EFFECT OF THE MYXOVIRUS RESISTANCE PROTEIN, MX1, ON SECRETION BY A UTERINE CELL LINE.

A Thesis in
Animal Science
by
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ABSTRACT

In cattle, substantial embryonic loss occurs during the peri-implantation period resulting in losses approaching half a billion dollars annually. During the peri-implantation period the conceptus must signal to the maternal uterus to maintain pregnancy. In ruminants, this signal is interferon tau (IFNT), which blocks the endometrial luteolytic mechanism, allowing the corpus luteum (CL) to continue producing progesterone and support pregnancy. Interferon-stimulated genes (ISGs) are upregulated by IFNT during this period and are thought to play crucial roles in supporting conceptus growth, differentiation, and embryo implantation. Two of these ISGs encode the myxovirus resistance proteins MX1 and MX2. Recently, MX1 was demonstrated to be a secreted protein that apparently regulated secretion of another protein, interferon stimulated gene-15 (ISG15). Interestingly, both of these proteins are secreted via one of the relatively uncharacterized “unconventional” secretory pathways leading to the hypothesis that MX1 is a regulator of unconventional secretion. This would be critical during early pregnancy because the conceptus is entirely dependent upon endometrial secretions for survival prior to attachment and placentation.

Methods for the quantification of the MX1 protein are only semi-quantitative, consisting of protein immunoblotting/western blotting, which can be quite variable from blot to blot. Therefore, the first objective of these studies was to develop a Meso Scale Discovery (MSD) assay for ovine MX1 (oMX1). This assay was validated by detecting known amounts of recombinant (r) oMX1 in different diluents over the range of 2.4-10,000 ng/mL and in bovine plasma over the range of 2.4-40,000 ng/mL.

Once this assay was developed and successfully measuring MX1 protein, we began the main objective of the present studies, which was to determine the effects of MX1 on secretion by
uterine epithelial cells using a combination of inhibition and overexpression assays. For the inhibition assay, morpholino antisense oligonucleotides were used to induce transient “knock-down” of the MX1 protein in a uterine epithelial cell line, oGE. The MSD assay was then used to quantify the inhibition of MX1 in oGE cell lysates. The assay quantified the MX1 reduction in lysates to be approximately 52% ± 52 ng of MX1/mL. Potentially regulated proteins included ISG15 and galectin-1 (LGALS1), which were both reduced with the inhibition of MX1. Interestingly, glalectin-15 (LGALS15) increased in secretions with the inhibition of MX1. These three proteins are all unconventionally secreted proteins. The secretion of the conventionally secreted protein, cathepsin L (CSTL), showed increased secretion of the 5-kDa active form/subunit with the inhibition of MX1 as well.

For the overexpression assay, an MX1 cDNA was cloned into a pCMV-Myc mammalian expression vector, which was used to overexpress the MX1 protein in oGE cells. Treatment with the overexpression plasmid resulted in robust overexpression of the MX1 protein in both lysates and secretions from these cells, but there was ultimately no difference in the unconventionally or conventionally secreted proteins examined.

As a second objective, we examined cellular proteins whose patterns of secretion change when MX1 is altered using 2D SDS PAGE and mass spectrometry. Samples from the overexpression and inhibition assays previously described were utilized in this study. In the MX1 inhibitions gels, 8 proteins were potentially identified that were inhibited when MX1 was inhibited (WD repeat-containing protein 38 (WDR38), serpin peptidase inhibitor (SERPIN), alpha-2-HS-glycoprotein (AHSG), triosephosphate isomerase-1(TPI1), peroxiredoxin-6 (GPX6), malate dehydrogenase 1B (MDH1B), ankyrin repeat and IBR domain-containing 1(ANKIB1), lim domain 7 (LMO7)), and 2 proteins were potentially identified in spots that increased when
MX1 was inhibited (cystatin E/M (CST6), toll-like receptor 1 (TLR1)). In the overexpression gels, 4 proteins were potentially identified in spots that were increased as well (SERPIN, glial fibrillary acidic protein (GFAP), lactate dehydrogenase B (LDHB), MDH1B). The quality score and the percent coverage (percent of matched peptides between the potential proteins and the peptides recovered from the chosen spot) varied for each of the potentially identified proteins.

The experiments in this thesis further define the role of MX1 in secretion and provide evidence that does not support the hypothesis that MX1 is a broad regulator of unconventional secretion. However, because some unconventionally secreted proteins but no conventionally secreted proteins were reduced when MX1 was inhibited, our hypothesis is partially supported and MX1 may regulate a specific pathway in unconventional secretion. Overall, the results presented here point to a more complex role of MX1 with some proteins of each class being affected. The free-floating preattached embryo is completely dependent upon the endometrial secretory milieu for survival prior to attachment and placentation. If MX1 has a role in regulating endometrial secretory processes during early pregnancy, it would therefore have a direct role in embryo survival. From this work, it will be important to; 1) determine if MX1 regulates secretion in any other tissues; 2) determine what specific proteins are regulated by MX1 and; 3) gather more evidence about the complex role MX1 possesses in regulating secretion. Understanding the factors associated with conceptus survival, including how the conceptus regulates endometrial secretion, could lead to techniques to reduce early embryonic mortality. Importantly, MX1 may play a direct role in regulating these functions by regulating endometrial secretory processes, necessary for embryo survival until placentation.
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Chapter 1

