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EXPRESSION AND REGULATION OF MX1 SPLICE VARIANTS IN REPRODUCTIVE TISSUES AND PERIPHERAL BLOOD IMMUNE CELLS OF SHEEP AND CATTLE

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ABSTRACT

Ruminant conceptuses produce interferon tau (IFNT) during early pregnancy which increases expression of the myxovirus resistance gene, MX1, in the uterus. The role of MX1 in pregnancy is not known, but its role in the immune response is well-characterized. Previous work demonstrated the existence of MX1 splice variants in cattle. We hypothesized that these variants might be differentially regulated or possess different biological activities in cattle and sheep.

In the first experiment we characterized the MX1 splice variants in ovine uterine epithelial cell lines. Sequence analysis of amplicons generated from an ovine endometrial cell line using a common set of MX1 primers revealed three splice variants that differed in their 5' regions. Full-length sequences for each splice variant were generated. Compared to the published sequences, one isoform contained an 18 base deletion (MX1b) and the other an insertion of 186 bases (MX1c). Computer-assisted translation of MX1b resulted in a protein lacking 6 amino acids near the amino terminus. An early stop codon in MX1c was followed by two secondary in-frame start codons. If internal initiation of translation occurred the first 30 amino acids of MX1a would be replaced with 27 unique amino acids. Sequence differences occurred at the boundary between putative exon 3 and exon 4, suggesting they are bono fide splice variants. Two-dimensional electrophoresis and Western blot analysis of oGE cell lysates with three different MX1 antibodies showed the existence of several isoelectric variants that could correspond to protein products from each variant.

To examine regulation of each variant, we developed quantitative PCR assays to distinguish each isoform. All three isoforms were identified in ovine uterine luminal

epithelial (oLE) and stromal (oSC) cell lines. Interestingly, only two of the isoforms, MX1a and MX1c, were detectable in glandular epithelial (oGE) cells and sheep endometrium. Concentrations of MX1c mRNA were roughly 1000-fold greater and did not change following treatment with IFNT in any cell line compared to MX1a and MX1b. However, concentration of MX1a strongly increased in all cell lines in response to IFNT as well as MX1b in oLE and oSC cells (> 300-fold at 6 hours; $P < 0.05$).

In the second experiment we quantified expression of MX1 variants in the endometrium and peripheral blood leukocytes (PBL) of ewes and cows. Only MX1a and MX1c were detectable in ewes. In the endometrium, MX1a concentrations were higher on Days 13, 15 17 and 19 of pregnancy when compared to cyclic Day 11 and peaked on Day 15 ($P < 0.05$). As with the cell lines, MX1c concentrations did not change ($P > 0.10$) according to pregnancy status. Similar to results from endometrial samples, the concentrations of MX1a in PBLs were 7-fold higher on Day 15 of pregnancy than Day 15 of the cycle ($P < 0.05$) and MX1c concentrations were not different ($P > 0.10$). We also examined concentrations of each variant in the PBLs of pregnant and bred, non-pregnant cows that were either treated with progesterone (CIDR) or not on Day 19 after insemination. Unlike the ewe studies, all three variants were detectable in all cows. Interestingly MX1a and MX1b were increased only in the CIDR open group which had high progesterone concentrations ($P < 0.05$) and were not different in any of the other groups including the no CIDR pregnant group ($P > 0.10$). Similar to the results from immortalized sheep cell lines, both sheep and cattle had MX1c concentrations 1000-fold higher than MX1a or MX1b.

Results suggest that three splice variants of MX1 mRNA are present in sheep and cattle and are differentially regulated by pregnancy status and IFNT. Unlike MX1a and MX1b, MX1c is highly abundant and is not regulated by IFNT or pregnancy. Two-dimensional electrophoresis and Western blot results suggest that the MX1 variants characterized here could be translated into proteins with different biological activities.

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LIST OF ABBREVIATIONS

2’5’ Oligoadenylate synthase.....OAS

Beta2-microglobulin B2MG

Central-interacting domain .....CID

Corpus Luteum..... CL

Estradiol E2

Estrogen receptor alpha..... ER1

Exon splicing enhancer ..... ESE

Exon splicing siliencer.....ESS

Follicular stimulating hormone..... FSH

Gamma-activated factor.....GAF

Glandular Epithelium..... GE

Gonadotropin releasing hormone..... GNRH

Interferon..... IFN

Interferon receptors.....IFNAR

Interferon regulatory factor..... IRF

Interferon-stimulated gene ..... ISG

Interferon-stimulated response elements .....ISRE

Interferon-stimulated transcription factor 3 gamma ISGF3

Interferon tau.....IFNT

Intron splicing enhancer.....ISE

Intron splicing silencer.....ISS

Janus kinase ..... JAK

Leucine Zipper ..... LZ

Luminal epithelium ..... LE

Luteinizing hormone ..... LH

Messenger ribonucleic acid ..... mRNA

Oxytocin ..... OXT

Oxytocin receptor ..... OXTR

Peripheral blood leukocyte ..... PBL

Pregnancy associated glycoprotein ..... PAG

Pregnancy specific protein B ..... PSPB

Progesterone P4

Progesterone Receptor ..... PGR

Prostaglandin F2 alpha PTGF2A

Signal Transducer and Activator of Transcription ..... STAT

Stroma ..... S

Tyrosine Kinase 2 ..... TYK2

Chapter 1

Review of Literature

Introduction

Over the last 50 years the dairy industry in the US has changed dramatically. Milk production per cow has increased approximately 20% and dairy herd size has increased so that nearly 30% of all dairy cows are on farms with over 500 cows (Lucy, 2001). During the same period, however, reproductive efficiency has declined. First service conception has decreased from 65% to nearly 40%, the number of services per conception has risen, and days open has increased from ~110 to ~150 days (Lucy, 2001; VanRaden *et al.* 2004). Reproductive efficiency is critical for the profitability of the dairy industry and the average cost of pregnancy loss was estimated to be $555, however this number is highly dependent on milk production and stage of pregnancy when the loss occured (De Vries, 2006). Because lactation is initiated by pregnancy, lifetime milk production depends on the frequency with which cows become pregnant. Therefore, reproductive efficiency is critical for the profitability of the dairy industry and low reproductive efficiency results in significant economic losses.

One major cause of low reproductive efficiency is early embryonic loss. Early embryonic deaths, those occurring before day 30, accounted for 75 to 80% of pregnancy loss in cattle and approximately 40% of total embryonic loss occurred between days 8 and 17 of pregnancy (Thatcher *et al*. 1990; 1994). During the first 30 days of pregnancy several critical events occur including rapid growth of the conceptus, maternal recognition of pregnancy, attachment and placentation. Maternal recognition of pregnancy is a term coined by R.V. Short that is used to describe the biochemical

signaling between the early embryo and the maternal system that results in maintenance of corpus luteum (CL) function and progesterone (P4) production (Spencer and Bazer, 2004). In ruminants, such as cattle and sheep, this is accomplished through the production and secretion of interferon tau (IFNT) by the mononuclear trophectoderm cells of the early conceptus (Bazer *et al*. 1991). Interferon tau binds to type I IFN receptors on the uterine epithelium and inhibits pulsatile endometrial production of prostaglandin F2-alpha (PTGF2A; Bazer *et al*. 1997). Pulsatile release of PTGF2A by the endometrium causes regression of the CL and a rapid drop in P4 as a prelude to a new ovulation and another attempt at pregnancy (McCracken *et al*. 1999). The critical period for embryo signaling to the uterus occurs between days 10 and 21 in cattle and sheep (Spencer and Bazer, 2004). This period coincides with the period of the highest embryonic loss in these species. One interpretation of this is that a portion of the losses are due to inadequate signaling between the embryo and the uterus. This, of course, leaves open the possibility of determining how to supplement or enhance this signaling to reduce early embryo loss and improve reproductive efficiency.

Reproductive Cycle

Estrous Cycle of Cattle and Sheep

The estrous cycles of cattle and sheep are similar. Because sheep have comparable reproductive physiology, are easy to handle, have low maintenance costs, and are readily available they are often used as a model for reproductive physiology of other mammals including cattle (Bazer *et al*. 1997). Some differences between the species include, length of the estrous cycle, age of puberty, and seasonality. In cattle the estrous cycle ranges in length from 17 to 24 days with an average of 21 days. However, in sheep

the range is 13 to 19 days, with an average of 17 days. Cattle are polyestrous animals with cycles repeating throughout the year, while ewes are seasonally polyestrous, short-day breeders. In general, cyclicity in sheep is initiated as the day length (photoperiod) begins to decrease in the fall and in most breeds will cease in the spring as hours of daylight increase. Breeds that were developed closer to the equator show less seasonality than those developed in the northern hemisphere. Heifers reach puberty between 9 and 24 months with an average of 11 months depending on the breed. Puberty in sheep is reached at an average of 7 months with a range of 4 to 14 months. Although there are differences in length and frequency of the estrous cycle and age of puberty, sheep and cattle do have similar phases and hormonal profiles throughout the estrous cycle.

The estrous cycle consists of two major phases, the follicular phase and the luteal phase, and is regulated by the nervous system and the endocrine system. The follicular phase is the period from regression of the CL of the previous cycle to ovulation (~Days 15 to 22 in cows). During this phase follicles are actively growing the ovary and there is high production of estradiol (E2) by the follicles. The luteal phase is the period from ovulation to regression of the CL (~Days 0 to 14). The primary ovarian structure during the luteal phase is the CL, which produces high amounts of P4.

Hormonal and Neural Control

The hypothalamus in the brain drives reproductive cyclicity with the production and release of gonadotropin releasing hormone (GNRH; Karsch *et al*. 1997). This neuropeptide is produced by the surge and tonic centers of the hypothalamus and is transported to the pituitary through the hypophyseal portal system, a specialized circulatory adaptation (Kaiser *et al*. 1997). Through the portal system GNRH is

transported to the anterior pituitary gland where it binds receptors on the gonadotrope cell and modulates the synthesis and secretion of the gonadotropins; luteinizing hormone (LH) and follicle stimulating hormone (FSH; Kaiser *et al*. 1997). These two glycoproteins travel through the systemic circulation to act on ovarian cells and regulate steroidogenesis and gametogenesis (Kaiser *et al*. 1997). The responses differ depending upon the structures present on the ovary. If a follicle is present, FSH causes follicular growth and maturation as well as E2 synthesis and LH stimulates ovulation (Kaiser *et al*. 1997). Luteinizing hormone also causes CL formation and P4 production (Kaiser *et al*. 1997). The steroid hormones produced by the ovary are released into the circulatory system and have effects on many tissues including the hypothalamus, pituitary, uterus, and the mammary gland (Kaiser *et al*. 1997). In the hypothalamus, sex steroids regulate GNRH production (Kaiser *et al*. 1997; Karsch *et al*. 1997; Petersen *et al*. 2003). Estradiol modulates GNRH release and indirectly LH and FSH release by the pituitary (Karsch *et al*. 1997). Progesterone also has a regulatory role due to its suppression of GNRH secretion and indirectly LH and FSH release by the pituitary (Bazer *et al*. 1997). The uterus also changes drastically depending on the dominant steroid hormone. High E2 concentrations cause enhanced motility of the uterine myometrium, while high P4 prepares the uterus for pregnancy by inhibiting myometrial contractions and increasing endometrial secretion.

Follicular Phase

The follicular phase of the estrous cycle is the time from regression of the CL (luteolysis) to ovulation of the follicle. The follicular phase can be further divided into two distinct stages: proestrus and estrus. Proestrus, as the name suggests, is the period

immediately preceding estrus. This stage begins at the onset of luteolysis and is marked by a rapid decline in circulating concentrations of P4 and increased E2 production by follicles.

Estrus (Day 0) is the period of sexual receptivity that ends with ovulation of the follicle. During estrus, behavior of the female changes significantly, with increased activity and interest in males. Estrus has a range of 6 to 24 hours in the cow, while the ewe has a slightly longer estrus with a range of 18 to 48 hours. Estrus, however, is highly variable in duration and intensity and is affected by environmental factors such as temperature, season, and types of housing and flooring. (Britt *et al*. 1986; Vailes and Britt, 1990; Dransfield *et al*. 1998; VanRaden *et al*. 2004)

Because there is no CL, P4 levels are low during estrus and thus GNRH secretion is not inhibited. This allows for increased basal secretion of GNRH that stimulates release of LH and FSH from the anterior pituitary. Follicle stimulating hormone causes the development of follicles which produce high amounts of E2. At low concentrations, E2 negatively reduces GNRH secretion, however, when E2 concentrations in the blood reach a threshold a large quantity of GNRH is released from the surge center of the hypothalamus (Petersen *et al*. 2003). The surge of GNRH stimulates the anterior lobe of the pituitary to secrete the preovulatory surge of LH (Petersen *et al*. 2003). The surge of LH activates LH receptors and causes a series of events that act together to cause the weakening of the follicular wall and eventually ovulation. Fibroblasts of the theca interna and the tunica albuginea elongate and dissociate from one another, cells of the theca interna and granulosa layers break apart, and hydrostatic pressure remains unchanged (Espey, 1994). Weakening of the follicular walls and no change in pressure

eventually leads to ovulation. The LH surge also causes a decline in E2 synthesis while P4 synthesis increases (Espey, 1994). This suggests that transformation of the follicular cells to luteal cells (luteinization) begins even before ovulation and that P4 is important for ovulation (Espey, 1994).

Luteal Phase

The luteal phase is the period from ovulation to luteolysis and can also be divided into two stages; metestrus and diestrus. Metestrus begins immediately following ovulation and ends with the formation of a functional CL. During ovulation, blood vessels rupture and a clot is formed in the collapsed follicle. This structure, called the corpus hemorrhagicum, is present for a short time after ovulation, when luteinization occurs. Luteinization is the term used to describe when the cells of the follicle are transformed into luteal cells and begin to produce P4 (Stocco *et al*. 2007). Ovulation causes the basement membrane between the theca and granulosa cells to break down and blood vessels begin to develop a vascular network (Niswender *et al.* 1994). The theca and granulosa cells of the follicle mix and are transformed into small and large luteal cells, respectively (Hoyer and Niswender, 1985). During development of the CL, the small luteal cells increase in number, while large luteal cells increase in size with little change in number (Niswender *et al.* 1994). Concentrations of E2 and P4 are relatively low during this stage resulting in decreased concentrations of LH and FSH.

The longest stage of the estrous cycle is diestrus. This is the period when the CL is fully functional and produces large amounts of P4. Small and large steroidogenic luteal cells gain LH receptors and are stimulated by LH to produce P4 (Stocco *et al*. 2007). Under the influence of high P4 concentrations the uterus becomes quiescent and

highly secretory, this is essential for survival of the embryo (Bazer and First, 1983; Bazer *et al*. 1997). In the uterus, P4 also regulates expression of its own receptor as well as estrogen receptor alpha (ESR1) and oxytocin receptor (OXTR). Production of luteolytic PTGF2A pulses by the luminal epithelium (LE) and superficial glandular epithelium (sGE) are coordinated by P4, E2, and oxytocin (OXT; Spencer, 1995; Spencer and Bazer, 2004). Early in the luteal phase, P4 acts through endometrial P4 receptors (PGRs) to block ESR1 and OXTR expression in the LE and sGE, leaving ESR1 and OXTR undetectable Days 5 to 11 (Spencer and Bazer, 2004). However, after prolonged exposure to P4, the PGR is down-regulated, removing the block on ESR1 and OXTR formation. Estrogen can then up-regulate formation of ESR1 and OXTR in the endometrium. Oxytocin from the pituitary and the CL can then bind OXTR in the endometrium and induce the pulsatile release of PTGF2A from the LE and sGE (Spencer and Bazer, 2004). Prostaglandin F_2 alpha is secreted in a pulsatile manner by the uterine endometrium into the uterine vein and by counter-current exchange is transported into the ovarian artery, where it can have a direct effect on the CL and cause luteolysis (McCracken *et al.* 1971; 1999). If a conceptus is present, it must prevent luteolysis by altering production of pulses of PTGF2A, directing PTGF2A away from the CL, or producing luteotrophic substance(s). Hormones involved in signaling differ between species, but in sheep and cattle this is accomplished through the production and secretion of IFNT by the conceptus (Bazer, 1991).

Maternal Recognition of Pregnancy

Pregnancy is maintained in response to interactions between the conceptus and the endometrium, which interrupts luteolysis. The signals for maternal recognition of

pregnancy differ among species. In humans, chorionic gonadotropin is secreted by the trophoblast and acts directly on the CL via the blood to promote continued P4 production (Roberts *et al*. 1997). Pig conceptuses produce E2 which causes PTGF2A, secreted from the endometrium, to be redirected away from the uterine vasculature and into the lumen of the uterus (Bazer and Thatcher, 1977; Spencer and Bazer, 2004). Therefore, less PTGF2A reaches the circulatory system, and thus can not cause regression of the CL.

In ruminants, the oocyte is fertilized in the oviduct, moves to the uterus as a morula, develops into a blastocyst, hatches from the zona pellucida, then becomes filamentous and begins to rapidly elongate (Spencer *et al.* 2007; 2008). Secretion of IFNT begins immediately after hatching, increases as the blastocyst elongates, reaches maximal levels when attachment to the uterus is initiated and decreases as implantation proceeds (Imakawa, *et al*. 2004). Interferon tau secretion signals the presence of a conceptus to the maternal system and prolongs the life of the CL (Bazer, 1991). Interferon tau is a type I IFN and binds the type I IFN receptor (composed of IFNAR1 and IFNAR2 subunits) on the endometrial cells of the uterus. In a paracrine fashion, IFNT acts on the endometrial LE and superficial GE to suppress transcription of ESR1 and indirectly inhibiting OXTR formation (Spencer and Bazer, 2007). Without the presence of OXTR, OXT is unable to cause the pulsatile release of PTGF2A that is necessary for luteolysis. The CL is saved from destruction and continues to produce P4 throughout pregnancy.

In addition to altering PTGF2A secretion, IFNT also up regulates expression of many other genes in the uterus (Vallet *et al*. 1991; Charleston & Stewart, 1993; Spencer & Bazer, 1993; Ott *et al*. 1998; Johnson *et al*. 2000; Choi *et al*. 2001 & 2003; Chen *et al*.

2007; Gifford *et al*. 2008), peripheral blood (Yankey *et al*. 2001; Han *et al*. 2006; Gifford *et al*. 2007 & 2008), and the CL (Chen *et al*. 2006; Gifford *et al*. 2008). Recent work has provided evidence to support the hypothesis that expression of IFN-stimulated genes (ISG) in extrauterine tissue during early pregnancy may be due to endocrine release of IFNT into the uterine vein (Oliveira *et al*. 2008). If this is the case, then up-regulation of ISG in the systemic immune system in response to pregnancy may provide a method for the semiallogeneic conceptus to evade rejection by the maternal immune system, while keeping the dam at low risk for disease (Gifford *et al*. 2007). Additionally, increased expression of specific ISGs in the peripheral blood may provide an opportunity for the development of an early blood diagnostic for cows that fail to conceive following insemination (Yankey *et al*. 2001; Han *et al*. 2006; Gifford *et al*. 2007). Numerous tests are available for pregnancy detection. Transrectal ultrasonography can be performed accurately at approximately Day 27; however accuracy at this stage depends a great deal on training and experience of the technician (Hansen and Galligan, 2007). In addition, cost of the ultrasound equipment is too high for many dairy producers. Rectal palpation is relatively inexpensive and can be used to determine pregnancy as early as 28-35 days (Hansen and Galligan, 2007). However, palpation this early is not recommended because mechanical manipulation of the uterine tract can cause the pregnancy to be lost (Hansen and Galligan, 2007). Both ultrasound and rectal palpation require trained technicians, but there are other choices for dairy producers that do not have these skills. The BioPRYN ™ assay detects pregnancy specific protein B (PSPB), a glycoprotein produced by binucleate cells of the trophoblast as early as Day 21 and continues throughout pregnancy (also, known as pregnancy associate glycoproteins or PAGs; Hansen and Galligan, 2007).

To assay for PSPB, a blood sample is collected at least 100 days after a previous calving and 30 days after insemination and is shipped to a laboratory for analysis. This assay is sensitive, but shipping samples is inconvenient and costly. On farm milk P4 assays are also available, however, accuracy of pregnancy diagnosis is only ~80% (Shearer, 2003).

An earlier more accurate assay that could detect open cows at Days 18 to 20, the period when ISGs have been shown to be increased in the blood (Yankey *et al.* 2001; Han *et al*. 2006; Gifford *et al*. 2007 & 2008), would allow for rapid rebreeding. Previous work has provided the basis for development of such a diagnostic (Yankey *et al*. 2001; Gifford *et al*. 2007). If a test was available for Days 18 to 20 cows, the use of resynchronization programs after an open diagnosis would allow for reinsemination to occur in approximately 23 days, which is 10 to 40 days earlier than would occur with current methods (Han *et al*. 2006; Gifford *et al*. 2007). This could potentially reduce costly days open and improve the efficiency of reproduction in dairy cows (Gifford *et al*. 2007).

