulation of oMx1 and oMx2. For example, comparative promoter analysis between oMx1 and oMx2 will shed more light main transcription factors that govern their apparent cell-specific expression. In addition, identifying binding partners, knock-down by siRNA or dominant-negative experiment, overexpression will enhance our understanding of the functions of oMx2 cytoplasmic and nuclear forms.

The second series of experiments were conducted to clone and characterize the 5' region of oMx1 gene. To clone the oMx1 promoter and define the transcription start site (TSS), 5'RACE was conducted which generated a

single PCR product that was cloned and sequenced. The oMx1 5' RACE sequence analysis revealed additional nucleotide sequence that was missing in GenBank published sequences (X66093 and AF399856). This new finding was utilized to delineate the oMx1 gene exon-exon boundaries. It was predicted that the oMx1 gene contained 16 exons and 15 introns which is similar to Mx genes in human and bovine (Gerardin et al., 2004; Tazi-Ahnini et al., 2000). Mapping the oMx1 exon-exon structure enhanced our ability to clone the 5' region of the gene. Sequence analysis of the oMx1 promoter/enhancer region revealed the presence putative binding sites for several cytokine and steroid transcription factors such as ISRE, IL6-RE, NF- κ B, PR and ER half sites, AP-1, SRF and Sp1. Finding of ER and PR binding sites in the oMx1 promoter confirmed our previous work that showed steroids are permissive for oMx1 stimulation by IFN. In oGE cells and by utilizing transient transfection system, we demonstrated that oMx1 promoter was responsive to IFN- τ treatment. The oMx1 promoter responsiveness to IFN response was mediated by the 2 proximal ISRE sites but not by the intronic ISRE site. The importance of the 2 proximal ISRE sites for oMx1 promoter activation by IFN- τ is consistent with results for the human MxA and mouse Mx1 promoters (Hug et al., 1988; Ronni et al., 1998). Although an intronic ISRE site is found in human MxA, this is the first study to demonstrate that this site may not be required for IFN- τ induction. In addition, serial deletions revealed the presence of an enhancer (at -920 to -715) and a suppressor (-715 to -437) sequence in the 5' region of the oMx1 gene. Deletion of the enhancer region resulted in approximately 50% reduction of the oMx1 promoter response to IFN- τ

stimulation. Subsequent deletion which eliminated a suppressor binding site(s) restored the full-response of the promoter to IFN- τ . In human breast cancer cell line; T47D, treatment with IFN- τ failed to stimulate the transcriptional activity of the oMx1 promoter constructs which appeared to be constitutively activated. For example, the full-length oMx1 promoter showed ~ 100-fold and 4-fold luciferase activity in non IFN-treated T47D and oGE cells, respectively. In T47D cells and in the absence or presence of IFN- τ , the pattern of luciferase activity of oMx1 promoter was similar to oGE cells following IFN- τ treatment which may suggest that T47D cells secrete IFN or IFN-like substances that activate the promoter.

Further experiments are needed to define key transcription factors that bind the enhancer and silencer region of the oMx1 promoter. The apparent activation of oMx1 promoter in T47D cells will be a useful model to study the regulation of Mx genes in the absence of IFN stimulation. Future experiment will be focused on identifying transcription factors that bind the oMx1 upstream enhancer and suppressor region by DNase footprinting, chromatin immunoprecipitation assay (ChIP), and electrophoresis mobility shift assay (EMSA). Identified transcription factors will be characterized in vivo animal model (i.e. cyclic and pregnant ewes). In addition, characterization of the identified steroid receptor putative binding sites in the oMx1 promoter will be carried out by ChIP and EMSA to test if these sites are functional. Additional experiments will be conducted to confirm these finding and to address the steroid co-regulation of oMx1 expression such as co-transfection of oMx1 promoter with ER and PR overexpression plasmid in oGE cells.

The presented findings will enhance our understanding of the endocrinology and gene regulation during the maternal recognition of pregnancy in pigs and sheep. Understanding the biology of the fetal and maternal interaction is an important step toward improving fertility in farm animals and humans.

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