Review of the literature

Introduction

In cattle, substantial embryonic loss occurs during the peri-implantation period (25-40%), resulting in losses approaching half a billion dollars annually (Sreenan and Diskin, 1986; Humblot, 2001; Moore et al., 2005). The peri-implantation period is the time during early pregnancy that, regardless of species, a biochemical dialogue between the developing conceptus (embryo and associated membranes) and the maternal uterus must be established. The losses during the peri-implantation period are thought to occur due to inadequate conceptus signaling, failure of the uterus to respond appropriately to conceptus signaling or both (Bazer et al., 2011). To improve reproductive efficiency, it is crucial to have a better understanding of the interaction between the conceptus and its uterine environment during this period of high embryonic loss. This understanding should lead to the development of strategies to reduce these losses and maximize reproductive efficiency and profitability in production animal agriculture. In viviparous mammals, the conceptus begins this biochemical dialogue with the uterine endometrium when it enters the uterus and spends time “free-floating” in the uterine lumen prior to firm attachment and placentation. If this communication does not commence the corpus luteum (CL) will regress and the pregnancy will be lost. In domestic ruminants, the conceptus produces interferon tau (IFNT), an antiluteolysin that acts on the endometrium to alter the luteolytic signal and maintain the CL and progesterone production (Barros et al., 1991; Ott et al., 1992; Bazer et al., 1997; Spencer et al., 1999; Mann and Lanning, 2001; Binelli et al., 2001; Spencer and Bazer, 2002). The conceptus also alters uterine secretory function and this is
imperative because the conceptus is entirely dependent upon the endometrial secretory milieu for survival prior to attachment and placentation (Gray et al., 2006). It is established in ewes in which uterine gland development is blocked, that ovulation, fertilization, and conceptus growth to blastocyst hatching occur unimpaired, however, the blastocysts fail to elongate (Gray et al., 2002). This is postulated to be due to inadequate or inappropriate secretion by the uterus that lacks uterine glands. Although we now know that pregnancy and IFNT both alter expression of a large number of endometrial secretory proteins, we know very little about the mechanism regulating secretion by the uterine endometrium (Vallet et al., 1991; Mirando et al., 1991; Ott et al., 1998; Spencer et al., 1998; Hansen et al., 1999; Johnson et al., 1999a; 1999b; 1999d; 2000; 2001; Stewart et al., 2001a; 2002; Gray et al., 2002; Choi et al., 2003; Kim et al., 2003; Gray et al., 2004; Song et al., 2005; Gray et al., 2006; Song et al., 2006b; 2007; Bazer et al., 2010). Any defects in secretory processes have the potential to affect a large number of secretory components, therefore this requires a better understanding of the processes of endometrial secretion that are necessary to ensure survival of the conceptus. We now know that the ruminant conceptus alters synthesis and secretion of many endometrial proteins, but the mechanisms by which the conceptus regulates endometrial secretion remain poorly described. Approaches for reducing the incidence of early embryonic mortality in livestock, humans, and even endangered wildlife species could arise from understanding the basic mechanisms associated with conceptus survival during the critical period of maternal recognition of pregnancy.

The maternal recognition of pregnancy signal in ruminants, IFNT, not only acts as an antiluteolysin, but also upregulates a number of IFN-stimulated genes (ISG) in the endometrium (Spencer and Bazer, 1996). Two of these genes encode the myxovirus resistance proteins MX1 and MX2 (Ott et al., 1998; Assiri et al., 2007). Through studying the temporal and spatial
expression, regulation, and function of the MX1 protein, there is evidence that it may play a role in regulating the process of uterine secretion. The MX1 proteins are one of the earliest and most strongly upregulated of the ISGs in the endometrium, are expressed in the human, rodent, and mare uteri, and are regulated in the ewe, cow and sow uteri during early pregnancy (Chang et al., 1990; Ott et al., 1998; Hicks et al., 2003; Kim et al., 2003; Ozaki et al., 2005; Chen et al., 2007). The MX1 protein is also the only classical interferon stimulated gene known to be expressed in the luminal and superficial glandular epithelium during early pregnancy. Therefore we hypothesize that MX1 is a mediator of unconventional secretory processes in endometrial epithelial cells (Johnson et al., 2002; Kim et al., 2003; Toyokawa et al., 2007b). Understanding key mechanisms characteristic of successful reproduction in both humans and animals will lead to the knowledge to enhance both reproductive health and fertility (Bazer et al., 2011). Therefore, the role of MX1 in unconventional secretion by uterine epithelial cells is the focus of the research presented in the following chapters.

The estrous cycle

Ruminants undergo estrous cycles that are characterized by “heat” or short periods of sexual receptivity termed estrus. Ewes and cows have similar, although not identical, physiology during the estrous cycle. In general, there is somewhat more information about sheep and throughout this section, information will be presented for both these species. The ewe has an average estrous cycle of 17 days, with a range from 13 to 19 days, and cattle have an average estrous cycle of 22-23 days, with a range from 17 to 24 days. Cattle are classified as polyestrus because their cycles repeat throughout the year, while sheep are classified as seasonally polyestrus, exhibiting recurring estrous cycles in the fall and winter months followed by variable
periods of anestrus in the late spring and early summer months in the northern hemisphere. As
day length begins to decrease in the late summer and early fall, cyclicity in sheep is initiated and
will discontinue again in the spring when daylight hours increase. Sheep reach puberty between
4 and 14 months of age, with an average of 7 months, while heifers reach puberty much later,
between 9 and 24 months with an average of 11 months, which is breed dependent.