Interferons

Classification of Interferons

Interferons are members of the cytokine superfamily that have vital roles in the immune system (Roberts *et al*. 1997). Issacs and Lindenmann (1962) were the first to identify IFN which was so named because it interfered with virus replication. Since then, much has been learned about the role that IFNs play in the immune system as well as cell growth and differentiation. Interferons can be classified into three groups, Type I, II, and III according to their amino acid sequence (Randall and Goodbourn, 2008). Type I IFNs include IFNT, as well as alpha, beta, delta, epsilon, kappa, omega, nu, and zeta (Petska,

2007). The only Type II IFN is IFN gamma. Type III IFNs, previously known as IFN-like cytokines, include IFN lamda-1, -2, and -3 also referred to as interleukin-28A (IL28A), IL28B, and IL29, respectively (Randall and Goodbourn, 2008). All three groups stimulate antiviral responses in cells, although the roles of Type I IFNs omega, epsilon, delta, and kappa are not well defined (Randall and Goodborn, 2008). Interferons alpha and beta are induced directly in response to viral infection and induce an antiviral response in target cells (Randall and Goodbourn, 2008). The sole type II IFN, IFN gamma, is secreted by activated T cells and natural killer cells (Randall and Goodbourn, 2008). Finally, type III IFNs are induced in direct response to viral infection and elicit a comparable antiviral response to IFNs alpha and beta (Randall and Goodbourn, 2008). Each type of IFN signals through specific receptors. All Type I IFNs share the type I receptor consisting of subunits IFNAR1 and IFNAR2 (Petska, 2007). IFNAR2 is the major ligand binding domain (Petska, 2007). However, each Type I IFN interacts with the receptor differently explaining differential activities of each IFN (Petska, 2007).

In ruminants, the trophectoderm cells that later become the placenta produce IFNT during the peri-implantation period of early pregnancy (Imakawa, *et al*. 2004). Interferon tau was discovered through efforts to identify the key factors released by the conceptus that acts as the signal for maternal recognition of pregnancy (Roberts *et al*. 2004). Interferon tau is a unique IFN produced only by the trophectoderm of ruminants that not only signals for pregnancy recognition in the mother, but also has antiviral properties (Roberts *et al*. 2004).

Interferon Signaling Pathway

Prior to IFN signaling the cytoplasmic domain of IFNAR1 is associated with tyrosine kinase 2 (TYK2) and IFNAR2 is associated with Janus kinase (JAK1) and signal transducer and activator of transcription 2 (STAT2; Randall and Goodbourn, 2008). STAT2 is also weakly associated with STAT1 (Randall and Goodbourn, 2008). Once IFNT binds, IFNAR1 and IFNAR2 dimerize and their conformation changes so that TYK2 is able to phosphorylate tyrosine 466 on IFNAR1, creating a docking site for STAT2 (Randall and Goodbourn, 2008). STAT2 is then phosphorylated by TYK2 on tyrosine 690, and JAK1 phosphorylates STAT1 on tyrosine 701 (Randall and Goodbourn, 2008). Phosphorylated STAT1 can homodimerize or heterodimerize to STAT2. Dimerization with STAT2 creates a nuclear localization signal and inactivates the dominant constitutive nuclear export of STAT2 (Randall and Goodbourn, 2008). Interferon regulatory factor 9 (IRF9) associates with the heterodimer forming the IFN-stimulated transcription factor 3 gamma (ISGF3) complex (Spencer *et al.* 2007). ISGF3 transactivates genes with IFN-stimulated response elements (ISRE) on their promoters, including STAT1, STAT2, IFN-stimulated gene 15 (ISG15), beta2-microglobulin (B2MG), 2'5' oligoadenlyate synthase (OAS) and the MX genes (Assiri *et al*. 2006; Spencer *et al*. 2007).

Another transcription factor complex, gamma-activated factor (GAF) is formed by homodimerization of STAT1 (Randall and Goodbourn, 2008). GAF translocates to the nucleus and activates transcription of genes containing a gamma-activation sequence (GAS) elements, such as IFN regulatory factor 1 (IRF1; Spencer *et al*. 2007). IRF1 is a transcription factor which can also bind and transactivate genes with an ISRE as well as an IRF-response element (IRFE) (Spencer *et al*. 2007; Randall and Goodburn, 2008).

The signaling pathway described above occurs in the stroma (S) and the deep GE of the endometrium, however IFNT signaling occurs differently in the LE and sGE (Spencer *et al.* 2007; 2008)

Interferon regulatory factor-2 (IRF2), a transcriptional repressor, is present in the LE and sGE and prevents transcription of some ISGs by binding to ISREs and IRFEs (Choi *et al*. 2001; Spencer *et al*. 2007). In these cell types, IFNT activates an unknown cell signaling pathway that results in induction of some ISGs, including cathespin L, cystatin C, galectin 15, and MX1 (Johnson *et al*. 2002; Song *et al*. 2006 & 2005; Gray *et al*. 2006).

Interferon Stimulated Genes

Many ISGs are known to be increased by IFNT during pregnancy recognition. Several of these genes have known function in the immune system, but it is not known what role they may play in reproduction. However, it has been postulated by many that these genes are involved in preparing the maternal system for pregnancy (Charleston and Stewart, 1993; Hansen *et al*. 1999; Johnson *et al*. 2002; Hicks *et al*. 2003; Roberts *et al*. 2008; Gifford *et al*. 2008). A few of the experiments examining ISGs in the uterus, CL and peripheral blood leukocytes (PBLs) are reviewed below.

As previously described, IFNT signaling is carried out by the ISGF3 complex consisting of STAT1, STAT2, and IRF9 and the GAF complex, which is comprised of a STAT1 homodimer that transactivates production of IRF1. However, in some cell types IFN signaling is repressed by IRF2. Choi *et al*. (2001) examined the expression of STAT1, STAT2, IRF1, IRF2, and IRF9 in the uterine endometrium throughout the estrous cycle and in response to pregnancy and intrauterine administration of IFNT.

Their results showed that STAT1, STAT2, IRF1, and IRF9 were detectable in cyclic ewes at low levels in all cell types. During early pregnancy expression of STAT1, STAT2, IRF1 and IRF9 increased by Day 15 throughout the stroma (S) and GE of the endometrium. Interestingly, IRF2 expression was detected in the LE and superficial to middle GE but was undetectable in the S throughout the estrous cycle. During the estrous cycle there was no change in mRNA expression of IRF2 in the LE and superficial GE, however, concentrations of IRF2 did increase in the LE and superficial GE on Day 15 of pregnancy. Choi *et al.*, further showed that the increase of STAT1, STAT2, IRF1 and IRF9 during early pregnancy was attributed to IFNT because results from intrauterine infusion of recombinant ovine IFNT were analogous to those seen during early pregnancy. However, IRF2 expression did not increase in response to IFNT infusion alone suggesting that IFNT alone does not regulate IRF2. These results support the hypothesis that IRF2 represses transcriptional activity of the ISGF3, by blocking of ISREs, as described above in IFN signaling.

Expression of major histocompatiblilty complex (MHC) class I molecules are also increased by IFNT. The MHC class I molecules are present on the cell membrane of most somatic cells and play an important role in differentiating between self and nonself by presenting foreign antigenic peptides to cytotoxic T lymphocytes (Choi *et al*. 2003). The MHC class I molecule is made up of a heavy alpha chain associated with a light beta chain, termed beta2-microglobulin (B2MG; Choi *et al.* 2003). Expression of both MHC class I molecules increased in the endometrial S and GE on Days 14 through 20 of pregnancy, but were absent in the LE and sGE (Choi *et al*. 2003). It was further shown that neither molecule was detected on the trophectoderm on Day 20 (Choi *et al*. 2003).

The lack of these molecules on the trophectoderm, LE and sGE, during early pregnancy may be critical for the prevention of immune rejection of the conceptus (Choi *et al*. 2003).

Recently, microarray data from our lab showed a novel ISG, receptor transporter protein – 4 (RTP4), was up regulated in PBLs during early pregnancy in cows. Gifford *et al.,* (2008) further showed that RTP4 was expressed in the CL, PBLs and the GE and S of the endometrium and that RTP4 expression was increased during the time of maternal recognition of pregnancy. The role of RTP4 in reproduction is not known, however, it belongs to a family of receptor transporters that transport certain G protein-coupled receptors from the trans-Golgi network to the cell surface (Gifford *et al.* 2008).

Interferon stimulated gene 15 (ISG15; previously known as ubiquitin cross reactive protein, UCRP) is a ubiquitin homolog that is induced by IFNT and early pregnancy in ruminants (Hansen *et al*. 1999; Johnson *et al*. 2002; Han *et al.* 2006; Spencer *et al*. 2008). It was detected in the uterine flushings of Day 18 pregnant cows and was also up regulated in the PBLs during early pregnancy (Austin *et al*. 1996; Hansen *et al*. 1999; Han *et al*. 2006; Gifford *et al.* 2008). ISG15 becomes conjugated to cellular proteins and is thought to modify their function (Hansen *et al.* 1999). In addition, ISG15 is a secreted cytokine (Hansen *et al.* 1999); however its extracellular functions are not well defined.

Two myxovirus resistance or MX proteins, MX1 and MX2, have been identified in sheep and cattle. Both are up regulated in response to IFNs and pregnancy in the endometrium (Ott *et al*. 1998; Hicks *et al.* 2003; Assiri, 2006) and in the PBLs (Yankey *et al*. 2001; Stevenson *et al.* 2007; Gifford *et al*. 2007). Characterization of the structure,

regulation and function of MX proteins is a central focus of research in our laboratory and this thesis will focus on the MX1 protein.

MX Proteins

Overview of known MX Proteins

The MX allele was initially described due to its ability to confer resistance to infection by a member of the orthomyxoviridae virus family in mice (Issacs and Lindenman, 1962). MX1 proteins were shown to confer specific resistance to the influenza A virus (Chang *et al*. 1990; Palm *et al*. 2007). MX proteins have been identified in every species examined including; mice, rats, fish, birds, humans, a variety of other mammals, and even yeast (Horrisberger and Gunst, 1991; Leong *et al.* 1998; Lee and Vidal, 2002; MacMicking, 2004). In mice, two proteins exist, MX1 and MX2. Mouse MX1 is located in the nucleus and confers resistance to orthomyxoviruses that replicate in the nucleus, whereas MX2 is found in the cytoplasm and blocks replication of bunyaviruses (MacMicking, 2004). Humans also have two MX proteins, MXA and MXB. However, both of the human MX proteins are located in the cytosol and only MXA has been shown to possess antiviral activity (MacMicking, 2004), while MXB is hypothesized to play a role in normal cellular function (King *et al.* 2004). MXA inhibits replication of viruses including the orthomyxomaviruses, paramyxomaviruses, rhabdoviruses, togaviruses, picornaviruses, bunyaviruses, and hepatitis B (MacMicking, 2004). Two MX proteins, MX1 and MX2, have also been identified in sheep and cattle (Horisberger *et al.* 1988: Charleston & Stewart, 1993; Gerardin *et al*. 2004; Assiri, 2006), although their precise roles in antiviral defense have not been determined.

MX Protein Structure and Function

MX proteins belong to a superfamily of IFN inducible dynamin-like GTPases, including the p47 family, the p65 guanylate binding protein family, and very large inducible GTPases (Flohr *et al*. 1999; MacMicking, 2004; King *et al*. 2004). All MX proteins are large, with a molecular weight between 70 and 100 kDa, have a relatively low affinity for GTP, and a high intrinsic rate of GTP hydrolysis (Flohr *et al*. 1999). All MX proteins also share three conserved domains; an N-terminal tripartite GTP-binding motif, a central-interacting domain (CID), and a C-terminal leucine zipper (LZ) motif (Flohr *et al*. 1999; MacMicking, 2004).

Pitossi *et al*, (1993) introduced mutations into the tripartite GTP binding motif elements of human MXA and mouse MX1 and examined the mutant proteins for antiviral activity, GTP-binding capacity, and GTPase activity. They found that a mutated GTP binding motif abolished antiviral activity. Later, it was also shown that the CID was necessary for GTPase activation and interaction with viral targets by human MXA (Flohr *et al*. 1999). An antibody that bound to the CID region of MXA, inhibited the association of MXA with nucleocaspids and neutralized the antiviral activity of MXA against influenza A virus and vesicular stomatitis virus (Flohr *et al.* 1999). These results suggested that the epitope bound by the antibody in the CID was critical to MXA function.

Antiviral Properties of MX Proteins

The first MX protein was discovered in 1962 by Lindenmann in an inbred strain of mice called the A2G strain, which had a high degree of resistant to the influenza A virus (Horisberger *et al*. 1983; Palm *et al*. 2007). The influenza A virus is lethal to all other inbred strains of mice (Chang *et al*. 1990). Since their discovery much research has

been done on the roles of MX proteins in combating viral infection. Mice have evolved two MX proteins that localize differently in the cell and control the viruses that replicate in the separate locations (Haller and Kochs, 2002). Nuclear MX1 confers resistance to the orthomyxoviruses, which replicate in the cell nucleus (Haller and Kochs, 2002). Cytoplasmic MX2 inhibits replication in the cytoplasm of viruses such as the bunyavirus (Haller and Kochs, 2002). Humans and most other vertebrates express only cytoplasmic forms of MX proteins (Haller and Kochs, 2002). In humans only MXA is known to possess antiviral activity, to date, MXB has not been shown to have these properties (Haller and Kochs, 2002). MXA inhibits replication of a wide spectrum of viruses, irrespective of where viral genome replication and virus assembly occurs (Haller and Kochs, 2002). MXA interacts with viral nucleocapsids and prevents transport of viruses to the location of transcription and replication and redistributes viral components away from replication sites (Haller and Kochs, 2002).

Role of MX Proteins in Cellular Function

Although most MX proteins have antiviral properties, not all MX proteins confer resistance to virus, including human MXB and rat MX3 (Haller and Kochs, 2002). We and others have hypothesized that MX proteins, in addition to having antiviral properties, may have roles in cellular function in the absence of viral infection. MX proteins are members of the dynamin family of large GTPases that are involved in vesicle trafficking, endocytosis, and have been implicated in cell migration and invasion (Accola *et al*. 2002; Krutchen and McNiven, 2006). Dynamins oligomerize into high order structures (Haller and Kochs, 2002) and in 2002 Accola *et al.* demonstrated that human MXA also had the ability to self assemble into rings and rod-like structures in a GTP-dependent manner.

Conventional dynamins contain a pleckstrin homology (PH) domain that mediates binding to lipids, however, MXA lacks a PH domain (Accola *et al*. 2002). Surprisingly, without a PH domain, MXA was shown to associate with lipid droplets and tubulate membranes *in vitro* (Accola et al. 2002). Human MXA was also shown to interact with dynamin (Accola *et al*. 2002; Jatiani and Mittal, 2004). Over expression of MXA in cells disturbs trafficking along the endocytic pathway and may accomplish this by interacting with dynamin (Jatiani and Mittal, 2004). Also, it was demonstrated that human MXB is involved in nuclear import and cell cycle progression (King *et al*. 2004).

Recently, work from our lab identified MX1 in uterine flushings of sheep and expression of MX1 was increased in uterine flushings from Day 17 pregnant sheep as compared to Day 15 cyclic and pregnant ewes (Toyokawa *et al*. 2007a). It was further shown that an ovine uterine cell line, oGE, secreted MX1 and that treatment with an inhibitor of the classical secretory pathway did not inhibit this secretion (Toyokawa *et al*. 2007a). These results suggested that MX1 was secreted via unconventional secretory pathways (Toyokawa *et al*. 2007a). A follow up study used small interfering RNA to knock down MX1 expression and examined the role of MX1 in regulating secretion. MX1 knockdown in oGE cells caused a decrease in the amount of ISG15 secretion, although, there was no reduction in the secretion of B2MG (Toyokawa *et al*. 2007b). Because ISG15 is secreted via unconventional secretory pathways and B2MG is secreted conventionally, it was concluded that MX1 may have a regulatory role in the pathway of unconventional secretions (Toyokawa *et al*. 2007b). These intriguing results support the hypothesis that MX proteins are important for cellular functions in the absence of viral infection.

MX1 in Reproduction

MX1 was first identified in the uterus of pregnant sheep by Charleston and Stewart (1993). Five years later Ott *et al.* (1998) described MX1 expression in the uterus throughout the estrous cycle and during early pregnancy. That work showed that MX1 expression in the endometrial epithelium was regulated during the estrous cycle in the absence of a conceptus or IFNT. This was interesting because previously, MX expression was thought to be exclusively regulated by virus or IFN. In that study, MX1 expression was greatest in the LE and shallow glands coincident with the time these tissues express PR and are exposed to high concentrations of P4 (Ott *et al*. 1998). These results were interpreted to suggest that MX1 expression was regulated by P4 in the endometrium (Ott *et al.* 1998).

During early pregnancy, MX1 expression increased first in the LE, followed by the GE, S and myometrium (Ott *et al*. 1998). Later, work showed that MX1 mRNA expression increased in peripheral blood leukocytes of early pregnant sheep (Days 15 – 30: Yankey *et al*. 2001). This was the first evidence that pregnancy increased expression of ISGs outside of the uterus in response to pregnancy. Previously, the effects of IFNT were thought to be confined to the uterus (Bazer *et al*. 1997).

Uterine MX1 is not unique to ruminants; it was also shown to be expressed in mice (Chang et al. 1990), and in gilts and mares (Hicks *et al*. 2003) during the estrous cycle. MX1 expression also increased in gilts (Hicks *et al*. 2003) during early pregnancy. These results suggest that uterine MX1 expression during early pregnancy may be a general phenomenon (Hicks *et al.* 2003). As described previously, MX1 is secreted by endometrial cells via unconventional pathways during early pregnancy, a time when

uterine secretions are vital to conceptus growth and development (Toyokawa *et al*. 2007a). Also, MX1 was implicated in modulation of the secretion of other proteins known to be secreted through unconventional pathways (Toyokawa *et al*. 2007b)

Several unconventional secretory pathways exist that are mechanistically distinct from the well-characterized conventional secretory pathways. One of these is secretion of exosomes. Our unpublished data supports the hypothesis that MX1 is a component of secreted exosomes and may regulate some aspect of exosome secretion (K.E. Racicot). A definitive role for MX1 in establishment and maintenance of pregnancy has not been determined, however, these exciting results lead us to hypothesize that MX1 is a regulator of exosome secretion by the endometrium during early pregnancy. Secretions by endometrial glands are crucial for the survival of the embryo (Gray *et al*. 2002).

MX1 Isoforms

In 1998, Ellinwood *et al.,* described two alleles of MX1 in cows (MX1 and MX1-a). The authors found MX1-a cDNA lacked 18 nucleotides and accounted for a predicted protein sequence of 648 amino acid residues compared to 654 in MX1 (Ellinwood *et al*. 1998). One of the MX1-a clones also lacked 13 nucleotides in the 3' untranslated region (UTR; Ellinwood *et al.* 1998). The authors concluded that the variants were alleles because only 2 of 8 animals tested lacked the 13 nucleotides, even though the 18 base pair difference occurred at putative exon boundaries (Mouse Exon 2 and 3; Ellinwood *et al.* 1998). It was not clear if the author also tested the coding region difference in these animals. The location of the 18 nucleotide differences (area between Exon 2 and 3) was further verified in 2004 when the bovine MX1 gene was analyzed (GenBank Accession No. AH012681; Gerardin *et al.* 2004). Because MX1-a was not identified in sheep those

authors suggested that MX1 was ancestral to MX1-a (Ellinwood *et al*. 1998). Alternatively, it was postulated that the lack of MX1-a in sheep may be the result of mutations around the splice donor site (Ellinwood *et al.* 1998). Later, Kojima *et al*., (2003) identified two other variants of MX1 in cattle. However, these authors concluded that all four of the variants (MX1, MX1-a, MX1A$_{-18}$B, and MX1B) were likely splice variants because of nearly identical sequences flanking differing regions and their presence in each breed examined (Kojima *et al*. 2003). Kojima *et al.* (2003) further showed that each splice variant contained consensus GTP binding and leucine zipper motifs, however, it was not determined if the variants were translated into functional proteins, or if each was regulated similarly by early pregnancy and IFNT. Kojima *et al.* (2003) also sequenced the first three introns of the bovine MX1 genome (GenBank Accession No. AB060171); however the unique sequences found in bMX1A-18B and bMX1B were not reported in the genomic sequence published by Gerardin *et al*. (2004; GenBank Accession No. AH012681).