The estrous cycle consists of two major phases based on the dominant structure apparent
on the ovary. The phases are the follicular phase, which is the period from corpus luteum (CL)
regression to ovulation, and the luteal phase, which is the period from ovulation to luteal
regression. The follicular phase is relatively short (~7 days in cattle or about 20% of the estrous
cycle). The growing dominant follicle on the ovary produces increasing amounts of estradiol (E₂)
at this time. On the other hand, the luteal phase (~14 days in cattle), compromises about 80% of
the estrous cycle and the dominant structure on the ovary at this time is the CL which produces
progesterone (P₄).

The estrous cycle can be further divided into four stages; proestrus, estrus, metestrus, and
diestrus. The follicular phase consists of proestrus and estrus, and the luteal phase consists of
metestrus and diestrus. During proestrus, ovulatory follicles are formed and E₂ production
increases, while estrus is the period of sexual receptivity characterized by peak E₂ secretion.
Metestrus is the period where the CL forms and P₄ begins to be secreted, and, finally, diestrus is
the period where P₄ secretion is detectable in the peripheral circulation. Diestrus is brought to an
end by the action of prostaglandin F₂α, secreted from the uterus, to regress the corpus luteum and
initiate another cycle.
Neural and hormonal control of the estrous cycle

Throughout the majority of the estrous cycle, gonadotropin releasing hormone (GnRH) is released in pulses, with the frequency and amplitude varying according to the estrous cycle stage (Clarke et al., 1987; Moenter et al., 1991). Gonadotropin releasing hormone is a decapeptide and is synthesized in neurosecretory cells of the hypothalamic preoptic area (Kaiser et al., 1997). The release of GnRH stimulates the release of the gonadotropins; luteinizing hormone (LH) and follicle stimulating hormone (FSH) which are required for changes in cyclic ovarian activity (Karsch et al., 1997). Luteinizing hormone stimulates androgen secretion in males, and in females stimulates ovulation and corpus luteum formation and function. Follicle stimulating hormone stimulates spermatogenesis in males and growth and maturation of ovarian follicles in females. Gonadotropin releasing hormone is transported to the anterior pituitary gland via the hypothalamic-hypophysial portal circulation where it binds its receptors on pituitary gonadotropes to initiate production and release of the gonadotropins LH and FSH (Baird et al., 1976; Kaiser et al., 1997; Knobil and Hotchkiss, 1988). These hormones enter the circulation where they act on the gonads to recruit follicles for ovulation (Richards and Midgely et al., 1976; Baird and Scaramuzzi, 1976). The gonads produce sex steroids that act on the hypothalamus, regulating GnRH and therefore regulate the gonadotropic hormones as well (Kaiser et al., 1997; Karsch et al., 1997). Estradiol indirectly controls LH and FSH release from the pituitary by acting on the surge center in the hypothalamus and causing increasing quantities of GnRH to be released. This in turn, causes release of the gonadotropins in a positive feedback loop (Moore et al., 1969; Baird et al., 1976). Progesterone, on the other hand, causes suppression of GnRH secretion, indirectly suppressing LH and FSH release by the pituitary (Karsch et al., 1997; Bazer et al., 1997). Progesterone can also cause direct suppression of LH and FSH at the level of the
pituitary (Batra and Miller; 1985; Phillips, et al., 1988). Through the tightly regulated communication between the ovary and the brain, the neural signal for ovulation occurs. During the follicular phase, there is a progressive increase in circulating $E_2$ that is responsible for signaling to GnRH neurons to cause a GnRH surge (Karsch et al., 1997). Males lack this gonadotropin surge center, and thus steroid hormone feedback tends to reduce gonadotropin secretion. The abrupt increase in GnRH causes a surge release of LH from the anterior lobe of the pituitary, and continued GnRH support is required for maintaining the LH surge (Karsch et al., 1997). The hypothalamic center responsible for the preovulatory LH surge is known as the surge center and releases only basal levels of GnRH until the threshold concentration of estrogen, without progesterone, is achieved. The large release of GnRH to this positive stimulus occurs only once during the estrous cycle. The other area of the hypothalamus responsible for releasing GnRH is referred to as the tonic center where GnRH is released in many small pulses with varying frequency and amplitude, referred to as an episodic profile (Constantin, 2011). Once the preovulatory LH surge occurs, multiple morphological and biochemical changes begin that result in ovulation and CL formation.

**Follicular phase**

The follicular phase of the estrous cycle is the time from regression of the CL (luteolysis) to ovulation of the follicle and can be further divided into proestrus and estrus. Proestrus begins at the onset of luteolysis and is marked by a rapid decline in circulating $P_4$ and increased $E_2$ production. The increased $E_2$ production by the follicles leads to estrus (Day 0). In the ewe, estrus is approximately 18 to 48 hours long and ends with ovulation and, in the case of the ewe, ovulation occurs approximately 26 hours after the LH surge (Talafha and Ababneh; 2011). In
cattle, estrus is slightly shorter, with a range from 6 to 24 hours, and ovulation occurs approximately 28 hours after the LH surge (Roelofs et al., 2010).

Follicular dynamics describes the processes of follicular growth, maturation, ovulation and degeneration which occur throughout the entire estrous cycle. A large pool of selectable follicles is always present on the ovary of the post-pubertal cow and they begin development in response to the changing concentrations of FSH and LH. The actual mechanism of selection remains poorly understood. Through the four processes; recruitment, selection, dominance, and atresia; many follicles are recruited, selected, become dominant, but ultimately undergo atresia at some point. In addition to E₂, inhibin is released from these follicles to selectively inhibit FSH from the anterior lobe of the pituitary (Bister et al., 1999). Follicles are recruited when FSH is high and inhibin and E₂ are low. Recruited follicles begin to grow and produce increasing amounts of E₂ and inhibin and will either be selected or undergo atresia. The selected follicles continue producing E₂ and inhibin, and may become the dominant follicle on the ovary or will undergo atresia (Bister et al., 1999). Dominance is defined as one or more large preovulatory follicles exerting inhibition on the other recruited and selected follicles. Dominance is characterized by low FSH and high LH and inhibin (Bister et al., 1999). In monotocous species (give birth to a single offspring) it is probable that only one follicle will become dominant, even though many follicles are initially recruited. However, in polytocous species (litter bearing) there are multiple dominant follicles. Follicles grow in waves and become atretic throughout the entire estrous cycle and during pregnancy. For example, a follicular wave is initiated after ovulation during metestrus (Days 3-5 in cattle), however all these follicles will undergo atresia as a result of P₄-induced suppression of GnRH. A second follicular wave typically occurs during diestrus. The dominant follicle from this wave will either go on to ovulate (if P₄ declines before
it becomes atretic) or will undergo atresia and will be followed by a third follicular wave that will generate the ovulatory follicle of the next cycle. The number of follicular waves within a cycle varies among and within a species, but cattle exhibit 2-3 follicular waves within a cycle. Once luteolysis is accomplished and a dominant follicle reaches its maximal size, secretion of $E_2$ reaches a threshold concentration to cause the preovulatory LH surge. Throughout the follicular phase, $E_2$ is not only important for causing the LH surge, but also increases blood flow to the female reproductive tract, the cervix and vagina to produce mucus, induces reproductive behavior, and initiates the growth of uterine glands which are important for the survival of the early embryo due to their secretion of histotrophe (Suarez and Pacey, 2006; Reynolds, 1986; Gray et al., 2002). The LH surge rapidly and transiently induces genes important for ovulation such as progesterone receptor (PGR), and cyclooxygenase-2 (PTGS2) (Park and Mayo, 1991; Natraj and Richards, 1993; Lim et al., 1997). These genes are known to be important because female knockout mice for PGR and PTGS2 will not ovulate even in response to exogenous hormones, but will form CL with trapped oocytes inside (Lydon et al., 1996; Lim et al., 1997). Physiological changes in the ovary leading to ovulation also include production of collagenase, increased contractions of the ovarian smooth muscle, increased blood flow to both the ovary and dominant follicle, breakdown of gap junctions between the granulosa cells and the oocyte, and the release of lysosomal enzymes (Russell and Robker, 2007). Granulosa cells of the follicle experience a down-regulation of the FSH and LH receptors after ovulation due to reduced expression of the genes encoding these receptors and internalization of engaged receptors (LaPolt et al., 1990; Nakamura et al., 1991). After ovulation occurs, luteinization, the process of the ovulated follicle differentiating into a CL, takes place (Stocco et al., 2006).
Luteal Phase

The luteal phase can be divided into metestrus and diestrus. Metestrus begins following ovulation in the ewe and lasts until the CL is formed and functioning, at which time diestrus, the longest stage of the estrous cycle begins. Diestrus is the period when the CL is fully functional, producing large amounts of P₄. After ovulation the follicle is reorganized into the corpus hemorrhagicum, composed of the collapsed follicle where a clot is formed after the rupture of blood vessels from ovulation. The theca and granulosa cells are then luteinized and E₂ synthesis declines while P₄ synthesis increases. This can be explained by decreased expression of 17β-hydroxysteroid dehydrogenases-1 (17βHSD-1) and prolactin receptor-associated protein 17β-hydroxysteroid dehydrogenases-7 (PRAP/17βHSD-7), the enzymes capable of converting estrone to estradiol (Risk et al., 2005; Nokelainen et al., 1998). The first enzyme, 17βHSD-1 is specific to granulosa cells and disappears after luteinization (Ghersevich et al., 1994a; Ghersevich et al., 1994b; Ghersevich et al., 1994c). The other enzyme, PRAP/17βHSD-7, found in many species including ruminants and humans, is present at low levels in the theca/interstitial cells of the CL, limiting the estradiol produced, which is most likely because high levels can terminate an early pregnancy (McLean et al., 1990; Parmer et al., 1992). Luteinization begins after ovulation; the basement membrane between the theca and granulosa cells breaks down and blood vessels begin developing a network (Niswender et al., 1994). At this time, theca and granulosa cells mix and become small and large luteal cells (Hoyer and Niswender, 1986). The CL grows until it reaches its maximal size and maximal P₄ production, about 8ng/mL, at the middle of the cycle, during diestrus (Wise et al., 1982). Approximately 80% of the CL consists of two distinct steroidogenic luteal cell types, termed the small and large luteal cells, because the most obvious difference between them is their size. Small and large luteal cells are present in the
cow, ewe, pig, rat, rabbit, monkey, and human (Lemon and Loir, 1977; Ursely and Leymarie, 1979; Koos and Hansel, 1981; Hoyer et al., 1986; Ohara et al., 1987; Hild-Petito et al., 1989; Miyauchi and Midgley, 1990; Nelson et al., 1992). The small cells originate from the theca interna cells and the large cells originate from the granulosa cells. The remaining 20% volume of the CL consists of vascular elements (endothelial cells, pericytes), macrophages, smooth muscle cells, and fibroblasts (Rogers et al., 1984; Farin et al., 1986). Lymphocytes are also present in the bovine CL, and increase at the time of luteal regression (Penny et al., 1999; Townson et al., 2002).