Alleles and Splice Variants

Two types of gene isoforms exist, alleles and splice variants. In diploid (2N) organisms (e.g. those with two sets of chromosomes), one copy of each chromosome is passed on from each parent (Lewin, 2004). One of the two copies is the paternal allele and the other is the maternal allele. Alternative mRNA splicing produces splice variants, another type of isoform in which some portions of the mature mRNA are common but others are different. Walter Gilbert was the first to propose that different combinations of exons could be alternatively spliced together to produce unique mRNA isoforms from a single gene (Modrek and Lee, 2002). Alternative splicing allows for the relatively small

coding (exon) portion of the genome to produce a relatively large proteome (Lewin, 2004). Most genes are made up of exons interrupted by non coding introns in initial DNA and pre-mRNA transcripts (Matlin *et al*. 2005). After transcription to mRNA, these introns are still present in the sequence, but through RNA splicing by the spliceosome, the introns are removed at consensus sequences form a chain of uninterrupted exons (Matlin *et al*. 2005). Removed introns are degraded quickly and the mature mRNA is ready for transport out of the nucleus so that translation can occur (Lewin, 2004). Alternative splicing of the exons and introns will therefore lead to distinct proteins with the possibility of unique functions. Alternatively, it may act as an on-off switch by the introduction of a premature stop codon (Smith and Valcarcel, 2000). Recent bioinformatics analyses have reported that the rate of alternative splicing in the human genome is higher (92-94%; Wang *et al*. 2008) than had previously been estimated: ~5% (Sharp, 1994), 35-59% (Modrek and Lee, 2002), and ~80% (Matlin *et al.* 2005). These results indicate that alternative splicing is ubiquitous and functionally more important than previously thought.

Alternative splicing is tightly regulated and often differs depending on cell-type or developmental stage of the cell. A 'strong' splice site is not enough to define an exon (Matlin *et al*. 2005). Exon and intron splicing enhancers (ESEs and ISEs, respectively), and silencers (ESSs and ISSs, respectively) have a role in activating and repressing splice sites and other regulators also exist (Matlin *et al.* 2005).

Summary

Early embryonic mortality is a major contributor to poor reproductive efficiency in cattle. Maternal recognition of pregnancy is a crucial event that occurs coincident with

the time of highest embryonic loss. It is generally accepted that miscommunication between the conceptus and the uterus is an important cause of embryonic loss. Understanding the signaling that occurs during early pregnancy may provide a way to decrease embryonic loss. In sheep and cattle, the signal for maternal recognition of pregnancy is IFNT, which acts by altering the pulsatile secretion of PTGF2A by the uterus that causes luteolysis. Signaling by IFNT also stimulates the production of a large number of ISGs, and it has been hypothesized that ISGs in the uterus, peripheral blood and CL may play a critical role in the establishment of pregnancy and development of the conceptus. One ISG, MX1, functions in the immune system as an antiviral protein. The expression of MX1 is regulated in the uterus during the estrous cycle of sheep (Ott *et al*. 1998) and is up regulated by IFNT during early pregnancy in sheep and cattle (Ott *et al*. 1998; Yankey *et al*. 2001; Hicks *et al*. 2003; Gifford *et al.* 2007). More recently, we presented evidence that MX1 is secreted by the endometrial epithelium (Toyokawa *et al*. 2007a) and that it may also regulate secretion of other unconventional secreted proteins (Toyokawa *et al*. 2007b). Recent description of MX1 splice variants in cattle (Ellinwood *et al.* 1998; Kojima *et al*. 2003) indicates there may be more complexity to MX1 regulation and function. However, it is not clear if the variants detected in cattle are translated into protein. Furthermore there are no data regarding the regulation of splice variant formation by IFNT during early pregnancy of sheep or cattle.

Objectives

The overall objectives of the studies described in this thesis are to characterize MX1 isoforms in immortalized ovine uterine cell lines, endometrial tissue of sheep, and

PBLs of sheep and cattle. We hypothesize that isoforms of MX1 are present in sheep and that the isoforms will be regulated similarly by IFNT and during early pregnancy. This work will increase our knowledge of MX1 involvement in pregnancy, as well as our understanding of the biochemical signaling between the conceptus and the dam that may lead to development of strategies to reduce early embryonic losses in ruminants.

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Chapter 2

Expression and regulation of MX1 splice variants in ovine uterine epithelial cell lines.

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Introduction

For successful establishment of pregnancy the conceptus (embryo and associated tissue) must signal its presence to the maternal system in time to prevent luteal regression. In ruminants, the trophoblast cells produce a unique interferon, interferon tau (IFNT; Bazer *et al*. 1991). Interferon tau is a member of the type I IFN family which also includes IFNs alpha, beta, delta, epsilon, kappa, omega, nu, and zeta (Petska, 2007). Ruminants produce IFNT during early pregnancy from days 10 to 21 (Spencer and Bazer, 2004). Interferon tau binds type I IFN receptors on the endometrial epithelium and blocks the luteolytic pulses of prostaglandin F_2 alpha (PTGF2A), thereby saving the corpus luteum (CL) from destruction (Bazer *et al.* 1997). The CL is then able to maintain production of progesterone (P4), which is necessary for pregnancy. In addition to altering production of PTGF2A, IFNT also up regulates a large number of genes in the uterus, CL and peripheral blood immune cells including 2'5'oligoadenylate synthetase (OAS; Johnson *et al.* 2001), beta2-microglobulin (B2MG; Vallet *et al.* 1991; Choi *et al*. 2003), IFN-stimulated gene 15 (ISG15; Johnson *et al*. 2000; Gifford *et al.* 2007), and the myxovirus resistance genes 1 and 2 (MX1 & MX2; Ott *et al.* 1998; Yankey *et al*. 2001; Hicks *et al.* 2003; Assiri, 2006; Stevenson *et al*. 2006; Gifford *et al.* 2007). Many of these IFN-stimulated genes (ISGs) have important roles in the immune system, but their roles in pregnancy are unknown. We and others have hypothesized that ISGs are important for establishing and maintaining pregnancy.

MX proteins are members of the dynamin superfamily of large GTPases (Haller and Kochs, 2002). Much is known about the functions of MX proteins in response to viral infection. However, there are some MX proteins, including human MXB and rat

MX3, that do not have antiviral activity and it has been proposed that MX proteins have function in uninfected cells (Toyokawa *et al.* 2007). Human MXB has been implicated in cell cycle progression (King *et al.* 2004) and human MXA has also been shown to have functions outside the immune system (Accola *et al.* 2002; Jatiani and Mittal 2004). Recently ovine MX1 (oMX1) was identified in uterine flushings of cyclic and pregnant ewes and oMX1 increased in flushings from Days 15 to 17 pregnant ewes (Toyokawa *et al.* 2007a). That report showed that oMX1 was secreted via an unconventional secretory pathway (Toyokawa *et al*. 2007a) and that by reducing oMX1 using small interfering RNA (siRNA), secretion of another unconventionally secreted protein, ISG15, was inhibited (Toyokawa et al. 2007b). These results suggest that MX proteins may have more varied functions than previously conceived. Different functions can arise from gene duplication and mutation generating proteins with distinct functions, but sharing many structural motifs. An example of this is MX1 and MX2 (MXA and MXB in humans). Variation in protein function can also occur from alternate splicing of mRNA transcripts to yield different protein structures from the same gene (Matlin *et al*. 2005).

In 1998, Ellinwood *et al.*, described two alleles of MX1 in cows (MX1 and MX1-a), and in 2003 Kojima *et al.*, reported the identification of two new variants of MX1 (MX1B and MX1A$_{-18}$B) in several breeds of cattle. Genomic analysis revealed that the MX1 variants were likely splice variants because of nearly identical sequences flanking differing regions and their presence in each breed examined (Kojima *et al.* 2003). However, our work analyzing two published bovine MX1 genomic structures (GenBank No. AH012681 and No. AB060171; Kojima *et al*. 2003; Gerardin *et al*. 2004) showed one sequence did not contain the unique sequences found in the MX1B and MX1A$_{-18}$B

(D.S. Clark and T.L. Ott, unpublished observation). It was further shown that each splice variant contained the necessary GTP binding and leucine zipper motifs characteristic of members of this superfamily of proteins (Kojima *et al.* 2003). However, it was not determined if the variants were translated into functional proteins, or if each was regulated similarly by early pregnancy and IFNT. Therefore, objectives of this study were: 1.) To identify MX1 splice variants in immortalized ovine uterine cell lines, 2.) to examine the regulation of each of the MX1 splice variants by IFNT in three immortalized ovine uterine cell lines, and 3.) to determine if each splice variant was translated into a full length MX1 protein.

Materials and Methods

Cloning

Cloning of MX1 splice variants was performed using RNA isolated from immortalized ovine endometrial cell lines (Johnson et al., 1999). A full length MX1 cDNA (GenBank Accession No. X66093) was amplified using gene specific primers (forward, CTGGGGACGGGTGGTGTTGG; and reverse CGGCTGCAGGCTGACCACTG) from mRNA isolated from immortalized ovine uterine cells. The PCR products were gel purified using GenElute Spin Columns (Sigma-Aldrich, St. Louis, MO) and subsequently cloned into PCR II-TOPO TA vector (Invitrogen, Carlsbad, CA). Plasmid sequencing was conducted using the Big Dye system (Applied Biosystems, Foster City, CA) at the Molecular Biology Core Laboratory (UI/WSU Center for Reproductive Biology). Sequence analysis was accomplished using the NCBI BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi), Vector NTI

(Invitrogen), ExPASy Proteomics Server (http://www.expasy.ch/), and the Biology Workbench from San Diego Super Computer (http://workbench.sdsc.edu/).

Cell Culture

For mRNA expression analysis, immortalized ovine uterine glandular epithelial (oGE), luminal epithelia (oLE), and stromal (oSC) cell lines (Johnson *et al.* 1999) were cultured in 6-well plates (Coster 3516; Corning Inc., Corning, NY) in Dulbecco's Modified Eagle's Medium (15.63 g/L DMEM; Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY) and 5% antibiotic antimycotic (ABAM; Gibco) under 5% CO_2 at 38.5ºC. Cells were washed with Hanks Balanced Salt Solution (HBSS; Sigma-Aldrich) before incubation with 10,000 U/mL of IFNT (provided by Fuller W. Bazer, Texas A&M University) in medium, or in medium alone for 0, 1.5, 3, 6, 12, and 24 hours. At harvest, cells were approximately 95% confluent. Each experiment was replicated three independent times with three wells per treatment per time in each experiment.

For protein expression analysis, oGE cells were incubated in medium containing DMEM with 10% FBS and 5% ABAM , or medium with 10,000 U/mL IFNT for 24 hours and were washed with HBSS (Sigma-Aldrich) before being lysed with Mammalian Protein Extraction Reagent (mPER, Thermo Scientific, Rockford, IL).

To determine if effects of IFNT were to activate transcription of the MX1 gene, actinomycin D was used to inhibit transcription. Briefly, oGE cells (70% confluent) were incubated in medium containing DMEM with 10% FBS and 5% ABAM or medium with 1 µg/mL of Actinomycin D (Sigma-Aldrich) dissolved in dimethyl sulfoxide (DMSO), or medium contain DMSO only. One hour later, 100 µL of medium or medium containing

10,000 U/mL of IFNT was added to wells and cells were incubated for 6 hours before harvesting (n=3 wells per treatment, experiment=1).

RNA Extraction and cDNA Synthesis

Ribonucleic acid was extracted and purified using TRIzol (Invitrogen) according to the manufacturer's recommendations. Quantity and quality of RNA was assessed by measuring absorbance at 260 and 280 nm and by agarose-formaldehyde gel electrophoresis. For cDNA synthesis, 1 μg of total RNA was incubated in 0.5 μL of RQ1 DNase (Promega, Madison, WI), 0.5 μL of Superscript III RT Buffer (Invitrogen), and brought to 7.5 μL total volume in nuclease-free water, then incubated at 37ºC for 30 min. One microliter of DNase stop solution (Promega) was added and samples were incubated at 65ºC for 10 min followed by freezing at -80ºC overnight. One microliter of 50 ng/mL random hexamers and 1 μL of 10 mM dNTP mix (Invitrogen) were added before incubating at 65ºC for 5 min. Master mix containing 1.5 μL Superscript III buffer, 4 μL of 25 mM MgCl2, 2 μL 0.1 M DTT, 1 μL of RNase OUT, and 1 μL of Superscript III RT (Invitrogen) for a total volume of 9.5 μL was added after at least one minute on ice. Samples were then incubated at 25ºC for 10 min, 50ºC for 50 min, and finally 85ºC for 5 min. Samples were stored at -20ºC.

Quantitative PCR

A TaqMan® assay was designed to distinguish between each of the three MX1 splice variants. A duplex assay was developed for Beta-actin (ACTB) and MX1a. Sample variation is decreased in duplex assays because there is no pipetting error between the two estimates of mRNA abundance. Duplex assays also save time and reagents. MX1b and MX1c were not measured together in a duplex assay, because

MX1b primers could anneal to and amplify MX1c. After validation (see below), reactions were completed using the following conditions: A working solution of cDNA was prepared by diluting cDNA samples 1:20 with nuclease-free water. Four microliters of this cDNA working solution was added to 16 µL of Master Mix containing 10 µL gene expression master mix (Applied Biosystems), 2 µL forward primer, 2 µL reverse primer, and 2 µL probe (single-plex assays; MX1b and MX1c). For duplex assays, 1 µL of each forward and reverse primer for each target and probes were added to 10 µL gene expression master mix (MX1a and ACTB). Table 1shows the concentrations of each primer and probe in the reactions. For amplification, the following times and temperatures were used; 50ºC for 2 min, 95ºC for 10 min, then 40 cycles of 95ºC for 15 sec and 60ºC for 1 min. Each sample was assayed in duplicate for ACTB and MX1 splice variants. All primers and probes were purchased from Applied Biosystems and are listed in Table 1. Quantification was accomplished using the Applied Biosystems 7500 fast real-time RT-PCR machine with ACTB as an internal control.

For quantitative PCR (qPCR) optimization and validation, all primer sets were tested across a primer concentration gradient (50, 300, and 900 nM final concentration in a 20 µL reaction). The concentration combination that yielded the lowest critical threshold (Ct) value was used for probe optimization across a probe concentration gradient (50, 100, 150, 200, and 250 nM final concentration). The primer probe concentration combination that yielded the lowest critical threshold (Ct) was then used for qPCR assay and validation studies. Each primer probe set was validated using a 6 log dilution curve of target cDNA. Purified MX1 splice variants had previously been generated and cloned into PCRII TOPO vector (Invitrogen). To determine if the cDNA

synthesis protocol had effect on the assays, a random cDNA was diluted 1:20 with nuclease free water and 1 µL of purified MX1 isoform in plasmid. This was then diluted 1:10 six times, and duplicates were assayed for each dilution. Standard Curve option on the Applied Biosystems 7500 Fast machine was used to calculate R^2, intercept and slope. Assay efficiency was then calculated as $10^{(-1/slope)-1}$. The efficiency of each primer set ranged between 90% and 105% (data not shown). Only Ct values that fell within the range of the 6 log dilution curve were accepted for sample data analysis.

Protein Extraction and 2D Electrophoresis

Protein extracts were filtered using Amicon Ultra 15 (Milipore Corporation, Billerica, MA) centrifuge tubes and resuspended in triple distilled H_20. Protein concentrations were quantified with the bicinchonic acid assay (Pierce), according to the manufacturer's instructions using bovine serum albumin (BSA) as the standard. Samples were then lyophilized and resuspended in Rehydration/Sample Buffer (8 M Urea, 2% CHAPS, 50 mM dithiothreitol, 0.2% (w/v) Bio-Lyte 4/7 ampholytes, and trace Bromophenol Blue; Bio-Rad). Samples were sonicated (Aquasonic 75T, VWR Scientific, West Chester, PA) at room temperature for 10 min before being centrifuged for 1 min at 20000 x *g*. Immobilized pH Gradient (IPG) strips (11 cm: pH 4 to 7, Bio-Rad) were rehydrated in the sample by active rehydration (50 V for 12 h) at 20ºC in a PROTEAN IEF cell (Bio-Rad). Immediately following rehydration, isoelectric focusing was done using a 3 step protocol: 1) 250 V for 15 min. 2) 8,000 V for 2.5 h. 3) 8,000 V for 50,000 Vhours. Following isoelectric focusing, IPG strips were equilibrated for second dimension electrophoresis by incubation in Equilibration Buffer I (6M urea, 2% SDS, 0.375M Tris-HCl, pH 8.8, 20% glycerol, 2% (w/v) DTT; Bio-Rad) for 10 min at

room temperature on a rocking platform, then in Equilibration Buffer II (6 M urea, 2% SDS, 0.375M Tris-HCl (pH 8.8), 20% glycerol, 0.5g iodoacetamide) for 10 min. Strips were rinsed in distilled water before being transferred to IPG wells of a 4% stacking, 10% resolving Criterion Tris-HCl gel (Bio-Rad) and separated in the second dimension under constant 200 V for 65 min. One gel of each duplicate was stained with 50 mL Coomassie brilliant blue R-250 staining solution (Bio-Rad) for 30 min at room temperature. The gel was destained with 50 mL of 38% ethanol and 7% glacial acetic acid (vol/vol) in water and photographed using Chemi-Doc XRS imager (Bio-Rad) using Quantity One software.

Proteins on the duplicate gel were transferred to Trans-Blot Transfer Membrane (0.2 micron; Bio-Rad) using a Criterion Cell apparatus (Bio-Rad) with stirring at 70 V (constant voltage) for 1 h at 4ºC. Following transfer, non-specific binding was blocked by incubating the membrane in blocking buffer (20 mM Tris, pH 7.5, 137 mM NaCl, 0.05% Tween 20) containing either 5% nonfat dried milk (polyclonal antibodies) or 5% bovine serum albumin (BSA; monoclonal antibody) with slow rocking at room temperature for 2 h. Membranes were placed in 50 mL of blocking buffer containing 2% nonfat dried milk or BSA and dilution of one of three MX1 antibodies: a carboxyl (No. 90621-3; 1:10,000), or an amino terminal (No. 90618-3; 1:10,000) rabbit polyclonal ovine MX1 peptide antiserum commercially prepared by Multiple Peptide Systems (Hicks *et al.* 2003; San Diego, CA), or a purified monoclonal MX1 antibody (1:1,000; unpublished data K.E. Racicot). The primary antibodies along with negative controls (pre-immune serum and mouse IgM; Product No. M5909, Sigma-Aldrich) were incubated on a rocking platform at 4ºC overnight. Following overnight incubation, the

membrane was washed four times with 100-200 mL of blocking buffer for 5 min each at room temperature. Blots were then incubated with 50 mL blocking buffer containing 2% nonfat dried milk or BSA and 1:200,000 dilution (wt/vol) of goat anti rabbit IgG (product No. 31460; Pierce), or a goat anti mouse IgG (Product No. 31430; Pierce) horseradish peroxidase conjugate on a rocking platform at room temperature for 1 hour. Following incubation with the secondary antibody, membranes were washed four times with blocking buffer for 10 min each at room temperature, and incubated with Supersignal West Femto Maximum Sensitivity Substrate chemiluminescent kit (Pierce) according to the manufacturer's instructions for 5 min to detect immunoreactive proteins. Signal was photographed using the Chemi-Doc XRS imager (exposed for 60-600 sec).

Statistical Analysis

Data for MX1 splice variant mRNA concentrations in ovine uterine cell lines are presented as least square means with the standard error of the relative fold-change from the mean time-point zero samples. Fold-change was calculated using the ΔΔ critical threshold method (reviewed by Kubista et al. 2006) with ACTB serving as the internal control. Data were analyzed using the GLM procedures in SAS (Version 9.1; SAS Institute, Cary, NC). Cell culture well was the experimental unit and fold-change was tested against time, treatment, and treatment × time. Each experiment was replicated 3 independent times.

Results

Ovine MX1 *splice variant sequence analysis*

Previous unpublished work from our lab identified distinct melting curves from MX1 amplicons in qRT-PCR analysis of mRNA from three different ovine uterine cell

lines (see Appendix A). These amplicons were cloned, sequenced and RACE analysis revealed the presence of 3 distinct splice variants that differed only in their 5' coding regions (FIG. 1). The sequence of one splice variant, designated MX1a, shared 95% homology to the sequence previously published by Charleston and Stewart (1993). MX1b lacked 18 nucleotides at positions 145–163. MX1c also lacked 18 nucleotides at positions 145-163, but in their place was an additional 186 nucleotides, making MX1c 168 nucleotides longer than MX1a and 186 nucleotides longer than MX1b.

Computer assisted translation of sequences showed all differences in amino acid sequences occur at the amino terminus (FIG. 2). MX1b differed from MX1a with the deletion of 6 amino acids 24–30. Premature stop codons were present in the unique region of MX1c making translation unlikely, however two secondary translational start codons were identified downstream, one of which is in the context of a consensus Kozak sequence (GTGATGG; Table 2). If this secondary start codon initiated translation of MX1c the first 30 amino acids found in MX1a and MX1b would be replaced with 23 amino acids at the amino terminus that are unique to MX1c. In the unique region are two stretches of arginines (MGGRRRRGRRRMRCLDGITDSMD) that bear some resemblance to a nuclear localization signal. Predicted molecular weights of the amino acid sequences were similar (~75 kDa), but isoelectric points showed modest differences ranging from 5.5 to 6.2 (Table 2).