As previously stated, the main steroid synthesized by the CL is P_4 and LH is the principle hormone that stimulates P_4 production (Harrison et al., 1987). A similar number of receptors for LH are present on large and small luteal cells in the ewe and cow (Harrison et al., 1987; Chegini et al., 1991). Receptors for LH increase in number as the CL forms, and in sheep the number of LH receptors is maximum in the mid-luteal phase, around day 10, of the estrous cycle (Diekman et al., 1978). Large luteal cells synthesize over 80% of the P_4 secreted throughout the midluteal phase (Niswender et al., 1985). However, small luteal cells respond to LH with a large increase in secretion of P_4, while LH has little or no effect on large luteal cell P_4 production (Niswender et al., 1994).

In addition to its effects on follicular dynamics in the ovary, the uterus is also a major target of P_4 action. The uterus becomes quiescent under the high P_4 conditions and also becomes highly secretory which is essential for survival of an embryo (Bazer and First, 1983; Bazer et al., 1997). In the uterus, P_4 regulates expression of its own receptor. When P_4 is high, it acts via progesterone receptors (PGR) to block the expression of the estrogen receptor alpha (ESR1) and in turn the oxytocin receptor (OTR) in the endometrial luminal epithelium (LE) and superficial
ductal glandular epithelium (sGE) Days 5-11 (Spencer *et al.*, 2007). Extended periods of high circulating concentrations of $P_4$ result in the loss of PGR from the LE and sGE after Day 11 and from the glandular epithelium (GE) after Day 13. Reduction in progesterone receptors in these tissues removes the inhibition of ESR1 expression, allowing for rapid increase in ESR1 starting on Days 12-13 in sheep (Spencer and Bazer, 2004). Estrogen from developing follicles and/or growth factors from the stroma presumably activate the ESR1 and stimulate expression of the OTR gene by Day 14 (Fleming *et al.*, 2006). Oxytocin from the posterior pituitary and/or the corpus luteum binds OTR in the endometrium between Days 14 and 16 to elicit pulses of prostaglandin $F_2\alpha$ (PGF$_{2\alpha}$) from the LE and sGE (McCracken *et al.*, 1999). Prostaglandin $F_2\alpha$ is secreted from the endometrium and enters into the uterine vein and ultimately the ovarian artery via a counter-current exchange, where it can stimulate regression of the CL (McCracken *et al.*, 1971; 1999). If a conceptus is present during this period, it can prevent luteolysis by acting on the endometrial LE and sGE to suppress transcription of the ESR1 and OTR genes (Vallet *et al.*, 1988; Spencer *et al.*, 1996; Fleming *et al.*, 2001). By inhibiting the transcription for the ESR1 gene in the LE and sGE, estrogen-induced transcription of the OTR gene is prevented and therefore oxytocin-induced luteolytic pulses of PGF$_{2\alpha}$ are altered and the CL is saved (Spencer *et al.*, 2007). In sheep and cattle, this is accomplished by production and secretion of interferon tau (IFNT) by the conceptus (Bazer *et al.*, 1991). More recently, it was demonstrated that IFNT is also detectable in the uterine vein, suggesting additional peripheral actions of this hormone (Oliveira *et al.*, 2008). If an oocyte is not fertilized, the CL regresses, allowing a new cycle to begin (Stocco *et al.*, 2006).
Maternal recognition of pregnancy

Establishment of pregnancy involves both implantation/attachment and maternal recognition of pregnancy. Maternal recognition of pregnancy, a phrase coined by Roger Short in 1969, is defined as the process where the conceptus signals its presence to the maternal system, prolonging the lifespan of the CL (Short, 1967; Bazer, 2004). This results in maintenance of luteal function and P₄ production. Most mammals require P₄ production to stimulate and maintain important uterine functions including early embryonic development, implantation, placentation, and successful fetal and placental development to term (Spencer and Bazer, 2004).

The maternal recognition of pregnancy signal from the conceptus may be luteotrophic, directly promoting luteal function, antiluteolytic, altering the pattern of uterine PGF₂α release which would cause the CL to regress, or a combination of both (Bazer et al., 2008).

Chorionic gonadotropin (CG), the luteotrophic signal in primates, acts directly on the CL, and in rodents, prolactin released from the anterior pituitary also acts in a luteotrophic manner, directly on the CL (Bazer et al., 2010). Cattle, sheep, pigs, and horses all maintain luteal function during early pregnancy by altering the pattern and/or the amount of PGF₂α secreted by the endometrium; a process termed antiluteolytic signaling (Bazer et al., 1998; Bazer et al., 2010). Rescue of the CL in swine was first described by the Endocrine-Exocrine theory published in 1977 by Bazer and Thatcher. The theory stated estrogen produced by porcine conceptuses between Days 11-15 after estrus is responsible for changing the direction of PGF₂α secretion towards the uterine lumen and away from the uterine vasculature (Bazer and Thatcher, 1977). In 1988 Gross and associates demonstrated that PGF₂α was secreted towards the uterine lumen or in an exocrine direction in pregnant gilts between Day 12 and Day 14, but was secreted towards the myometrium, or in an endocrine direction during this same period in cyclic gilts.
Gross et al., 1988). They demonstrated that PGF was still produced in the pregnant sow; it was
just rerouted away from the capillaries to the lumen. This redirection of PGF$_{2\alpha}$ secretion was
shown to be a uterine response to conceptus estrogen, produced by the blastocyst starting around
Day 11-12 of pregnancy (Perry et al., 1976 & Bazer, 1989). In the mare, however, there is no
known conceptus signal mediating luteal rescue. Through experiments that involved restricting
the conceptus to progressively smaller areas of the uterus, conceptus mobility was realized to be
necessary for recognition of pregnancy (McDowell et al., 1988). Furthermore, it was determined
that the concentration of PGF$_{2\alpha}$ is reduced in endometria from pregnant mares when co-incubated
with conceptus membranes (Sharp et al., 1989). Even though conceptus mobility and conceptus
membranes have both been implicated to contribute to maternal recognition of pregnancy and
luteal rescue in the mare, the exact signal from the conceptus is still unidentified.

When sheep are bred, fertilization of the oocyte occurs in the oviduct; the morula-stage
embryo will enter the uterus around Days 4-5, and a blastocyst will be formed by Day 6
(Guillomot, 1995; Spencer et al., 2004b). The blastocyst then hatches from the zona pellucida on
Day 7-8 and becomes tubular in shape by Day 11 (Spencer et al., 2007). The conceptus
elongates, reaching 25cm or more by Day 17 (Spencer et al., 2007). The blastocyst will not
elongate in vitro, suggesting the uterus is necessary for these crucial events to take place
(Heyman et al., 1984; Flechon et al., 1986). At the point where the conceptus is elongating from
a tubular to filamentous form the mononuclear trophoblast cells are synthesizing and secreting
IFNT. This occurs between Days 10 and 21 to 25, with maximal production between Day 14
and Day 16 (Bazer, 1992; Roberts et al., 1999). Rescue of the CL in ruminants occurs during
this time because IFNT binds type I IFN receptors on the endometrial epithelium and alters the
luteolytic pulses of PGF$_{2\alpha}$, saving the CL from destruction (Spencer et al., 1996; Roberts et al.,
Interferon tau alone is the factor produced by the conceptus that prevents luteolysis, acting in a paracrine manner on the endometrial LE and sGE to suppress the transcription of the ESR1 and OTR genes (Vallet et al., 1988; Spencer et al., 1996; Fleming et al., 2001). In cyclic sheep ESR1 and OTR expression increase and are detected in the LE and GE between Days 11 to 17, but this does not occur in pregnant or IFNT infused cyclic sheep (Spencer et al., 1996). By inhibiting the transcription for the ESR1 gene in the LE and sGE, estrogen-induced transcription of the OTR gene is prevented and therefore oxytocin-induced luteolytic pulses of PGF$_{2\alpha}$ are altered and the CL is saved (Spencer et al., 2007).

**Interferons**

Interferon tau is the only IFN known to act as a pregnancy recognition signal, but it is possible that other IFNs expressed during pregnancy in primates, ruminants, pigs and rodents affect other aspects of uterine receptivity to implantation, decidualization and/or placental growth and development (Bazer et al., 2008; 2009). Interferons are a group of secreted cytokines and are well studied for their role in eliciting antiviral effects (Randall and Goodbourn, 2008).

Based on their amino acid sequences, IFNs are grouped into type I, II, or III. Type I IFNs were discovered in 1957 and consist of a large group of molecules that include mammalian IFN-α (alpha), IFN-β (beta), IFN-κ (kappa), IFN-δ (delta), IFN-ε (epsilon), IFN-τ (tau), IFN-ω (omega), and IFN-ζ (zeta, also known as limitin) (Isaacs and Lindenmann, 1957; Oritani and Tomiyama, 2004; Hardy et al., 2004). Type III IFNs include; IFN-λ1, IFN-λ2 and IFN-λ3 (also called IL29, IL28A and IL28B respectively) (Vilcek, 2003). Finally, the type II IFN has a single member that is secreted by mitogenically activated T cells and natural killer (NK) cells and is
called IFN-γ or ‘immune IFN’ (Randall and Goodbourn, 2008). Type I IFN –α and –β along with type II and type III IFNs are induced directly by a viral infection (Randall and Goodbourn, 2008).

Interferons are known for their ability to block the replication of DNA/RNA viruses in vivo and in vitro and there are three extensively studied IFN-induced enzyme systems consisting of protein kinase R (PKR), 2′-5′ oligoadenylate synthetase/rnase (2′-5′ OAS/RNase), and Myxovirus GTPases (MX GTPases) (Lindenmann, 1962; Silverman, 1994; Williams, 1999; Garcia et al., 2006). The 2′-5′ OAS/RNase pathway is an innate immunity pathway that responds to pathogen-associated molecular patterns (PAMPS), dsRNA for example. Interferon signaling induces transcription of the OAS genes which synthesize 2-5A oligomers that activate RNase L. Overall, 2-5A signals innate immunity through RNase L, which cleaves within single stranded regions of RNA (Silverman, 2007). Protein kinase R is also activated by dsRNA and induced by interferon. It has pro-apoptotic functions due to the dsRNA binding domain and the c-terminal kinase domain. Through phosphorylating the eukaryotic transition initiation factor (EIF2A) mRNA translation of viral proteins is inhibited (Scheuner et al., 2006). Viruses with anti-IFN properties have been extensively described. Viruses can block the IFN system by targeting the IFN signaling cascade, directly inhibiting PKR and 2′-5′ OAS or expressing RNA binding proteins to prevent activation of PKR and 2′-5′ OAS (Haller et al., 2007). Remarkably, there is no known viral inhibitor of the MX proteins which are not post-translationally modified and their activity is not apparently modulated by dsRNA (Haller et al., 2007).