Steady-state concentrations of MX1 *Splice variants*

We designed and validated TaqMan® assays that were specific for each splice variant and showed no cross-reactivity with the others. Figures 3-5 depicts results from semi-quantitative RT- PCR analysis of MX1 splice variant concentrations in three ovine

uterine cell lines. Figures 3-5 depict the semi-quantitative real-time PCR analysis of MX1 isoform concentrations in three ovine uterine cell lines. All three MX1 splice variants were detected in oLE and oSC cell lines, while only MX1a and MX1c were detectable in oGE cells. In oLE cells (FIG. 3), a treatment × time interaction was detected for MX1a and MX1b. MX1a and MX1b increased (P>0.01) approximately 40 and 60 fold, respectively within 1.5 hours of treatment with IFNT. The two isoforms peaked at 12 hours over 400 fold and remained elevated 24 hours after treatment with IFNT. MX1a and MX1b also exhibited a treatment × time interaction in oSC cells (FIG.4). Both MX1a and MX1b increased (P>0.01) nearly 60 and 100 fold, respectively, within 1.5 hours. They then reached maximum concentrations at 6 hours, and remained elevated at 12 and 24 hours after IFNT treatment. Only MX1a was increased in oGE cell lines (FIG.5). As early as 1.5 hours after treatment MX1a had increased (P>0.01) over 50 fold. MX1a then peaked at 400 fold, 6 hours after IFNT treatment, and remained elevated at 12 and 24 hours. There was no change in the expression of MX1c (P>0.10) in any of the cell lines after treatment with IFNT. Surprisingly, however, steady-state concentrations of mRNA for MX1c were approximately 1000 fold higher than MX1a and MX1b (data not shown).

To determine if IFNT induced increases in steady-state concentrations of MX1 mRNA resulted from increased transcription of the MX1 gene, we treated cells with an inhibitor of transcription (actinomycin D) followed by IFNT. Figure 6 depicts the semi-quantitative RT- PCR analysis of MX1 splice variants in oGE cells after treatment with actinomycin D and IFNT. As expected, concentrations of MX1a increased after treatment with IFNT in wells with medium alone (~33 fold) or medium with DMSO (~40

fold). However, MX1a was not increased in oGE cells that were pretreated with actinomycin D one hour prior to addition of IFNT. Interestingly, concentrations of ACTB and MX1c mRNA were not reduced by this same treatment.

MX1 *Protein analysis*

Two dimensional SDS-PAGE and Western blot analyses of oGE cell lysates with three different MX1 antibodies was consistent with the existence of several isoelectic variants that could correspond to protein products of the different mRNA splice variants (FIG. 4-6). Each antibody gave slightly different patterns of MX1 immunoreactivity. Blots showed no evidence of expression of an MX1c protein (e.g. protein expressed at high concentrations that did not change after treatment with IFNT). The polyclonal amino terminal MX1 antibody (No. 90618-3) recognized several potential MX1 isoforms with a molecular weights near the predicted 75 kDa and pIs from 4 to 7 (FIG. 6). Expression of each of these isoforms increased after treatment with IFNT. The polyclonal carboxy terminal MX1 antibody (No. 90621-3) recognized several proteins that appeared to correspond with those detected by the amino terminal antibody at ~75 kDa (FIG. 7). Interestingly, this antibody also detected several smaller molecular weight proteins (~75-25 kDa). Expression of these smaller proteins also increased with treatment of IFNT. The pattern of protein immunolocalization exhibited by the monoclonal MX1 antibody most closely resembled that of the carboxy polyclonal antibody blots (FIG. 8). The monoclonal MX1 antibody recognized some proteins with molecular weight of approximately 75 kDa at several different isoelectric points, but also some smaller molecular weight proteins that were increased with IFNT treatment.

Discussion

The MX1 splice variants identified in immortalized ovine uterine cell lines in these experiments were similar to the bovine MX1 splice variants described by Ellinwood *et al*. (1998) and Kojima *et al.* (2003). Ovine MX1a had 95% homology to the oMX1 cDNA described by Charleston and Stewart (1993). Ovine MX1b and oMX1c splice variants described here also matched the bMX1-a and bMX1A$_{-18}$B sequences, respectively, identified by Ellinwood *et al*. (1998) and Kojima *et al.* (2003). The ovine MX1b sequence lacked 18 bases at positions 145-163, oMX1c also lacked the same 18 nucleotides and in their place was an additional 186 nucleotides. The fourth bovine MX1 splice variant (MX1B) characterized by Kojima *et al.,* (2003) was not identified in the ovine cell lines, although these studies were not designed to definitively determine if the bovine MX1B existed in sheep cells. Overall, these results suggest that the mRNA splice sites are similar between sheep and cattle in the MX1 gene.

Putative amino acid sequences revealed that all differences occurred at the amino terminus of the putative proteins. Ovine MX1b lacked 6 amino acids at positions 24-30. Premature stop codons (TGA) in the unique portion of MX1c (nucleotide positions 202-204, 208-210 and 238-240) were followed by two secondary, in-frame start codons (ATG; nucleotide positions 268-270 and 270-282), the second of which was in the context of a consensus Kozak sequence. If internal initiation of translation began at either of these sites it would cause replacement of the first 30 amino acids found in MX1a and MX1b with 27 and 23 unique amino acids. Kojima *et al.,* (2003) also predicted that this splice variant would have the same amino terminus and renew transcription at a

secondary start site, however this seems improbable. The other splice variant, bMX1B, described by Kojima *et al.*, (2003), was not identified in this study.

According to the scanning model of translation, the eukaryotic initiation factor 4F complex binds the 7-methylguanosine cap of mRNA and recruits 43S ribosomal complex (Short and Pfarr, 2002). The 43S ribosomal complex than scans the 5'UTR linearly in the 5'-3' direction until it reaches the first AUG surrounded by a Kozak sequence whereupon translation is initiated (Short and Pfarr, 2002). However, mRNAs have been shown to produce multiple protein products from more than one start codon, generally resulting from 'poor' strength of the first start codon which allows the ribosomal complex to pass and reach another downstream initiation codon (Kozak, 1999; Short and Pfarr, 2002). Three models exist to describe how ribosomes can bypass an upstream start codon 1) internal ribosome entry, 2) linear but leaky scanning and 3) non linear scanning (Short and Pfarr, 2002). Internal ribosomal entry occurs independent of the mRNA cap but requires ribosome binding to an internal ribosomal entry sequence element (IRES; Short and Pfarr, 2002), it is unknown if MX1c has an IRES.

In the case that the two unique splice variants (MX1c/bMX1A$_{-18}$B and bMX1B) identified by Kojima *et al.* (2003) were translated; both splice variants contain a unique sequence with repeated arginine residues (RRRRGRRRMR). This sequence does not match the consensus nuclear localization signal (NLS; KR[PAATKKAGQA]KKKK; Lange *et al*. 2007) but, because of its long stretch of positively charged arginines, there are some similarities to it. In the nucleus MX1 proteins may function differently or have altered antiviral activity. A nuclear form may also cause the protein to lose function. Studies by Zurcher *et al*., (1992) and Turan *et al*., (2004) showed, however, that MXA

(normally cytoplasmic) exhibited antiviral activity when induced to translocate to the nucleus using a rodent NLS. While the MX1c splice variant may be related to rodent MX1 which contains a NLS and has antiviral activity specific to viruses that replicate in the nucleus (Haller *et al.* 2007), we feel that this is unlikely because the rodent NLS is in the carboxy terminus of the protein (Melen, *et al*. 1992; Garber *et al.* 1993).

The NCBI conserved domain program (www.ncbi.nlm.nih.gov/Structure/) was used to identify the presence of conserved domains in the oMX1 splice variants. Each splice variant maintained the GTP binding motif, central interactive domain and leucine zipper that have been shown to be necessary for function of MX1 proteins in other species (Pitossi *et al*. 1993; Flohr *et al*. 1999). These results were intriguing because they suggest that the splice variants could code for proteins that possess functional properties.

Quantitative PCR analysis revealed that the two MX1 splice variants; MX1a and MX1b, were regulated by IFNT in oLE and oSC cell lines. Both MX1a and MX1b were rapidly and strongly up regulated by IFNT in oLE and oSC cells. MX1a was also up regulated in oGE cells, however, MX1b was not detectable. The immortalized ovine endometrial cell lines used in this study were developed and characterized by Johnson *et al*. (1999). In that report, the authors indicated that tissues for all three cell lines came from the uterus of one ewe. However, it was later revealed (G.A. Johnson, personal communication) that uteri from two ewes were used to derive the three cell lines. This opened up the possibility that MX1 splice variants were actually alleles. Interestingly, the MX1b splice variant was thought not to exist in sheep (Ellinwood *et al.* 1998). Our results and the results of Ellinwood *et al.* (1998) and Kojima *et al*. (2003), strongly support the hypothesis that these represent splice variants of the same gene. The 18 base

pair differences occurred at positions corresponding to an alternative splice site at the boundary between exons 2 and 3 of the mouse (Ellinwood *et al*. 1998; Kojima *et al*. 2003) and the bovine MX1 genomic sequence (D.S. Clark and T.L. Ott, unpublished observation). Also, the unique 186 nucleotides in MX1c are found in intron B with suspected splice sites flanking that region (Kojima *et al*. 2003). However, the genomic sequence described by Kojima *et al.* (2003) did not match another sequence published by Gerardin *et al.* (2004). Because we were able to clone a full length cDNA of MX1c the sequence described by Kojima *et al.* (2003) is likely the correct sequence.

It is interesting that concentrations of MX1c mRNA were very high and unchanged by IFNT treatment. One interpretation of these results is that the promoter of the ovine MX1 gene is constitutively active, and that regulators of MX1 splicing are induced to produce MX1a and MX1b after treatment with IFNT. This may explain how MX1 is rapidly up regulated after IFNT treatment and also explain some differences in the pattern of expression between MX1 and other ISGs in the endometrium. Unlike other ISGs, MX1 is up regulated by IFNT in the LE and superficial GE of the endometrium (Ott *et al.* 1998; Johnson *et al*. 2002). This is in contrast to most other ISG (Johnson *et al.* 2002; Choi *et al.* 2003). Instead, IFNT may modulate regulators of the spliceosome responsible for the MX1a or MX1b variants. Quantitative RT-PCR analysis of oGE cells after incubation with actinomycin D showed that IFNT did not induce accumulation of MX1a when transcription was blocked with actinomycin D, however, concentrations of ACTB and MX1c mRNA were not affected. This suggests that ACTB and MX1c mRNA have long half-lives and also that MX1c mRNA is not available for

rapid splicing into other splice variants. Although, it is possible that IFNT activates transcription of splicing regulators that were blocked by actinomycin D treatment.

Finally, results presented here related to high constitutive concentrations of MX1c would also occur if our mRNA samples were contaminated with genomic DNA. Due to an oversight in primer design, the primers used to amplify the MX1c variant did not span an exon-exon boundary. We feel that genomic DNA contamination is unlikely for the following reasons: 1) Samples were treated with DNase for one hour to destroy genomic DNA prior to RT-PCR. 2) In each batch of cDNA synthesis we included a negative control that lacked reverse transcriptase. Because these samples were negative for MX1c we believe that genomic contamination was not responsible for the MX1c results. To confirm that the assay was amplifying MX1c we used the MX1c probe and mismatched the MX1c primers with MX1a and MX1b primers. Using plasmid alone all combinations of the primer sets amplified MX1c equally (data not shown), however, when a cDNA product was examined only the validated MX1c assay resulted in amplified product of the expect size. We believe that there are a few possibilities for this: 1) the primers and probe were not designed to work together and it is possible that the mismatched assays were not as efficient, 2) it is also possible that there were interfering substances in the cDNA synthesis reaction that could cause the inefficient primer sets to not work well, 3) genomic contamination would also lead to this result, however as explained previously we have controlled for potential contamination by genomic DNA; and finally, 4) it is possible that there are abundant amounts of primary MX1 mRNA in the samples. To determine if primary MX1 mRNA is contributing to the high concentrations of MX1c found in our samples, we designed seven new PCR assays with primers that recognize

only MX1 intron B, as well as the intron-exon boundary and the unique exon-exon boundary of MX1c. In this experiment, none of the assays were able to amplify a product. A positive control of a plasmid containing full length MX1c proved that the exon-exon primer sets worked; however, there was no positive control for the assays that would recognize primary MX1 mRNA. This further complicates our previous results. Because we were able to clone a full length MX1c cDNA, we can not conclude that MX1c does not exist. However, the MX1 genomic sequence published by Gerardin *et al*. (2004) does not include the unique nucleotide sequences identified in MX1c by us and Kojima *et al.* (2003).

The putative protein products of the splice variants were predicted to be of similar size (~75 kDa), so identification of different oMX1 isoforms was not possible by one-dimensional SDS-PAGE and Western blotting. The predicted isoelectric points differed more dramatically, so two-dimensional electrophoresis was used to determine if there was evidence of MX1 isoforms with different isoelectric points that could represent translation products of each of the splice variants. Three MX1 antibodies were used for Western blotting to identify protein isoforms (Hicks *et al*. 2003; unpublished data K.E. Racicot). Two-dimensional electrophoresis of oGE cell lysates identified several possible isoforms of oMX1 that migrated at ~75 kDa. Various lower molecular weight proteins were also identified using the COOH and monoclonal antibody, and could be the result of proteolytic cleavage of MX1 or the products of premature termination of translation. These lower molecular weight isoforms were not detected with the amino terminal Ab. An earlier report by Horisberger *et al*., (1988) used a polyclonal antibody that recognized both human and mouse MX proteins to identify a similar protein in

bovine cells treated with IFN alpha. In that study the authors identified two possible isoforms with molecular weights and isoelectric points comparable to the human and mouse MX proteins (Horisberger *et al.* 1988). The authors were also able to identify these proteins by one-dimensional electrophoresis and Western blotting, however, we have not detected a similar set of proteins in our lab.

It still remains to be determined if the MX1b mRNA is translated into a fully functional protein. However, because the differences between MX1a and MX1b are minor it is likely that MX1b is functional. It would be interesting to determine if MX1b protein has altered function compared to MX1a. If, as our data and that of others suggest, these are true splice variants, it would be interesting to determine what regulates the relative ratio of each splice variant and if the variants are tissue specific or developmentally regulated. Kojima *et al.*, (2003) proposed that presence of MX1 splice variants may strengthen the resistance of cattle to viruses, including the influenza virus, which also occurs in sheep. It is also possible that MX1 splice variants may have developed different functions. Recent evidence from our lab suggests that MX1 proteins possess roles in regulation of 'unconventional' secretion by uterine epithelial cells (Toyokawa *et al.* 2007a, 2007b). It is possible that one variant may inhibit viral replication while others have functions outside the immune system. It would also be interesting to over express each of the splice variants and see if each regulated 'unconventional' secretion and possessed antiviral activity. Future research should examine functions of the splice variants.

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TABLE 1. Gene, primer sequence, probe fluorescence, sequence (5' to 3'), concentration and amplicon size for TaqMan sets used in quantitative real-time PCR assays

Gene	**Primer-Probe**	**Sequence (5' to 3')**	**Conc. (nM)**	**Amplicon Size (bp)**
Beta-Actin	Forward	GGGACCTGACGGACTACCT	300	63
	Reverse	CCGTGGTGGTGAAGCTGTAG	50	
	6FAM-MGBNFQ	TCCGTGAGGATCTTCA	200	
MX1a	Forward	CCAGTCTAAATGGAAGTGAAGATATGGT	900	87
	Reverse	GCACCTTCTCTTCATATTGGCTGTA	300	
	VIC-MGBNFQ	CTCTTGGACTCAGTTTCATG	250	
MX1b	Forward	GGTATCGAAGAACTTGATTCACCTGAA	300	102
	Reverse	GCACCTTCTCTTCATATTGGCTGTA	300	
	VIC-MGBNFQ	CTCTTGGACTCCATATCTT	250	
MX1c	Forward	GGCCACCTCATGTGAAGAGTT	900	94
	Reverse	CTCCGTCGTCCCCTTCTC	300	
	VIC-MGBNFQ	CCAGCATCAGAGTCTTT	250	

MX1a	91	**ATG**GTTCATT CTGACTTGGG TATCGAAGAA CTTGATTCAC CTGAATCCAG	140
MX1b	91	**ATG**GTTCATT CTGACTTGGG TATCGAAGAA CTTGATTCAC CTGAATCCAG	140
MX1c	91	**ATG**GTTCATT CTGACTTGGG TATCGAAGAA CTTGATTCAC CTGAATCCAG	140
MX1a	141	TCTAAATGGA AGTGAAGATA TGGTGAGGGA ACATGAAACT ----------	180
MX1b	141	TCTAAATGGA AGTGAAGATA TG-------- ---------- ----------	162
MX1c	141	TCTAAATGGA AGTGAAGATA TGCTGTTGAG AGGAGGAGTA TTTACTTTGT	190
MX1a	180	---------- ---------- ---------- ---------- ----------	180
MX1b	162	---------- ---------- ---------- ---------- ----------	162
MX1c	191	GCAGCTGTTG C**TGA**AGC**TGA** AACTCCAGTA CTTGTGGCCA CCTCATG**TGA**	240
MX1a	180	---------- ---------- ---------- ---------- ----------	180
MX1b	162	---------- ---------- ---------- ---------- ----------	162
MX1c	241	AGAGTTGACT CACTGGAAAA GACTCTG**ATG** CTGGGAGTG**A TG**GGGGGCAG	190
MX1a	180	---------- ---------- ---------- ---------- ----------	180
MX1b	162	---------- ---------- ---------- ---------- ----------	162
MX1c	191	GAGGAGAAGG GGACGACGGA GGATGAGATG TCTGGATGGC ATCACTGACT	240
MX1a	181	--------GA GTCCAAGAGC AACCTGTACA GCCAATATGA AGAGAAGGTG	222
MX1b	163	--------GA GTCCAAGAGC AACCTGTACA GCCAATATGA AGAGAAGGTG	204
MX1c	241	CGATGGACGA GTCCAAGAGC AACCTGTACA GCCAATATGA AGAGAAGGTG	290
MX1a	223	CGGCCCTGCA TTGATCTCAT CGACTCCCTG CGGTCCCTGG GCGTGGAGCA	272
MX1b	205	CGGCCCTGCA TTGATCTCAT CGACTCCCTG CGGTCCCTGG GCGTGGAGCA	254
MX1c	291	CGGCCCTGCA TTGATCTCAT CGACTCCCTG CGGTCCCTGG GCGTGGAGCA	340
MX1a	273	GGACCTGGCC CCCTGCCCGC CATCGCTGTT ATCGGGGACC AGAGCTCAGG	322
MX1b	255	GGACCTGGCC CCCTGCCCGC CATCGCTGTT ATCGGGGACC AGAGCTCAGG	304
MX1c	341	GGACCTGGCC CCCTGCCCGC CATCGCTGTT ATCGGGGACC AGAGCTCAGG	390

Table 2. Nucleic acid sequence of MX1 splice variants. Sequences before and after this region are identical. Bolded letters represent potential start (ATG) and stop codons (TGA). Secondary initiation sites for MX1c are bolded in the unique region. The Kozak sequence surrounding the second potential start site is boxed. Shaded nucleotides show probe binding sites for MX1a and MX1b qRT-PCR.

TABLE 3. Computer generated translation of the amino terminus of oMX1 isoforms. All isoforms are identical following this region.

MX1a	MVHSDLGIEELDSPESSLNGSEDM**VREHET**ES
MX1b	MVHSDLGIEELDSPESSLNGSEDM- - - - - - - ES
MX1c	MLGVMGGRRRRGRRRMRCLDGITDSMDES

TABLE 4. Theoretical molecular weight and isoelectric point of MX1 isoforms.