Interferon-induced viral defense mechanisms are transcriptionally regulated. Type I IFNs are produced in response to viral infections by the host cell first recognizing PAMPS by their pattern recognition receptors (PRRS) such as toll-like receptors (TLRs). The main PAMPs of
viruses appear to be nucleic acids, like double stranded RNA (dsRNA) and single stranded RNA (ssRNA), but there are also glycoproteins and CpG nucleotides (Boo and Yang, 2010). Type I interferons and antiviral proteins are both induced by activated transcription factors, such as interferon regulatory factor-3, 5, 7 (IRF-3, 5, 7), and nuclear factor-KB (NF-κB) (Boo and Yang, 2010). For IFN-β, specifically, RNA helicase retinoic acid-inducible gene I (RIG-1) and/or melanoma differentiation-associated gene (MDA5) bind dsRNA and ssRNA. Both RIG1 and/or MDA activate IKK-like kinases IKKE and tank binding kinase-1 (TBK-1) which phosphorylate interferon regulatory factor -3 (IRF-3) causing it to homodimerize and move into the nucleus to recruit transcriptional co-activator cyclic adenosine 3’, 5’-monophosphate response element binding protein (CREB) to initiate IFNβ mRNA synthesis (Haller et al., 2007). Interferon regulatory factor -7 is expressed at very low amounts in most cells and acts in a positive feedback loop leading to amplification of IFN gene expression. For example, activated IRF-7 with IRF-3 stimulates expression of numerous IFNα genes leading to an IFNα response (Haller et al., 2007).

The type I IFNs all share the same receptor complex whereas the type II and type III IFNs bind to distinct receptors. The type I receptor is composed of two subunits, IFNARI and IFNAR2, that mediate cell signaling via the classical Janus activated kinases (JAK)-signal transducer and activator of transcription (STAT)-IRF signaling pathway and tyrosine kinase 2 (TYK2) pathways (Darnell et al., 1994). When type I interferons bind to their receptor, JAK1 and TYK2 (Janus-family tyrosine kinases) are activated which leads to the phosphorylation of STAT1 and STAT2. The STAT families of proteins are latent cytoplasmic transcription factors. The phosphorylated STAT1 and STAT2 can now form a stable heterodimer. This also creates a novel nuclear localization signal (NLS) (Banninger and Reich, 2004) and the dimers are
transported into and retained in the nucleus (Reich and Liu, 2006). The STAT1-STAT2 complex associates with interferon regulatory factor-9 (IRF-9), also termed p48, in the nucleus to form a heterotrimer, interferon simulated gene factor 3 (ISGF3) (Randall and Goodbourn, 2008). Phosphorylated STAT1 can also homodimerize to form gamma activated factor (GAF) which also translocates to the nucleus (Stark et al., 1998). In the nucleus, both ISGF3 and GAF activate genes containing interferon stimulated response elements (ISRE) and gamma activated sequences (GAS), respectively (Stark et al., 1998). Type I and III receptors activate more than 300 IFN-stimulated genes that have antiviral, antiproliferative, and immunomodulatory functions (Der et al., 1998; de Veer et al., 2001).

**Interferon tau**

Interferon tau was first termed trophoblastin when it was discovered to be the antiluteolytic protein in early pregnancy of sheep and later termed protein X when it was observed that sheep conceptus cultures produce it (Martal et al., 1979; Godkin et al., 1982). Interferon tau was later termed ovine trophoblast protein 1 (OTP-1) when it was discovered to be the sole anti-luteolytic factor produced by sheep conceptuses and finally, oTP-1 was re-named IFNT when it was discovered to be a type I interferon (Godkin et al., 1984; Imakawa et al., 1987; Vallet et al., 1988). Bovine trophoblast protein 1(bTP-1), the major secretory product by cow conceptuses Day 16-25, was identified and shown to be homologous to IFNT (Helmer et al., 1987). This unique interferon is produced by trophoblast cells of the ovine and bovine conceptus before the trophoblast makes firm attachment to the uterine wall and begins to form a placenta. Interferon tau has not only been identified in cattle and sheep, but also in muskoxen, goats, gazelle, giraffe, and deer (Leaman and Roberts, 1992). Interestingly, cattle possess several IFNT, estimates ranging from four to at least ten, but it is unclear which represent a distinct gene
and which represent allelic forms (Leaman and Roberts, 1992; Ryan et al., 1993; Alexenko et al., 2000). Other ruminant species also possess several IFNT, except for the giraffe, which may have a single gene and a group of closely related genes (Roberts, 2007).

Besides altering the release of the luteolytic factor and rescuing the CL, IFNT possesses potent antiviral, antiproliferative, and immunomodulatory activities similar to other interferons.