Gene	**Molecular Weight (Da)**	**Isoelectric Point**
MX1a	75,768.32	5.54
MX1b	74,860.40	5.61
MX1c	75325.24	6.20

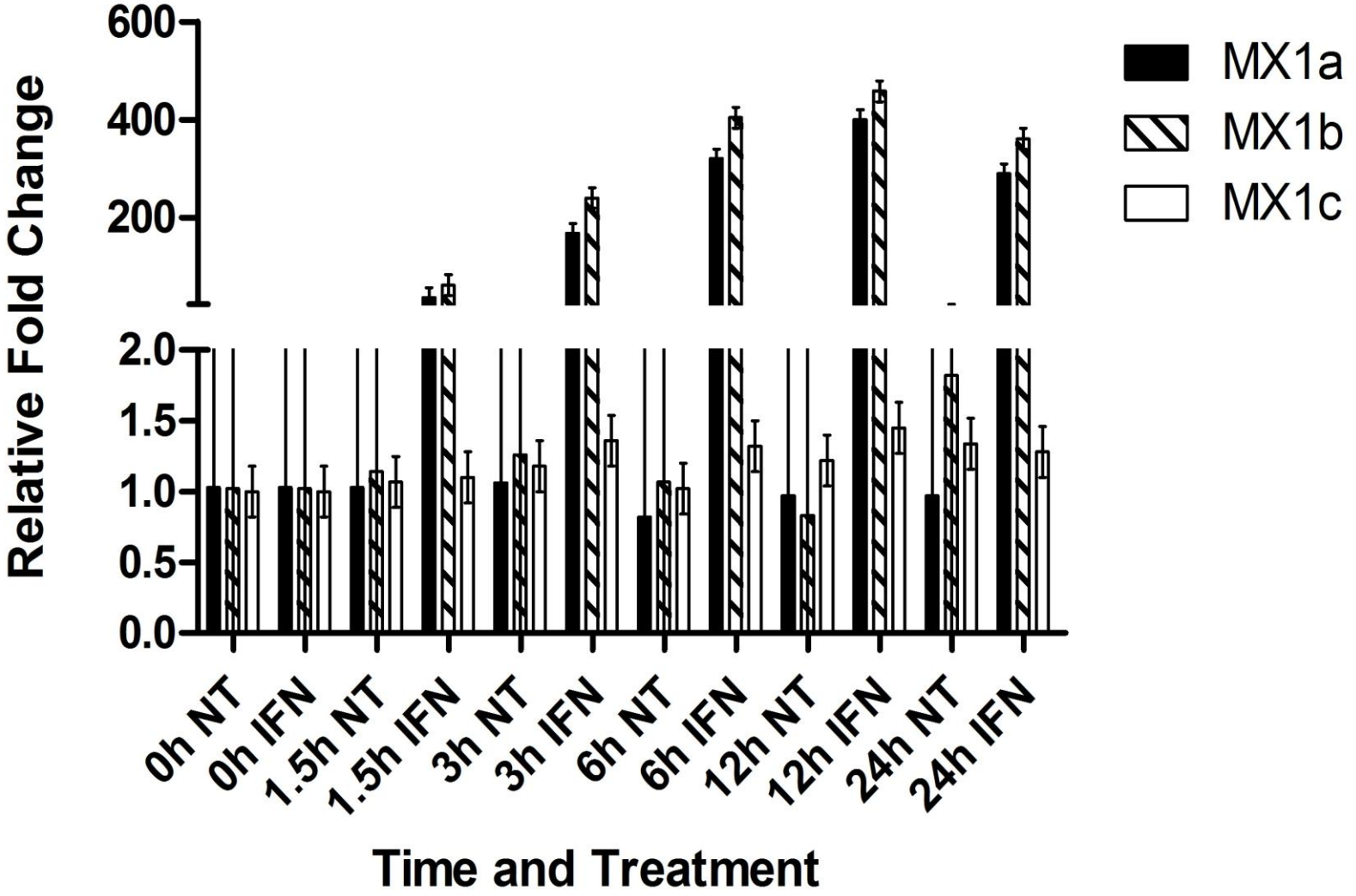

600
400
200
2.0
1.5
1.0
0.5
0.0
Relative Fold Change
MX1a
MX1b
MX1c
0h NT
0h IFN
1.5h NT
1.5h IFN
3h NT
3h IFN
6h NT
6h IFN
12h NT
12h IFN
24h NT
24h IFN
Time and Treatment

FIG. 1. Representative graph of steady state mRNA concentrations for MX1a (closed bars), MX1b (striped bars), and MX1c (open bars) determined using quantitative PCR in IFN treated and non-treated oLE cells 0, 1.5, 3, 6, 12, and 24 hours after treatment with IFNT (n = 3 wells per treatment). Bars represent the average fold change from time 0 calculated using the ΔΔ critical threshold method IFNT increased concentrations of MX1a and MX1b mRNA($P < 0.01$), but did not affect concentrations of MX1c. Error bars represent SEM.

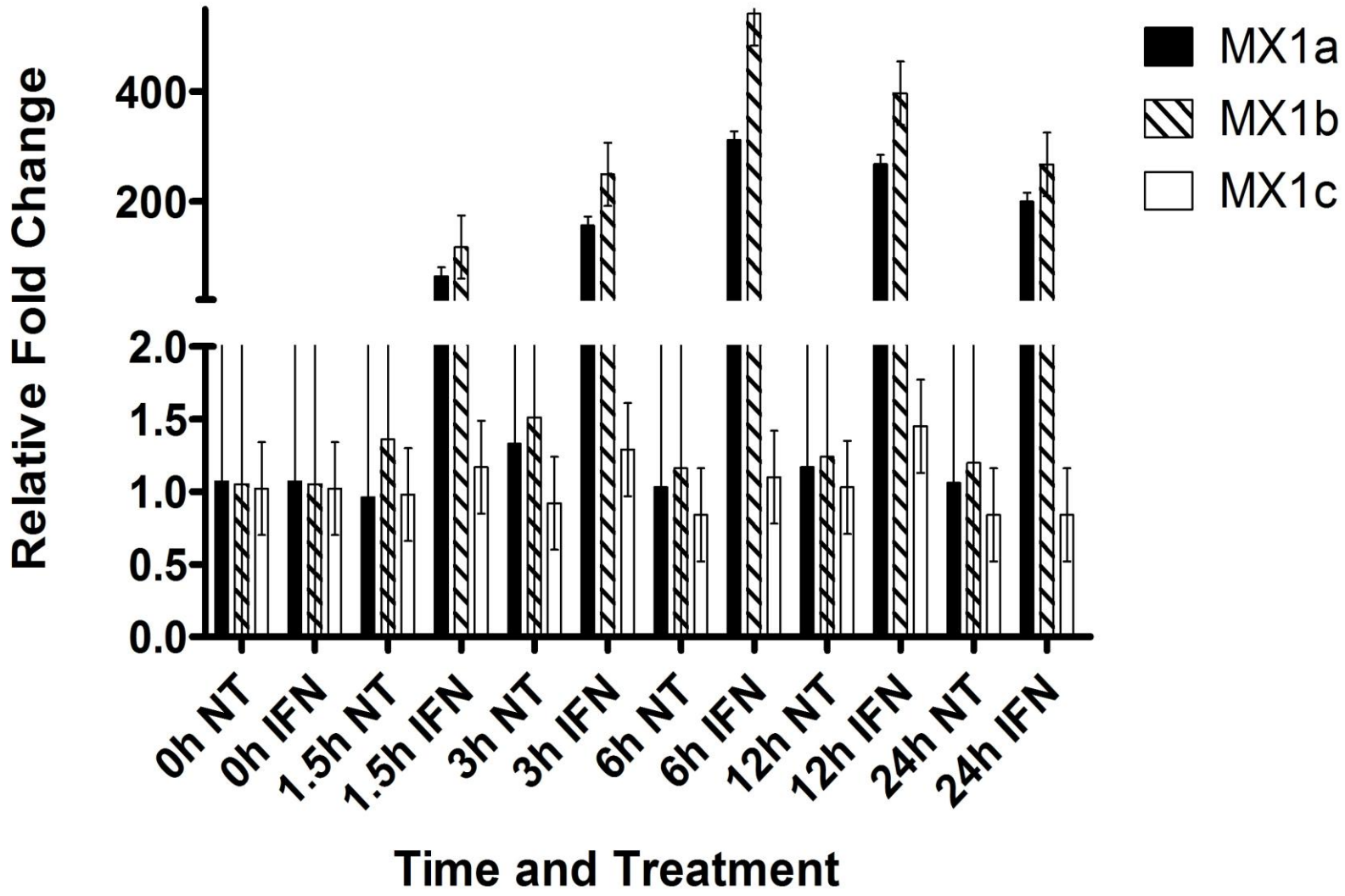

Relative Fold Change
400
200
2.0
1.5
1.0
0.5
0.0
MX1a
MX1b
MX1c
0h NT
0h IFN
1.5h NT
1.5h IFN
3h NT
3h IFN
6h NT
6h IFN
12h NT
12h IFN
24h NT
24h IFN
Time and Treatment

FIG. 2. Representative graph of steady state mRNA concentrations for MX1a (closed bars), MX1b (striped bars), and MX1c (open bars) quantified using quantitative PCR in IFN treated and non-treated oSC cells 0, 1.5, 3, 6, 12, and 24 hours after treatment (n = 3 wells per treatment). Bars represent the average fold change from time 0 calculated using the ΔΔ critical threshold method. Error bars represent SEM. IFNT increased concentrations of MX1a and MX1b mRNA($P < 0.01$), but did not affect concentrations of MX1c. Error bars represent SEM.

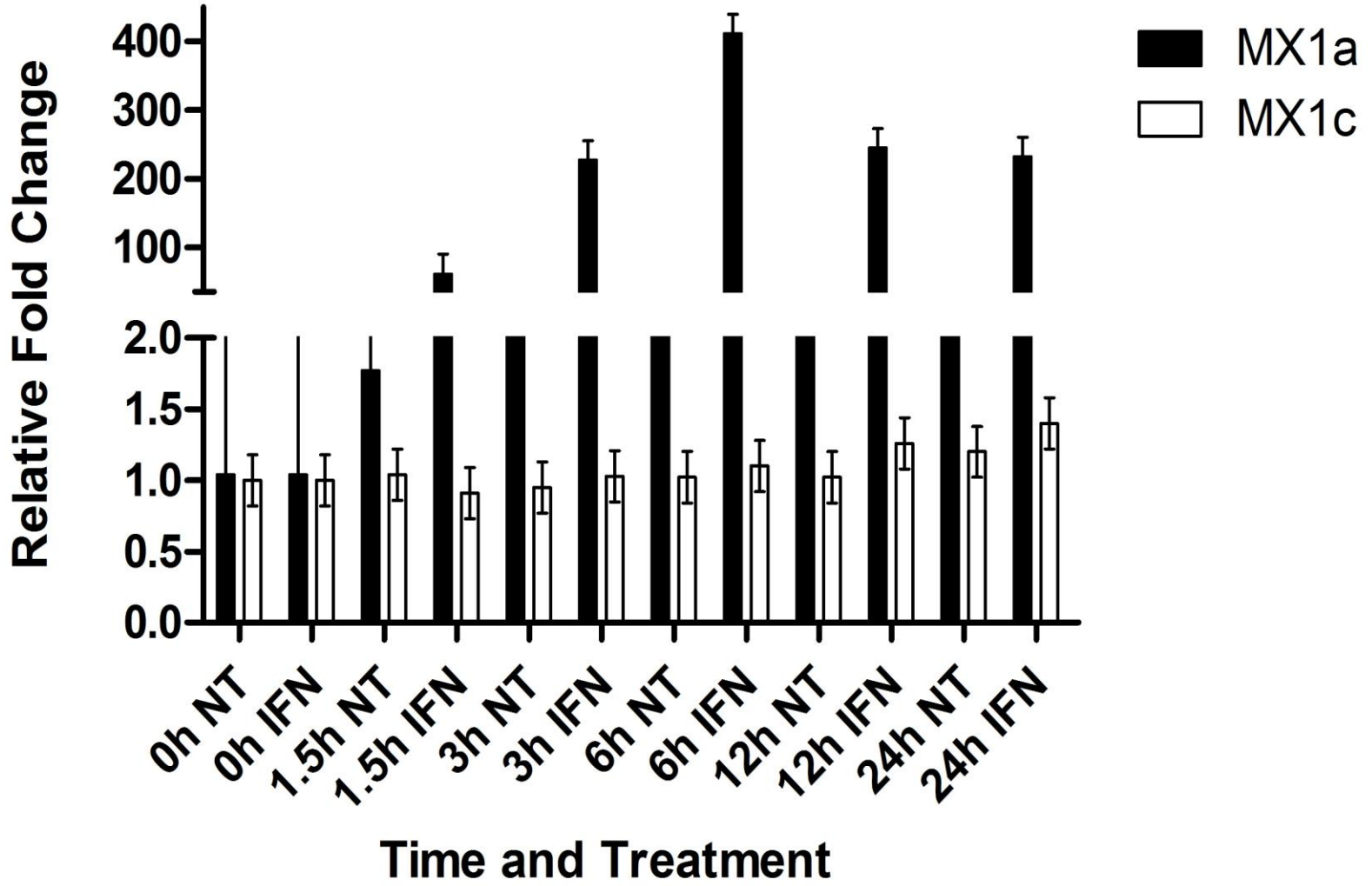

Relative Fold Change
400
300
200
100
2.0
1.5
1.0
0.5
0.0
MX1a
MX1c
0h NT
0h IFN
1.5h NT
1.5h IFN
3h NT
3h IFN
6h NT
6h IFN
12h NT
12h IFN
24h NT
24h IFN
Time and Treatment

FIG. 3. Representative graph of steady-state mRNA concentrations for MX1a (closed bars) and MX1c (open bars) quantified using quantitative PCR in IFNT treated and non-treated oGE cells 0, 1.5, 3, 6, 12, and 24 hours after treatment (n = 3 wells per treatment). Bars represent the average fold change from time 0 calculated using the ΔΔ critical threshold method. Error bars represent SEM. IFNT increased concentrations of MX1a mRNA ($P < 0.01$), but did not affect concentrations of MX1c. Error bars represent SEM.

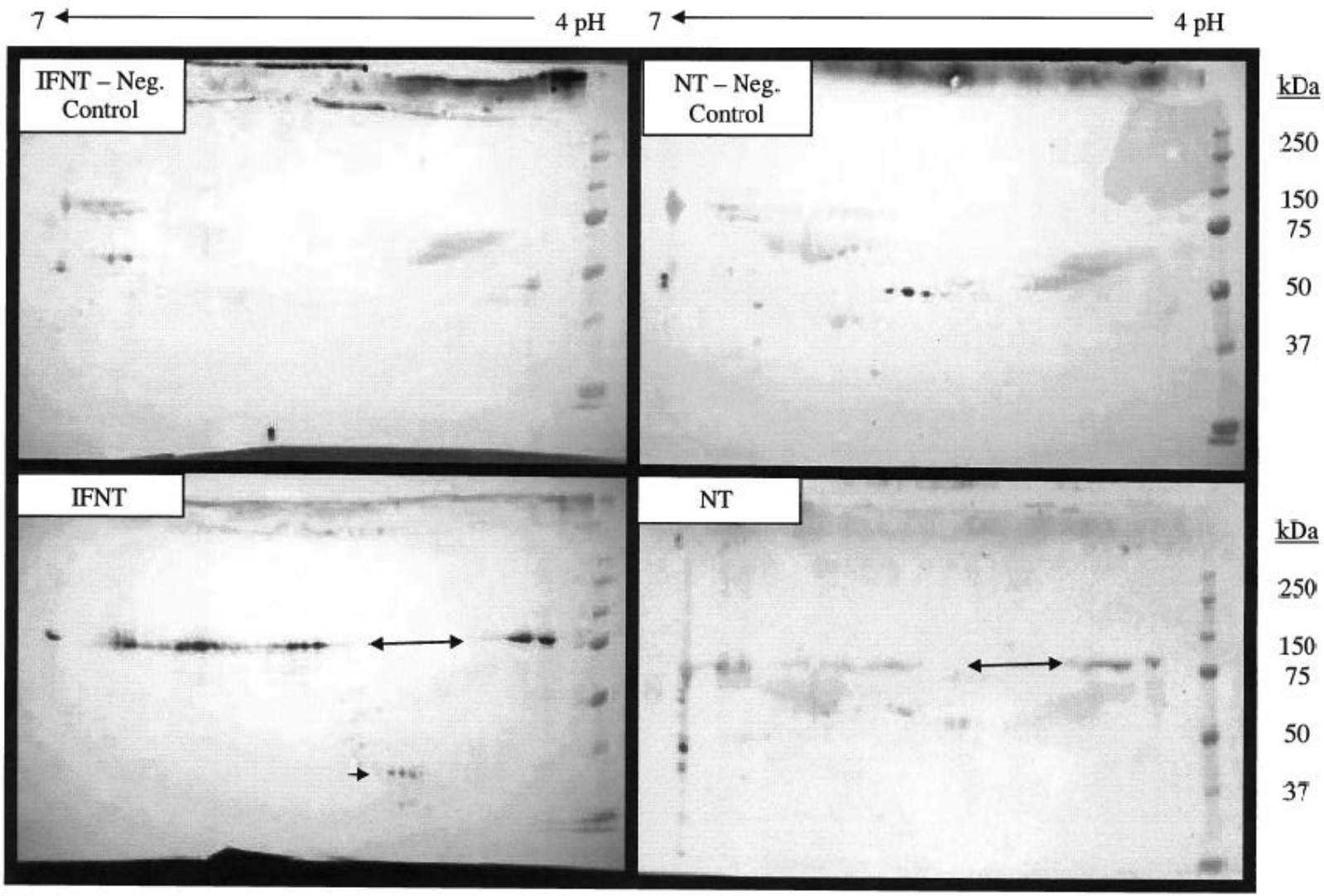
MX1a
MX1c
Relative Fold Change
50
40
30
20
10
2.0
1.5
1.0
0.5
0.0
NT-NT
NT-IFNT
DMSO-NT
DMSO-IFNT
Act.D-NT
Act.D-IFNT
Treatment

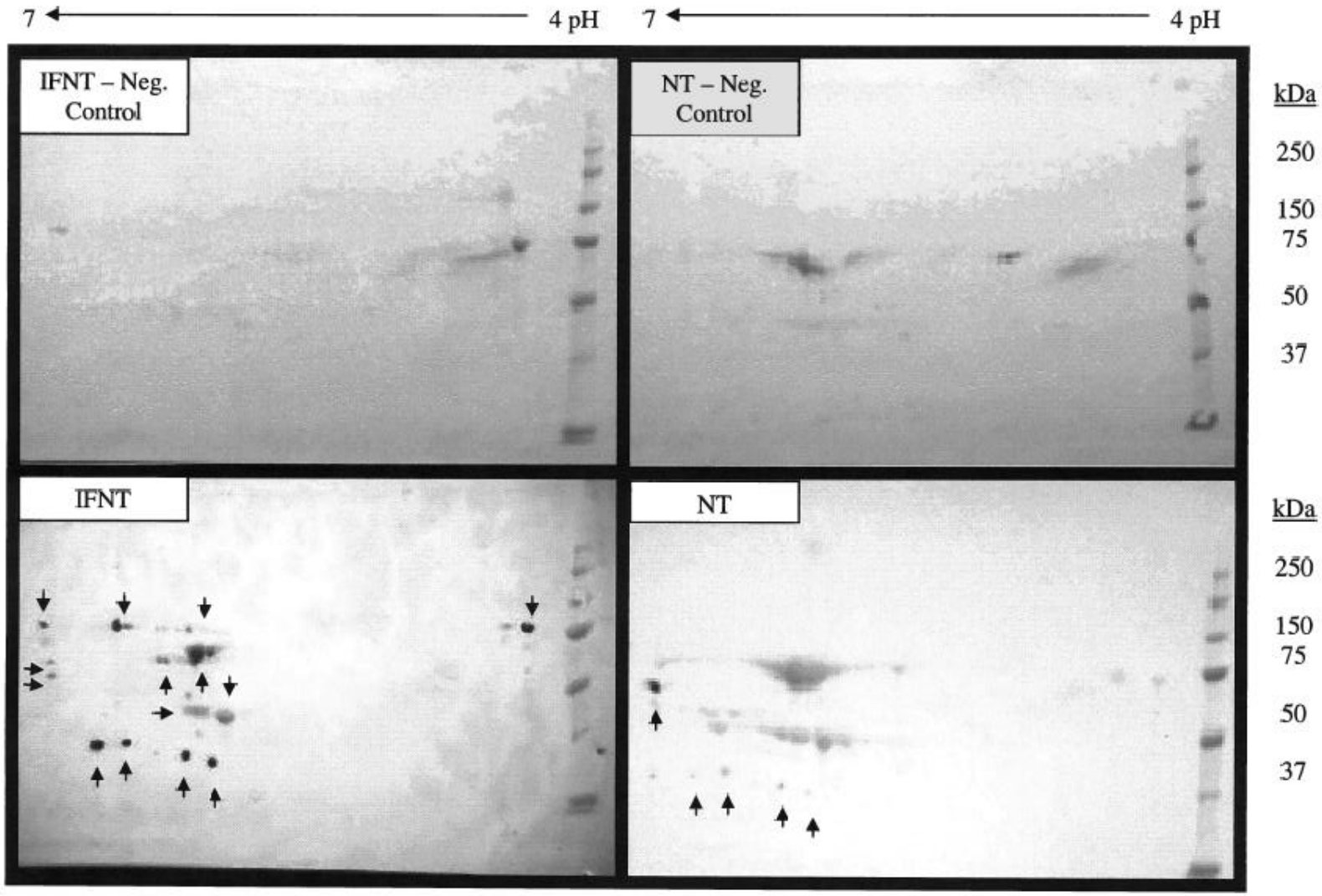
FIG. 4. Steady-state mRNA concentrations for MX1a (closed bars) and MX1c (open bars) quantified using quantitative PCR in oGE cells treated with actinomycin D for 1 hour before IFNT treatment (n = 3 wells per treatment). Bars represent the average fold change from non-treated controls calculated using the ΔΔ critical threshold method. Actinomycin D inhibited the increase of MX1a mRNA concentrations.

FIG. 5. Two-dimensional SDS-PAGE and Western blot analysis with polyclonal amino terminal MX1 antibody (No. 90618-3). Molecular weight markers are shown on the right side in kilodaltons. Cell lysates treated with (A, C), or without IFNT for 24 hours (B, D). Pre-immune serum was used as a negative control (A, B). Several immunoreactive proteins with molecular weights ~75 kDa are identified by arrows along with some smaller proteins.

FIG. 6. Two-dimensional SDS-PAGE and Western blotting with polyclonal carboxy terminal MX1 antibody (No. 90621-3). Molecular weight markers are shown on the right side in kilodaltons. Cell lysates treated with (A, C), or without IFNT for 24 hours (B, D). Pre-immune serum was used as a negative control (A, B).

FIG. 7. Two-dimensional SDS-PAGE and Western blotting with monoclonal MX1 antibody. Molecular weight markers are shown on the right side in kilodaltons. Cell lysates treated with (A, C), or without IFNT for 24 hours (B, D). Mouse IgM was used as a negative control (A, B). Several immunoreactive proteins are identified by arrows.


7
4 pH
7
4 pH
IFNT – Neg. Control
NT – Neg. Control
kDa
250
150
75
50
37
IFNT
NT
kDa
250
150
75
50
37


7
4 pH
7
4 pH
IFNT – Neg. Control
NT – Neg. Control
IFNT
NT
kDa
250
150
75
50
37
kDa
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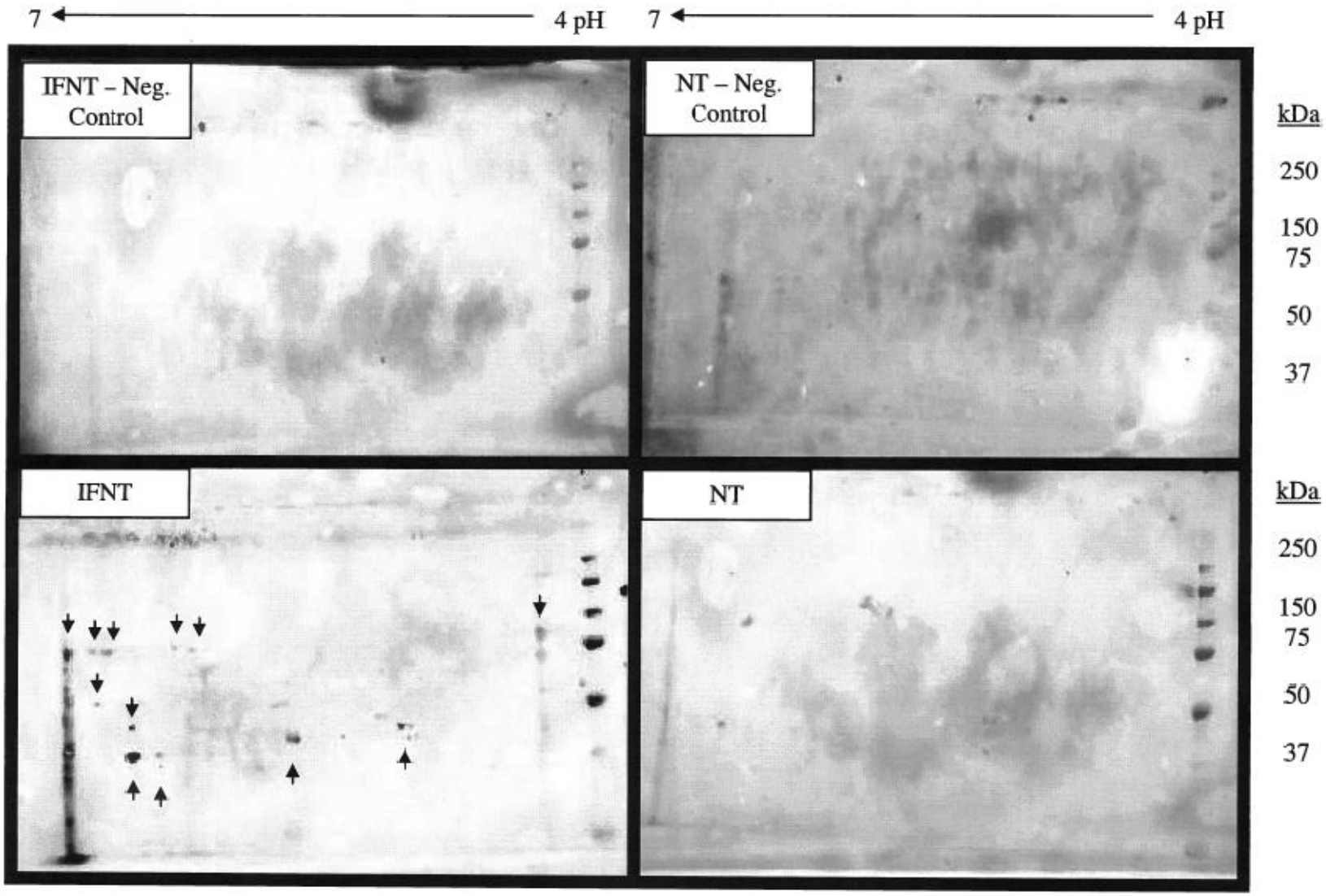
150
75
50
37


7
4 pH
7
4 pH
IFNT – Neg. Control
NT – Neg. Control
IFNT
NT
kDa
250
150
75
50
37
kDa
250
150
75
50
37

Expression of MX1 splice variants in endometrium and peripheral blood leukocytes of pregnant and cyclic ewes, and peripheral blood leukocytes of pregnant and bred, non-pregnant cows

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Introduction

During maternal recognition of pregnancy in ruminants such as cattle and sheep, interferon tau (IFNT) is secreted by the trophoblast cells of the blastocyst and signals its presence to the mother (Bazer *et al.* 1991). Interferon tau is a type I IFN, which binds to type I IFN receptors in the uterus and alters expression of a large number of genes and ultimately leads to protection of the corpus luteum (Spencer and Bazer, 2007). Maximal production of IFNT in sheep occurs from days 14 to 16 (Farin *et al*. 1997) and in cattle is from days 16 to 19 (Helmer *et al*. 1987). IFNT rescues CL function by blocking the pulsatile production of prostaglandin F_2 alpha (PTGF2A). It does this by inhibiting the expression of oxytocin receptors (OTRs) in the luminal and superficial glandular epithelium (Spencer and Bazer, 2007). In the absence of OTR, oxytocin is unable to cause pulses of PTGF2A, the luteolysin, and the corpus luteum (CL) is maintained and continues to produce progesterone (P4). In addition to blocking luteolysis (Spencer and Bazer, 2007), IFNT also induces the expression of many other genes in the endometrium (Ott *et al*. 1998, Choi *et al*. 2003 Gifford *et al*. 2008), CL (Gifford *et al*. 2008), and periperhal blood leucocytes (PBLs; Yankey *et al.* 2001, Han *et al.* 2006; Gifford *et al*. 2007, 2008). A few of the interferon stimulated genes (ISGs) include; 2',5'-oligoadenylate sythetase (OAS; Johnson *et al*. 2001), interferon-stimulated gene 15 (ISG15; Hansen *et al*. 1999; Johnson *et al*. 2000; Gifford *et al*. 2007), beta2-microglobulin (B2MG; Choi *et al*. 2003), and the myxovirus resistant genes MX1 and MX2 (Ott *et al.* 1998; Yankey *et al*. 2001; Hicks *et al.* 2003; Stevens *et al*. 2006; Gifford *et al.* 2007). Not only are MX proteins up-regulated by type I IFNs, and viruses, but they

may also be modulated by progesterone (P4) (Ott *et al*. 1998). In the endometrium of cyclic ewes, MX1 mRNA was maximal when P4 concentrations were greatest (Ott *et al*. 1998).

The roles of MX proteins in the antiviral system are well characterized, however their role in reproduction and pregnancy establishment is unknown. Many recent studies have implicated MX proteins in roles outside the antiviral response. Human MXB has been implicated in nuclear import and cell cycle progression (King *et al*. 2004) and ovine MX1 has been shown to be secreted and suggested as a regulator of 'unconventional' secretory pathways (Toyokawa *et al*. 2007a & 2007b).

In 1998, Ellinwood *et al.,* described a variant of MX1 that the authors named MX1-a. Then in 2003, Kojima *et al.,* described two new MX1 variants in cattle, and concluded that all four were splice variants of the MX1 gene. However, the regulation of each splice variant by IFNT and pregnancy was not examined. Previously, we showed that three splice variants of MX1 (MX1a, MX1b, MX1c) were detectable in immortalized ovine uterine luminal epithelial (oLE) and stromal (oSC) cell lines, and that MX1a and MX1b were up-regulated by IFNT (Chapter 2). We further showed that unlike MX1a and MX1b, MX1c mRNA concentrations were much higher and not up-regulated by IFNT in any of the cell lines (Chapter 2). Two-dimensional electrophoresis and Western blotting provided evidence of multiple immunoreactive MX1 proteins, although there was no evidence of a protein similar in expression pattern to MX1c (high basal concentrations that did not change with IFNT treatment), although, there is some uncertainty related to the product amplified in the MX1c assay which was previously discussed (Chapter 2). Premature stop codons in the unique region of MX1c are expected to render the variant

untranslatable (Chapter 2). The objective of this study was to examine expression and regulation of MX1 splice variants in the endometrium and peripheral blood leukocytes during early pregnancy in sheep and cattle. Our working hypothesis is that MX1a and MX1b mRNA concentrations would increase in response to pregnancy in these tissues, and that, consistent with our previous studies (Chapter 2) MX1c mRNA would not be affected by pregnancy status.

Materials and Methods

Animals

Endometrial samples were previously collected (Gifford *et al*. 2008). Briefly, mature, crossbred ewes were randomly assigned to collection days and endometrial samples were collected on days 11, 13, and 15 of the estrous cycle and on days 11, 13, 15, 17, and 19 of pregnancy (n =4 samples per day per status). Tissues were snap-frozen.

For ewe blood collection; thirteen Dorset ewes were housed and maintained at the Pennsylvania State University Beef/Sheep Center. Starting in August of 2008, a ram equipped with an apron to prevent breeding was allowed access to ewes for 30 minutes twice a day for 24 days to stimulate estrous activity. Estrus was synchronized with two intramuscular injections of Lutalyse® (15mg Pfizer; New York, NY) administered 3 hours apart. Sheep were bled on the day of estrus (Day 0) and Day 15 of the cycle. During the subsequent estrus, the same ewes were mated to an intact ram of proven fertility on Day 0, and blood was collected on Day 0 and 15 after mating and processed as described below. Pregnancy was confirmed using transabdominal ultrasonography approximately 60 days after breeding.

Cow blood was previously collected (Gifford, 2008). Briefly, cows were housed and maintained at a commercial dairy (Kulp Family Dairy, LLC, Martinsburg, PA). Holstein cows were synchronized to ovulate (Ovsynch; Pursley et al., 1995) and inseminated (Day 0; n=14) or not inseminated (n=10). To ensure that blood P4 concentrations were elevated at Day 19, a CIDR® (EAZI-BREED; InterAg, Hamilton, New Zealand; Pfizer) was administered to 4 of the 14 inseminated cows on Day 17 and removed on Day 19. Blood samples were collected (coccygeal venipuncture) on Day 0 and 19. Pregnancy was confirmed via transrectal ultrasonography 40-45 days after breeding. After pregnancy diagnosis, cows were divided into the following groups: cows that did not receive a CIDR and were pregnant (NCP; n = 6); cows that did not receive a CIDR and were bred but not pregnant (NCO; n = 4); cows that received a CIDR and were bred but not pregnant (CO; n = 4); and cows that did not receive a CIDR and were not bred (NB; n = 10).

All animal procedures were approved by the Institutional Animal Care and Use Committee at Pennsylvania State University or the University of Idaho where experiments were completed.

Blood Sample Processing

Blood samples were collected into EDTA-containing tubes (Tyco Healthcare Group LP, Mansfield, MA), gently mixed by inverting 5 times and immediately placed on ice until processing. Tubes were centrifuged at 1,200 x *g* for 12 min at 4ºC. The buffy coat (leukocyte) fraction from each was collected and transferred to a 15 mL conical tube. Twelve to 15 mL of red blood cell lysis buffer (150mM NH4Cl, 10mM NaHCO3, 1mM EDTA, pH 7) was added to the buffy coat, and tubes were inverted

several times, followed by incubation at room temperature for 5 min to lyse the red blood cells. Samples were then centrifuged at 300 x *g* for 10 min at 4ºC to isolate peripheral blood leukocytes (PBL) and the supernatant was discarded. The PBL pellet was then re-washed with 5 mL of red blood cell lysis buffer, incubated at room temperature, and centrifuged at 300 x *g* for 10 min at 4ºC, and the supernatant was discarded. The PBL pellet was then washed with ice-cold 1X PBS and centrifuged at 300 x *g* for 10 min at 4ºC, and the supernatant was discarded. The PBL pellet was resuspended with residual PBS, and 2 mL of TRIzol (Invitrogen, Carlsbad, CA) was added to each sample. Cells were lysed by gently pipetting up and down and the samples were evenly divided between two 1.5 mL microcentrifuge tubes and stored at -80ºC until assayed.

RNA Extraction and cDNA Synthesis

As described previously (Chapter 2).

Quantitative PCR

As described previously (Chapter 2).

Statistical Analysis

For MX1 splice variant expression in cow and ewe PBL, data were analyzed using the MIXED procedures in SAS (Version 9.1; SAS Institute, Cary, NC) for repeated measures. Animal was the experimental unit and fold change of dependent variables was tested against status. Data are presented as least squares mean ± standard error of the mean of relative fold change from day 0 calculated by the ΔΔ critical threshold method (as reviewed by Kubista *et al*. 2006) with ACTB serving as the internal control.

For MX1 splice variant expression in endometrial tissue, data were analyzed using the MIXED procedures in SAS. Animal was the experimental unit and fold change

of dependent variables was tested against status, day and the interaction of status and day. Data are represented as least square means ± standard error of the mean relative fold change from Day 11 cyclic calculated by the ΔΔ critical threshold method with ACTB serving as the internal control.

Results

In ewe endometrium only MX1a mRNA concentrations increased ($P<0.05$) during early pregnancy. MX1a concentrations were elevated as early as Day 13 and peaked at Day 15 being ~17 fold higher compared to Day 11 cyclic (FIG. 1). MX1a expression remained elevated on Days 17 and 19.

Similar to the endometrium, only MX1a was up-regulated by pregnancy in sheep PBLs. Compared to Day 0, MX1a mRNA concentration was increased by pregnancy ~7 fold ($P<0.05$; FIG. 2). When comparing to Day 15 cyclic values to Day 15 pregnant MX1a was ~6 fold higher ($P<0.05$; FIG. 3).

All three MX1 splice variants were detectable in the PBLs of cows, however, their expression did not change in response to pregnancy ($P>0.05$). However, concentrations of both MX1a and MX1b increased ~3 and ~4 fold ($P<0.05$), respectively, in bred non-pregnant cows that received a CIDR on Days 17-19 (CO; FIG. 3.).

MX1c mRNA concentrations did not change ($P>0.1$) during early pregnancy, in any of the experiments but were ~1000 fold higher than MX1a and MX1b in both sheep and cattle (data not shown).

Discussion

Until recently, IFNT was thought to act locally on the uterine endometrium to block luteal regression and to prepare the uterus to support early conceptus growth,

development and attachment (Hansen *et al.* 1999). Work from our lab (Yankey *et al.* 2001) was the first to show that IFNT-stimulated genes (ISGs) also increased in PBL of pregnancy ewes. This novel observation was subsequently confirmed in cattle (Han *et al.* 2006; Gifford *et al.* 2007). We now know that many ISGs are regulated in the endometrium, CL, and PBLs of ruminants during maternal recognition of pregnancy (Ott *et al.* 1998; Johnson *et al*. 2000 & 2001; Yankey *et al*. 2001; Hicks *et al.* 2003; Han *et al*. 2006; Stevenson *et al*. 2006; Gifford *et al.* 2007, 2008;). One of the ISG up-regulated by IFNT is the myxovirus resistant gene, MX1. Ellinwood *et al.* (1998) and Kojima *et al*. (2003) recently described MX1 splice variants in cattle, however their presence in sheep and regulation by IFNT and pregnancy was not examined. Here, we studied MX1 splice variant expression in ovine endometrial cell lines, and determined that three variants identical to those found in cattle were present in LE and SC cell lines derived from the sheep uterus. Our analysis of one of these putative splice variants, MX1c, revealed that it was likely not translated due to an early stop codon in its unique region. In addition, we were not able to detect an immunoreactive protein with similar expression characteristics to MX1c mRNA expression using two-dimensional electrophoresis and Western blotting (Chapter 2). Furthermore, although a cDNA for MX1c was cloned from mRNA isolated from ovine uterine cell lines, we now question whether MX1c represents a mature mRNA. The primers used to amplify MX1c in these studies did not span an exon-exon boundary and thus could recognize both genomic DNA and pre-spliced mRNA. It is unlikely that our results reflect DNA contamination because controls to detect genomic contamination were negative and samples were treated with DNase. Therefore, the simplest explanation of our results is that the MX1c primers are detecting MX1c or a pre-

spliced mRNA for MX1. Future PCR assays using intron specific and MX1c specific (exon-exon) primers will allow us to determine whether pre-spliced mRNA is contributing to the high concentrations of MX1c detected.

We also showed that two of the splice variants, MX1a and MX1b, increased after IFNT treatment (Chapter 2). Here we examined regulation of these splice variants in the endometrium and PBLs during the estrous cycle and early pregnancy. The present study used sequence specific qRT-PCR TaqMan assays to demonstrate the presence of MX1 splice variants, in sheep endometrium and PBLs, as well as in cow PBLs.

In contrast to previous work using oLE and oSC cell lines, only MX1a was detectable in sheep endometrium and PBLs. However, this is in agreement with our results using the oGE cell line and results by Ellinwood *et al*. (1998) that found MX1b (labeled MX1-a) was not present in sheep, which the authors suggested was most likely due to a mutation around splice sites. It is possible that breed differences affect the relative abundance of these splice variants. The ewes sampled for these studies were of the Dorset breed, whereas the ewes from which the cell lines were established were Western Range ewes of primarily Rambouillet breeding (Johnson *et al.* 1999). Assay validation ensured the sensitivity for detecting MX1b (See Appendix A), and this was further supported by the fact that MX1b was detected in cow PBL samples.

Expression of MX1a increased as early as Day 13 in the endometrium of pregnant sheep and remained elevated through Day 19. This was consistent with our previous results using assays that did not differentiate between different MX1 splice variants (Ott *et al.* 1998). We compared MX1a concentrations in the PBLs of sheep to both their own Day 0 value and to their Day 15 cyclic value to account for differences in steroid

concentrations on different days of the estrous cycle. Both analyses showed MX1a mRNA expression was increased on Day 15 of pregnancy. These results are consistent with results from earlier studies that showed MX1 expression increased by Day 15 in PBLs of pregnant ewes (Yankey *et al*. 2001).

In this study, MX1c mRNA was not affected by pregnancy in endometrium or PBLs of sheep. This supports previous results that showed MX1c mRNA was not regulated by IFNT in oGE, oLE, and oSC cell lines (Chapter 2). Similar to sheep, concentrations of MX1c mRNA were not changed by pregnancy or P4 in PBL isolated from Holstein dairy cows.

We were also not able to detect an effect of pregnancy on MX1a and MX1b mRNA concentrations in PBL from dairy cows. While we expected to detect increases in these two splice variants, our recent results (Gifford *et al.* 2007) suggested that MX1 increased only about 2-fold on Day 20 of pregnancy in dairy cows. With the small number of samples analyzed here and the relatively small increase in expression level, it is likely that more cows would need to be sampled to detect differences at Day 19. Interestingly, both MX1a and MX1b were up-regulated in the PBLs of non-pregnant cows that received CIDRs from Day 17 through 19. Work from our lab using probes that did not differentiate between the splice variants of MX1 (Ott *et al.* 1998) showed that MX1 mRNA concentrations were modulated during the estrous cycle in the absence of conceptus-produced IFNT. We hypothesized that P4 influences MX1 expression, because high P4 concentrations coincided with increased MX1 expression in the uterine endometrium and because IFNT could not increase uterine MX1 mRNA in the absence of P4 (Ott *et al.* 1999). Also, putative P4 and estrogen response element half sites were

identified on the ovine MX1 gene promoter enhancer region (Assiri *et al*. 2007). It is likely that the sampling would need to occur on Day 20 or later to optimally detect pregnancy-induced increases in MX1 splice variants. It is important to point out, however, that concentrations of another MX family member, MX2 increased as early as Day 18 in pregnant dairy cows (Gifford *et al.* 2007). Clearly, more animals will need to be sampled and perhaps later sample times utilized to detect pregnancy-induced differences in MX1 splice variants in PBL of cattle.

Since the discovery of an MX protein in the uterus of sheep, it has been hypothesized that MX proteins have a physiological role in the endometrium, unrelated to virus defense (Charleston and Stewart, 1993; Ott *et al*. 1998). Human MXA was shown to interact with, and have characteristics similar to, dynamin (Accola *et al*. 2002). Transient overexpression of MXA disturbed trafficking along the endocytic pathway (Jatiani and Mittal, 2004). Human MXB was also implicated in nuclear import and cell cycle progression (King *et al*. 2004). More recently, ovine MX1 was detected in uterine flushings, and shown to be secreted from an ovine uterine cell line (oGE; Toyokawa *et al*. 2007a). It was further shown that oMX1 was secreted via an 'unconventional' secretory pathway and that siRNA knockdown of MX1 also decreased secretion of another unconventionally secreted protein, ISG15 (Toyokawa *et al.* 2007b). From these studies, the authors concluded that MX1 may have a regulatory role in the pathways of 'unconventional' secretion. Uterine gland secretions are critical for conceptus survival (Gray *et al.* 2006). It is conceivable that isoforms MX1 evolved to have unique functions with one form regulating unconventional secretory pathways, another with antiviral properties, and another modulating cell cycle progression.

In summary, two MX1 splice variants, MX1a and MX1c were detected in sheep endometrium and PBLs. However, only MX1a was increased in the endometrium and PBLs of pregnant sheep. In the PBLs of cows, MX1a and MX1b were not up-regulated by pregnancy at Day 19, however in P4 supplemented non-pregnant cows there was a modest increase in both splice variants. Similar to sheep, MX1c was not regulated in cow PBLs by pregnancy or by P4. Further studies should examine the effects of viral challenge on MX1 splice variant expression and the possible functions of these variants. Perhaps the most intriguing observation here is that the MX1 gene promoter appears to be constitutively active leading to sustained high levels of the MX1c splice variant that is apparently not translatable into a functional protein. However, we hypothesize that, in response to pregnancy and IFNT splicing could be altered to favor accumulation of the MX1a and, in some cases, MX1b variants. The biological regulation of this alternative splicing phenomenon will be the focus of future studies.

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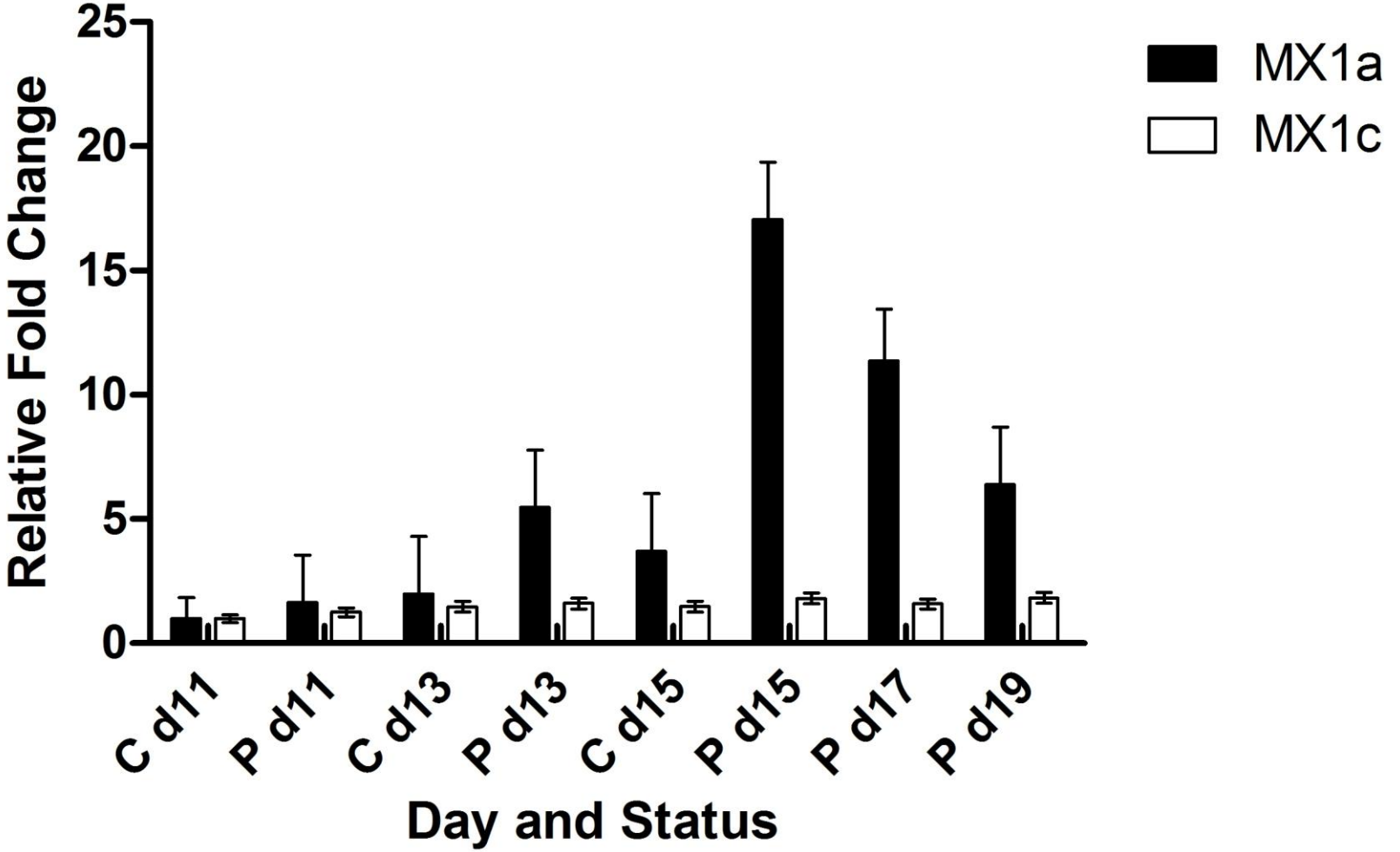
Relative Fold Change
25
20
15
10
5
0
MX1a
MX1c
C d11
P d11
C d13
P d13
C d15
P d15
P d17
P d19
Day and Status

FIG. 1. Fold change in MX1a (closed bars) and MX1c mRNA (open bars) concentrations in ovine endometrium for each splice variant compared to its own Day 11 cyclic value; error bars represent SEM. MX1a increased on day 13 of pregnancy, peaked approximately 17-fold on day 15 of gestation and remained elevated on days 17 and 19 ($P < 0.05$).

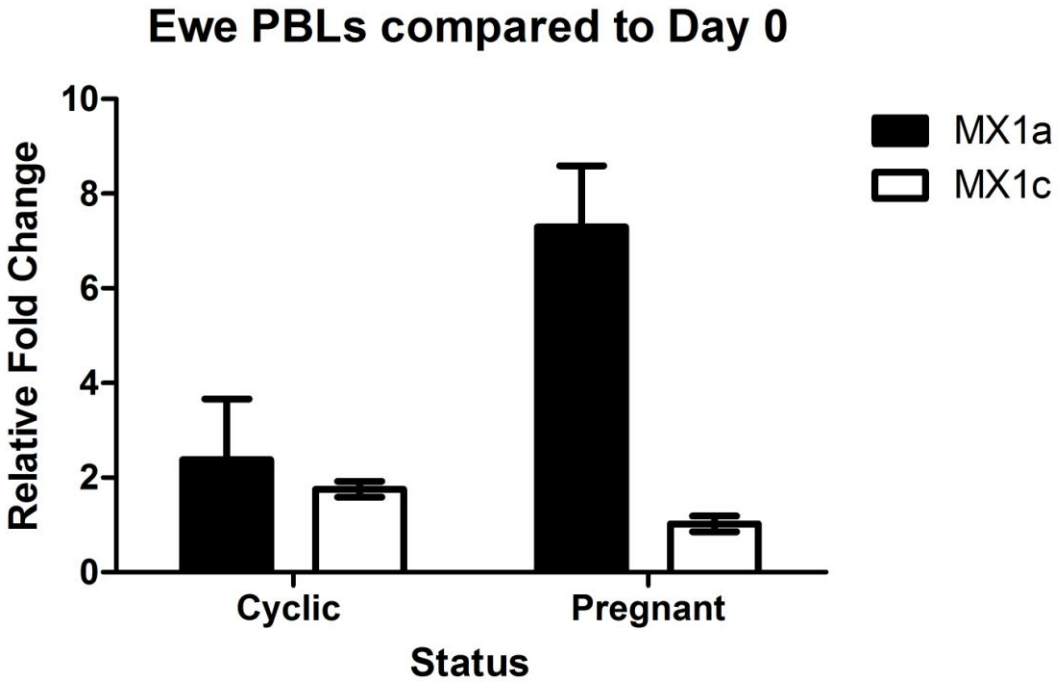

FIG. 2. Fold change in MX1a (closed bars), MX1c mRNA (open bars) concentrations in ovine peripheral blood leukocytes relative to their Day 0 value; error bars represent SEM. The MX1a splice variant increased approximately 7-fold on Day 15 of gestation. ($n = 13$; $P < 0.05$). MX1b was not detectable in these samples.

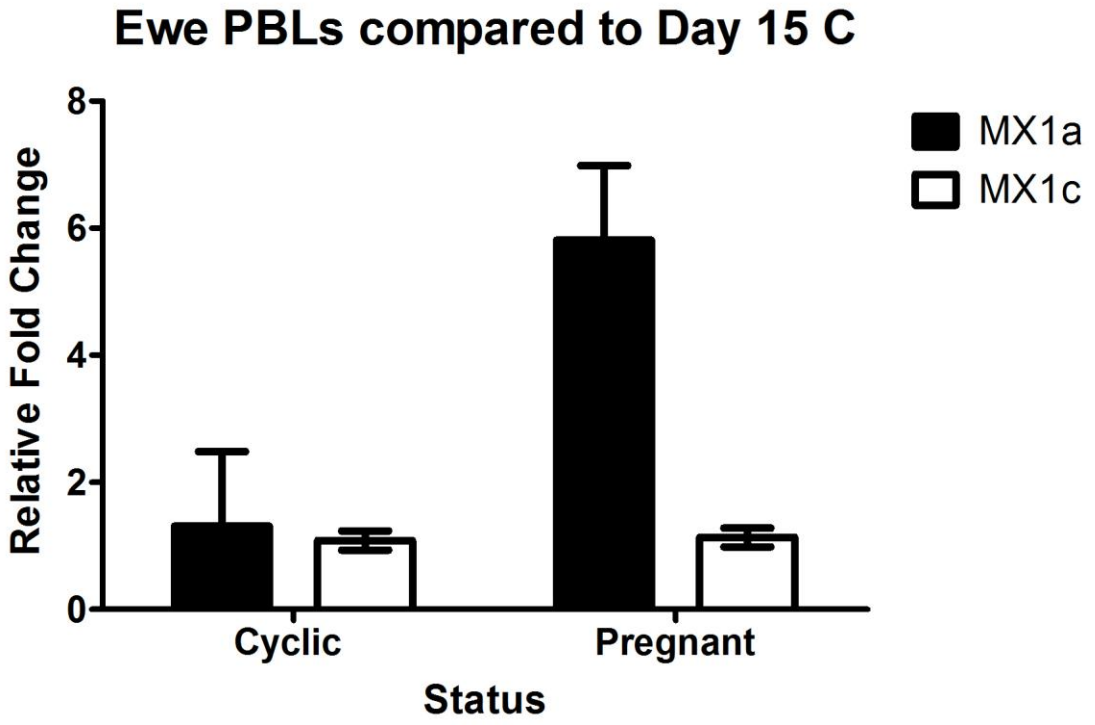

FIG. 3. Fold change in MX1a (closed bars), MX1c mRNA (open bars) concentrations in ovine peripheral blood leukocytes relative to their Day 15 value; error bars represent SEM. MX1a increased approximately 6-fold on Day 15 of gestation. ($n = 13$; $P < 0.05$). MX1b was not detectable in these samples.

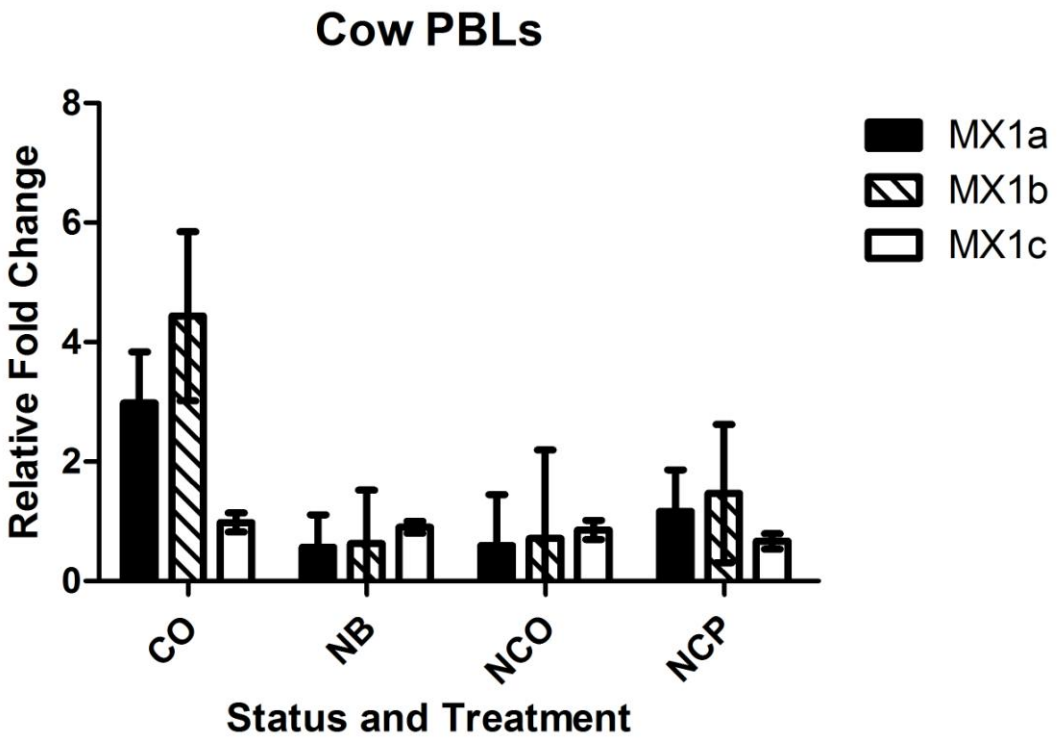

FIG. 4. Fold change in MX1a (open bars), MX1b (striped bars) and MX1c mRNA (closed bars) concentrations in bovine peripheral blood leukocytes relative to their Day 0 value; error bars represent SEM. MX1a and MX1b splice variants increased approximately 3- and 4-fold, respectively on Day 19 in CO cows (CO=CIDR Open; NB=Not Bred; NCO=No CIDR Open; NCP=No CIDR Pregnant; $P < 0.05$).

Chapter 4

Summary and general discussion

The dairy industry has seen a dramatic decline in reproductive efficiency over the last 50 years (Lucy, 2001). A primary cause of low reproductive efficiency is poor heat detection. To address challenges associated with heat detection, especially on large dairies, there has been development and implementation of numerous heat detection aids (tail chalk, pedometers, etc.) and synchronization programs (Ovsynch, etc.) that help dairy producers identify cows in estrus or synchronize ovulation. Some producers are opting to greatly reduce or eliminate the need for heat detection by combining estrous synchronization with timed artificial insemination protocols. However, even with all these new tools, reproductive efficiency remains low. Research presented in this thesis address another contributor to infertility; early embryo loss. It has been estimated that 75-80% of all embryonic losses are early (before Day 30) and that the majority of early embryonic losses, roughly 40%, occur between Days 8 and 17 of pregnancy (Thatcher *et al*. 1990 & 1994). Presently there are few tools available to address this problem. The best approach is to identify these losses as early as possible so that bred, non-pregnant, cows can be resynchronized and reinseminated in a timely manner. This can be accomplished by implementing better heat detection following insemination, or by utilizing early pregnancy diagnosis with trans-rectal ultrasonography or BioPRYN® (BioTracking, Moscow, Idaho) blood pregnancy test. Clearly more research is needed on the signaling between the developing conceptus and the dam that is responsible for establishment and maintenance of pregnancy.

Research from our laboratory focuses on early embryonic losses that we hypothesize occur from inadequate communication between the conceptus and the maternal system. In particular, we study interferon stimulated genes (ISGs) and their

importance in preparing the maternal system for establishing and maintaining pregnancy. This is because the first essential signal from the conceptus to the maternal endometrium is the type I IFN, IFNT. Interferon tau regulated expression of a large number of maternal genes in the uterus and peripheral tissues. One of these ISGs is the myxovirus resistant gene, MX1. The function of MX1 is well characterized in the antiviral system, however, we and others have postulated that MX1 has functions outside the immune system, in normal cellular processes (Charleston and Stewart, 1993; Hicks *et al*. 2003; Gifford *et al*. 2007; Toyokawa *et al.* 2007a; 2007b). Previous unpublished work from our lab suggested that uterine cells express splice variants of the MX1 mRNA following treatment with IFNT. Using quantitative RT-PCR we consistently detected three amplicons. After ruling out contamination and non-specific binding of primers, these amplicons were cloned and sequences revealed the amplicons to be splice variants of MX1 (T.L. Ott and K.A. Williams, unpublished). Evidence that the MX1 gene may yield several mRNA variants was further strengthened by work in cattle that identified four MX1 variants (Ellinwood *et al.* 1998; Kojima *et al*. 2003). Three of the 4 were identical to the amplicons cloned from our sheep cell lines. Because proteins possessing different functions can arise from alternative splicing of mRNA we wanted to examine the regulation and expression of MX1 splice variants further.

Previous work from our labs quantifying steady-state concentrations of MX1 used primers that did not differentiate between MXI splice variants (Ott *et al.* 1998; Yankey *et al*. 2001; Hicks *et al.* 2003; Gifford et al. 2007). The first objective of this work was to develop sensitive real time assays to quantify each splice variant. Therefore, we designed TaqMan® assays specific for each splice variant. The probes were designed such that

they recognize the unique regions of each splice variant. Stringent validation was done to ensure the assays only recognize specific splice variants and did so at similar efficiencies (See appendix A). Using these assays we were able to perform quantification of MX1 isoforms in ovine luminal epithelial (oLE), glandular epithelial (oGE), and stromal (oSC) cell lines. In oLE and oSC cells, we were able to detect each of the three splice variants, however only MX1a and MX1c were detectable in oGE cell. Because these cell lines were developed from two different sheep (G.A. Johnson, personal communication), results suggested the possibility that there were animal to animal differences in regulation of MX1 mRNA splicing or that there were different alleles of the MX1 gene. Importantly we found that only MX1a and MX1b were up-regulated when treated with IFNT, while MX1c was not affected. Most surprisingly, we discovered that concentrations of MX1c were ~1000 fold higher than either of the other splice variants and were similar to beta-actin (ACTB). However, these results are not conclusive due to an error that occurred when designing the assay for MX1c that was unnoticed until late in the project. The primers for MX1c were contained within one exon leading to the possibility that the assay could not only detect MX1c, but also genomic DNA contamination and primary MX1 mRNA transcript. We do not believe that genomic contamination is responsible for the results because we include a negative control (sample without reverse transcriptase) also we treat samples with DNase to degrade DNA contaminants, these controls were negative in all assays. To determine whether the MX1c variant was actually a pre-spliced message, we also used primers from MX1a and MX1b assays to detect MX1c. When these mismatched assays were tested against an MX1c cDNA cloned into a plasmid, each assay amplified at equal critical threshold

values. This confirmed that the mismatched primer assay could detect spliced MX1c in samples. However, when we attempted to amplify MX1c out of cDNA samples the mismatched assays were unable to amplify a product. There are two likely explanations for this: 1) Mismatched assays were unable to amplify MX1c in the TaqMan assays because they were not specifically designed to work together; or. 2) The primers used in the original MX1c assay recognized pre-spliced mRNA. To test which of these explanations was correct we designed three primer sets. One primer set specifically measures the MX1c splice variant and contain the unique exon-exon boundary. The second set of primers recognizes sequences within intron B and amplifies primary MX1 mRNA and undegraded spliced introns. Finally, the third set recognizes both intronic and exonic sequences, and is also an assay for primary MX1 mRNA transcript. None of the assays were able to amplify a product. A plasmid containing full length MX1c was used as a positive control and proved that the exon-exon primer sets worked; however, there was no positive control for the assays that would recognize primary MX1 mRNA. Previously we were able to clone a full length MX1c cDNA, so we cannot conclude that the MX1c variant does not exist. However, the unique nucleotide sequence identified in MX1c by us and Kojima *et al*. (2003) was not contained in the MX1 genomic sequence published by Gerardin *et al*. (2004).

Computer assisted translation of the MX1c splice variant revealed that it may not be translated due to three in-frame premature stop codons (TGA; See Table 1-Chapter 2) in its unique region. Although, translation could still occur from a downstream start codon if internal ribosome entry, linear but leaky scanning or non-linear scanning occurred (Short and Pfarr, 2002). Two secondary start codons were found downstream of

the final stop codon and the second of the two had a consensus Kozak sequence (Gs at -3 and +4), making initiation from that site more likely. If translation were to occur from this ATG, we predicted that we would detect an abundant immunoreactive MX1 protein that was not induced by IFNT. We were unable to use one-dimensional SDS-PAGE and Western blotting because predicted molecular weights were all ~75 kDa. Instead, we subjected oGE cell lysates to two-dimensional SDS-PAGE and Western blotting with three antibodies specific to MX1 that were developed in our lab (Hicks *et al*. 2003; unpublished data K.E. Racicot). Results from these Western blots provided some answers, but more questions remain. Not surprisingly, we did not detect a protein with expression similar to MX1c (high basal levels that were unchanged by IFNT). However, we did detect several other potential splice variants that were up-regulated by IFNT. An amino terminal polyclonal antibody recognized several proteins with a molecular weight of ~75 kDa and isoelectric points from 4 to 7. The carboxy terminal polyclonal and monoclonal antibodies recognized distinct proteins at ~75 kDa along with several smaller molecular weight proteins (~25-75 kDa). There are a number of post-translational modifications that can result in differences in molecular weights and isoelectric points, including proteolytic cleavage and glycosylation. Future experiments should examine these isoforms to determine if they resulted from alternative mRNA splicing or from post-translational modifications of the known MX1 protein. One way this could be done is through mass spectrometry.

From these results we hypothesized that the MX1 promoter is constitutively active, producing high amounts of MX1 transcripts (in the form of MX1c or primary mRNA transcript). Interferon tau treatment, instead of or in addition to activating the

promoter, may regulate splicing factors to alternatively splice MX1 into a functional transcript. This may also explain differences in MX1 expression in the endometrium when compared to other ISGs. In the luminal and superficial glandular epithelium IRF2 is present and blocks transcription of ISGs by binding to and blocking interferon stimulated response elements (Spencer *et al.* 2007). However, unlike many other ISGs, MX1 is highly expressed in these cell types (Ott *et al.* 1998; Johnson *et al.* 2002).

In the second chapter we examined expression of MX1 splice variants in endometrium and PBLs of pregnant and cyclic ewes, and PBLs of pregnant and bred, non-pregnant cows. Interestingly, we were unable to detect MX1b in any of the sheep sampled. This was not entirely surprising based on the *in vitro* results where we were also unable to detect MX1b in one of the three cell lines (oGE). It is possible that alternative splicing of MX1b is specific to certain species, breeds, animals, cell types or developmental stages. However, we anticipated that we would detect MX1b in endometrial tissue. The ewes sampled for these studies were of the Dorset breed, whereas the ewes from which the cell lines were established were Western Range ewes of primarily Rambioullet breeding (Johnson *et al.* 1999). Kojima *et al.* (2003) identified each splice variant in each of the tissues examined (skeletal muscle, heart, brain, lung, liver, spleen, kidney, ileum, ovary, uterus, chorio-allantois, thymus, bone marrow, lymph node, and PBLs) and in eight different breeds of cattle, but they did not discuss relative abundance or regulation of the variants. Although a powerful and convenient way to increase our understanding of uterine physiology, using immortalized cell lines can give results inconsistent with *in vivo* results and may account for differences in sheep (Bazer and Salmonsen, 2008).

Expression of MX1a in the endometrium and PBLs of sheep were consistent with past research that showed total MX1 mRNA was increased in the endometrium of pregnant ewes on Days 13 through 19 (Ott *et al*. 1998), and in the PBLs of pregnant ewes on Days 15 through 30 (Yankey *et al*. 2001a), although the probes used in those studies did not differentiate between the isoforms. In both the endometrium and the PBLs, MX1c mRNA concentration was not changed, but consistent with *in vitro* results were present in much higher concentrations than MX1a. This was also consistent with previous results that showed MX1c was not regulated by IFNT in immortalized ovine cell lines.

Contrary to results from sheep, all three MX1 splice variants were detected in each of the cows sampled. However, the PBLs of pregnant cows at Day 19 after insemination showed no change in expression of any of the three splice variants. Recent research on MX1 expression in the PBLs of pregnant cows showed that MX1 increased by only ~2 fold on Day 20 and no change was detected on Day 18 of pregnancy (Gifford *et al*. 2007). However, using more animals (n=21), another study was able to detect increases in bred dairy cows on Day 18 and 20 (Yankey, 2001b). Another study showed that on Day 17 of pregnancy expression of MX1 was undetectable in beef heifers even though a large number of animals were sampled (n=70; Yankey, 2001b). It is likely that more animals would need to be sampled and perhaps later sample times utilized to detect pregnancy-induced differences in MX1 splice variants in the PBL of cattle. Interestingly, both MX1a and MX1b were increased in the PBLs of non-pregnant cows that received CIDRs from Day 17 through 19. Previously, we showed that MX1 expression was modulated throughout the cycle and was at highest concentrations when P4 was highest

and it was hypothesized that P4 also influenced MX1 expression (Ott *et al.* 1998). Furthermore, IFNT was not able to increase uterine MX1 expression in ovariectomized ewes unless the ewes were treated with estrogen, P4 or estrogen plus P4 (Ott *et al.* 1999). We further showed that several P4 response element half sites were located on the promoter/enhancer gene of MX1 (Assiri *et al.* 2007)

The presented findings have enhanced and complicated our understanding of signaling between the conceptus and dam by describing changes in gene expression induced by IFNT. Here we describe regulation of three MX1 splice variants by IFNT and early pregnancy. This knowledge is important to understanding the function of ISGs during early pregnancy and possibly combating early embryonic loss in ruminants. Understanding the biochemical communication between the conceptus and the maternal system is important to improving fertility.

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Appendix A:

Validation of qRT-PCR TaqMan assays for MX1 splice variants

MX1 splice variants were first identified while quantifying expression of MX1 in multiple ovine cell lines using a SYBR green assay. The product from the glandular epithelial (oGE) cells consistently had a lower melting temperature than luminal epithelial (oLE) and stromal (oSC) cells (FIG. 1). Multiple peaks indicate unique products were amplified. We cloned and sequenced the products as described in Chapter 2 and designed three TaqMan assays with probes that bound the specific region of each splice variant.

Each primer probe set was validated using a 6 log dilution curve. Purified MX1 isoforms had previously been generated and cloned into PCRII TOPO vector (Invitrogen; unpublished K.A. Williams). To determine if the cDNA synthesis protocol had an effect on the assays, a random cDNA was diluted 1:20 with nuclease free water and 1 µL of purified MX1 variants in plasmid. This was then diluted 1:10 six times, and duplicates were assayed for each dilution. Standard Curve option on the Applied Biosystems 7500 Fast machine was used to calculate R^2, intercept and slope. Assay efficiency was then calculated as $10^{(-1/slope)-1}$. The efficiency of each primer set ranged between 90% and 105%. Only Ct values that fell within the range of the 6 log dilution curve were accepted for sample data analysis. Assays were validated for each isoform and beta-actin each time the source of the sample was changed. To validate that each assay only recognized the unique splice variant they were designed for, purified MX1 variants in plasmid were diluted 1:10 six times and PCR was performed with assays designed for other variants. All showed no increase in fluorescence (data not shown).

One absolute standard curve was also completed for each assay. Quantity of MX1 variants in plasmid assessed by measuring absorbance at 260 and 280 nm and diluted to 5 ng/uL. One µL of purified MX1 variants in plasmid was then diluted 1:10 six times, and duplicates were assayed for each dilution. Standard Curve option on the Applied Biosystems 7500 Fast machine was used to calculate R^2, intercept and slope. Assay efficiency was then calculated as $10^{(-1/slope)-1}$. Figures 2-4 show results from these assays.

Late in the project it was realized that the assay for MX1c was designed within one exon. When possible we design PCR assays that contain exon-exon boundaries to eliminate the possibility of amplifying contaminating genomic DNA. Prior to cDNA synthesis all samples are treated with DNase to degrade any genomic DNA contamination. During cDNA synthesis a negative control (no reverse transcriptase) is also included and showed no amplification. However, because, MX1c was at such high concentrations we wanted to further confirm the validity of our assay. To do this we performed PCR with the MX1c probe and mismatched MX1c forward primers with MX1a and MX1b reverse primers, and MX1c reverse with MX1a and MX1b forward primers. When these mismatched assays were tested against full length MX1c in plasmid all amplified the product with similar efficiency (Ct= ~18, data not shown). However, when tested against cDNA product, the mismatched assays were unable to amplify MX1c. There are two possibilities for this result: 1) Interfering substances present in cDNA synthesis could obstruct the mismatched assays or 2) pre-spliced MX1 mRNA is present. To determine which is correct, we designed new PCR assays that will amplify

MX1c and contain an exon-exon boundary and also primers to amplify intronic and intron-exon regions.

FIG. 1. Semi-quantitative PCR melting curves for MX1 amplicons from three cell lines oGE (78°C), oLE and oSC (79°C). Amplicons from oGE cells had consistently low melting temperatures compared to oLE and oSC amplicons, suggesting differences in nucleotide composition between amplicons

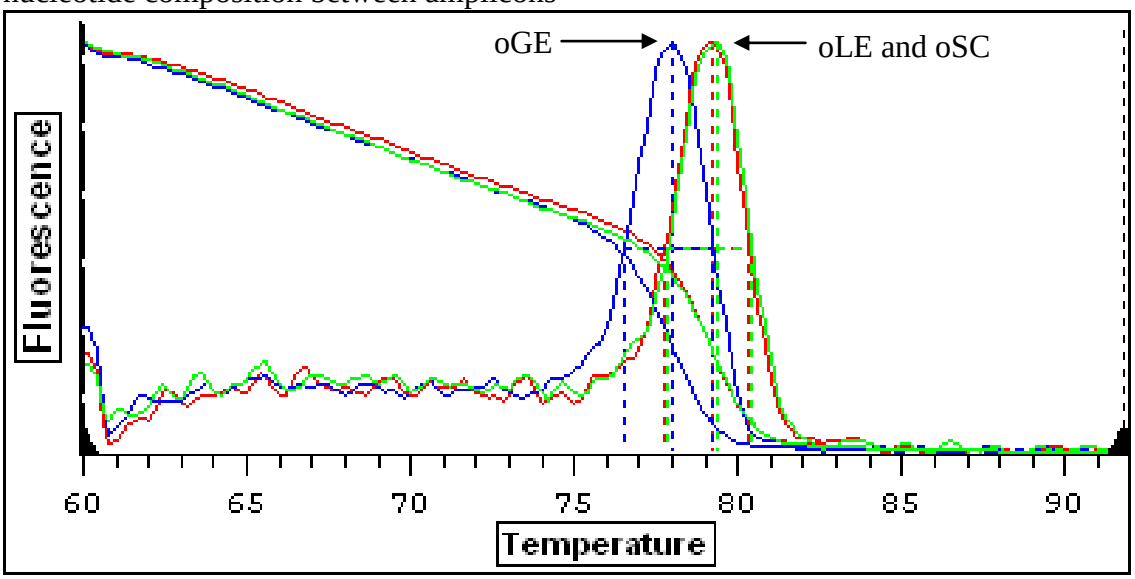


FIG. 2. Absolute standard curve for MX1a TaqMan PCR assay. Efficiency=1.086

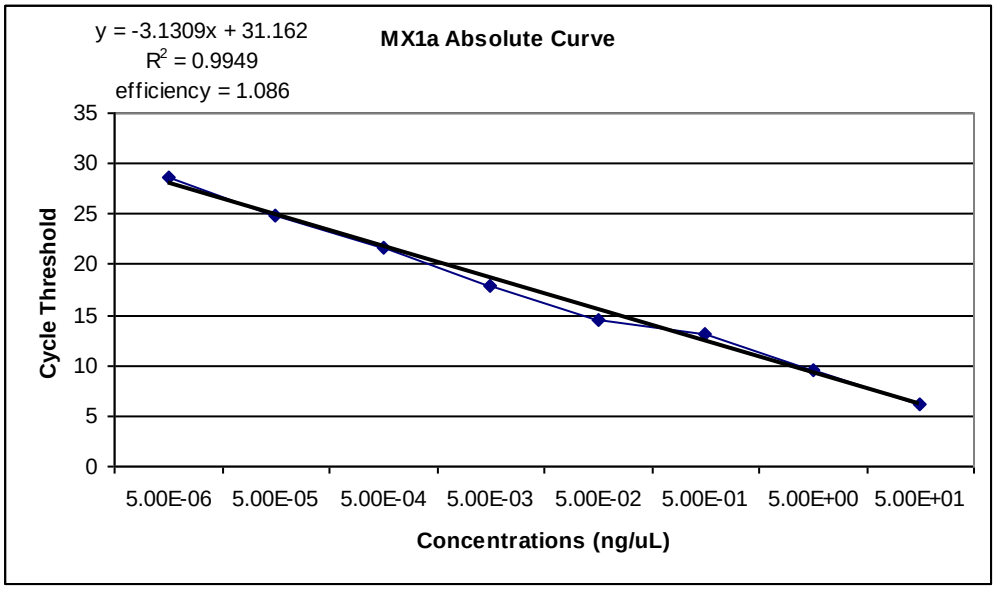


FIG. 3. Absolute standard curve for MX1b TaqMan PCR assay. Efficiency=1.008

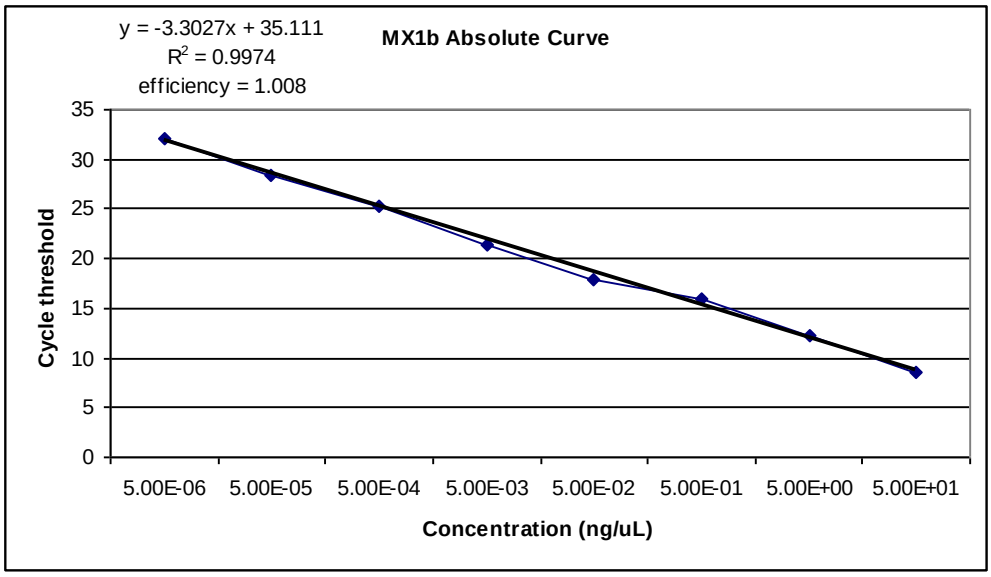
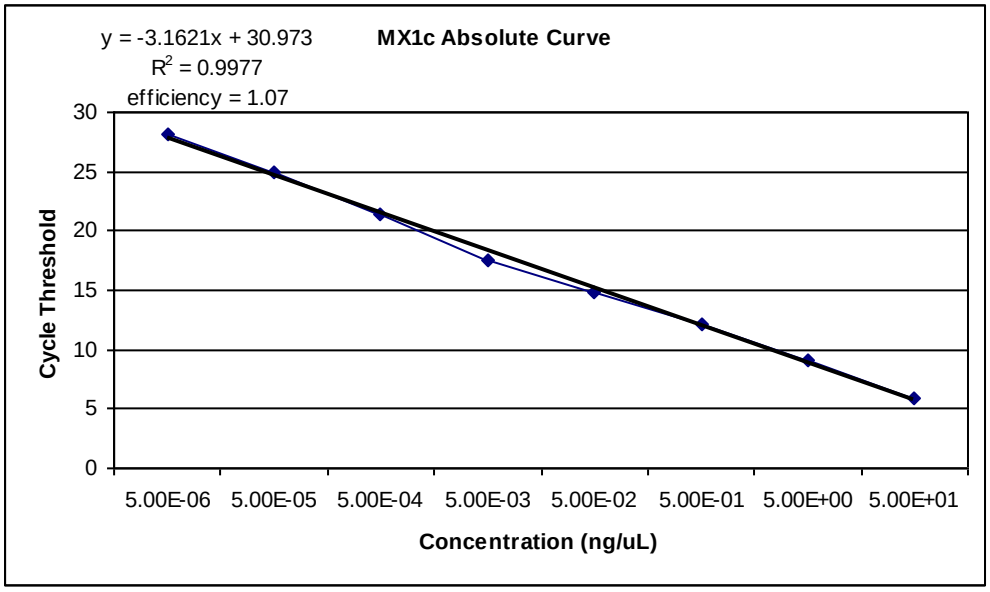


FIG. 4. Absolute standard curve for MX1c TaqMan PCR assay. Efficiency=1.07



Appendix B:

MX1 mRNA expression in pigs challenged with swine influenza H1N1 virus

Dr. Lester Griel (Department of Veterinary and Biomedical Sciences) performed a study to examine strategies to reduce aerosol spread of viral infections. Three separate groups of 4 pigs each were inoculated intranasally with 5 x 10 pfu of swine influenza H1N1 virus. Although the study was not designed for gene expression analysis, we assayed for steady-state concentrations of MX1 (only one MX1 is known in pigs) mRNA in the peripheral blood leukocytes of pigs challenged with swine influenza H1N1 virus at 0, 24, 48, and 96 hours after inoculation. Semi-quantitative PCR assays were optimized and validated as described previously (Gifford *et al.* 2006 & 2007). The primers used are listed in Table 1. The following times and temperatures were used for amplification: 95ºC for 5 min, and 40 cycles of 95ºC for 30 s, 59ºC for 30 s, and 72ºC for 15 s. Results were highly variable and there was no overall change in MX1 expression from 0 h during any of the time periods (Figure 1). Generally, however, pigs that did show increased MX1 levels also showed signs of infection when the necropsies were performed. None of the pigs showed outward symptoms of influenza infection during the study. It is likely that the intranasal inoculations were not effective on all pigs and may be the cause for high variability and no changes.

Table 1. Gene, primer orientation, primer sequence (5' to 3'), concentration, and amplicon size for primer sets used in quantitative PCR assays.

Gene	Primer	Sequence (5'-3')	Conc. (nM)	Amplicon Size (bp)
MX1	For.	TTGGGCTTTCAGATGCTTCG	300	
	Rev.	CGATGTCGTATGGCTGATTGC	300	191
18S	For.	AAACGGCTACCACATCCAAG	350	
	Rev.	CGCTCCCAAGATCCAACTA	350	231

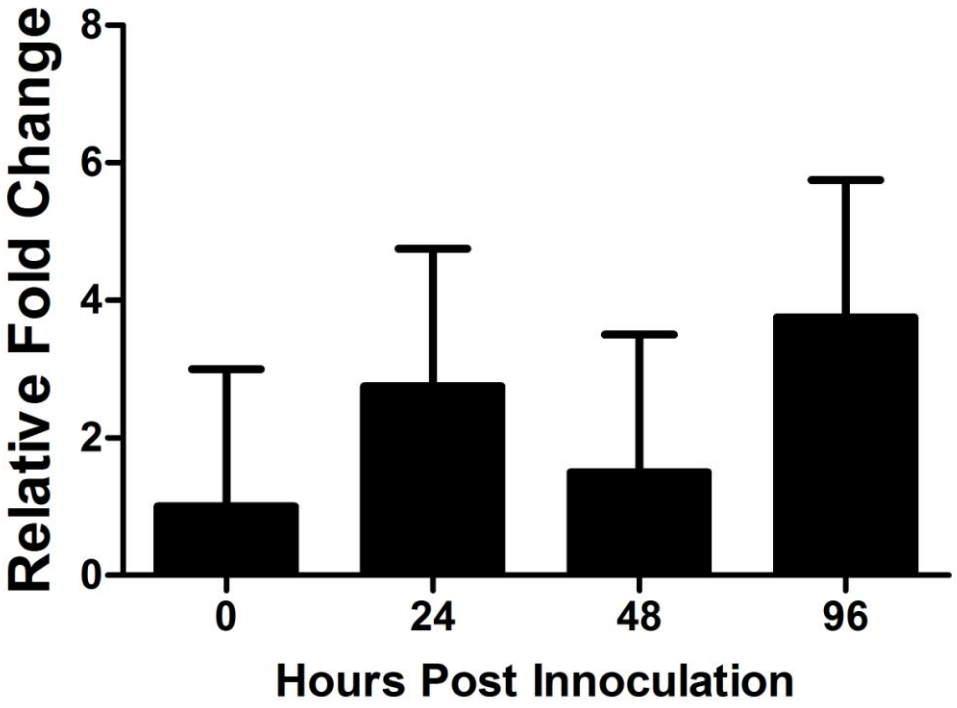


FIG. 1. Steady-state mRNA concentrations for MX1 in porcine peripheral blood leukocytes. Presented as relative fold change from time 0; error bars represent SEM. There was no effect of time after virus inoculation on MX1 mRNA concentrations (P>0.10)