Interferon tau binds with type I interferon receptors with similar affinity as other Type I IFN (Li, 1994; Roberts et al., 1997; Pontzer et al., 1997; Ott et al., 1997). It shares ~75% identity with IFN-ω (omega), most resembling this IFN. Both of these IFNs are 172 amino acids in length (Roberts et al., 1997). Interferon α is about six amino acids shorter, although it and IFN-β are also quite similar to IFNT (~50% and ~25% identity respectively; Roberts et al., 1997).

Interferon tau lacks introns, is minimally affected by classic inducers of interferons (like virus and double stranded RNA), and appears to be the most recently evolved mammalian type I gene family (restricted to ruminant ungulate species) (Bazer et al., 1997; Roberts, 2007).

Again, IFNT is unique in that it lacks viral inducibility, is restricted to embryonic trophoderm, and its synthesis is sustained at high levels over several days (Roberts, 2007). Related type I interferon genes generally have short lived expression and occur in response to virus and other pathogens in a variety of tissues (Roberts, 2007). Tissue and temporal expression control for IFNT lie in the 5’ flanking region where the expression appears to be under the control of two specific promoter regions (-358 to -322bp and -91 to -61bp; Roberts et al., 2008).

V-ets erythroblastosis virus E26 oncogene homolog 2 (avian) (ETS2) is identified as the activator of transcription for IFNT, however a combination of ETS2 and downstream distal-less homeobox 3 (DLX3) promote expression cooperatively (Roberts et al., 2008). Other transcription factors that participate in the upregulation and silencing of IFNT still remain
unclear (Roberts et al., 2008). However, there is evidence that maternal uterine secretory factors induced by rising P₄ may upregulate IFNT expression via activation of ETS2 (Roberts et al., 2008).

**Interferon stimulated genes**

In addition to antiluteolytic actions, IFNT is responsible for increasing the expression of a large number of ISGs important for endometrial differentiation and implantation of the conceptus (Hansen et al., 1999; Bazer et al., 2010). These include, STAT1 and STAT2 (Johnson et al., 1999a; 1999c; Stewart et al., 2001a), IFN regulatory factor-1 (IRF1; Spencer et al., 1998; Johnson et al., 1999a; 1999c; Stewart et al., 2001a), IRF9 (Stewart et al., 2002), ISG15 (Johnson et al., 1999a; 1999b; 1999c; 2000; Stewart et al., 2001a), MX (Ott et al., 1998), OAS (Mirando et al., 1991; Johnson et al., 2001), MHC class I polypeptide-related sequence (MIC) (Choi et al., 2003), beta-2 microglobulin (B2M) (Vallet et al., 1991; Choi et al., 2003), galectin-15 (LGALS15) (Gray et al., 2004), wingless-type MMTV integration site family member 7A (WNT7A) (Kim et al., 2003), cathepsin L (CTSL) (Song et al., 2005), Cystatin C (CST3) (Song et al., 2006b), radical S-adenosyl methionine domain containing 2 (RSAD2), and interferon-induced with helicase C domain 1 (IFIH1) (Song et al., 2007).

Interferon tau induces dimerization of type I IFN receptors and two transcription factor complexes, ISG factor 3(ISGF3; STAT1:STAT2:IRF9 complex) and gamma-activation factor (GAF; STAT1 homodimer) (Johnson et al., 1999a; 1999c; Stewart et al., 2001a; 2001b), are responsible for activating transcription of target genes. Gamma-activation factor regulates genes containing a GAF sequence, like IRF1 and, in contrast, ISGF3 regulates genes containing IFN-stimulated response elements (ISREs), like STAT1, STAT2, IRF9, and OAS (Reich et al., 1987; Levy et al., 1988; Decker et al., 1991; Shuai et al., 1992; Schindler et al., 1992; Pine et al., 2001).
Interferon regulatory factor-1 is a transcriptional activator that binds to both ISREs and IRF elements (Fujita et al., 1988; Harada et al., 1989; Karin, 1991; Stark and Kerr, 1992; Harada et al., 1994; Nguyen et al., 1997; Mamane et al., 1999) and is present in the uteri of sheep (Spencer et al., 1998). Interferon regulatory factor-2 is a potent transcriptional repressor (Senger et al., 2000) in the ovine uterus and is constitutively expressed in the ovine endometrial LE (oLE) and sGE (Choi et al., 2001). Interferon regulatory factor-2 increases during early pregnancy to prevent induction or increased expression of classical (STAT1 dependent) IFNT-stimulated genes in the oLE and sGE (Choi et al., 2001). By this mechanism, classical IFNT stimulated genes are expressed by endometrial stromal cells and middle to deep uterine GE cells in the uteri of sheep (Johnson et al., 2000; 2001; Choi et al., 2001; 2003). There are a novel set of genes, however, in the uterine LE and sGE that are expressed through the combined action of P₄ and/or IFNT using an as yet unidentified signaling pathway. These genes, interestingly, lack both P₄ receptors (PGR) and STAT1 response elements (Bazer et al., 2010). The myxovirus resistance gene, MX1, is one of these genes. The myxovirus resistance gene-1 is expressed in the LE and sGE during the estrous cycle and early pregnancy (Ott et al., 1998), even though IRF-2, the potent transcription repressor, is present during early pregnancy. Hence, MX1 is regulated in the ovine uterus by multiple induction systems and is more complex than other classical ISGs (Johnson et al., 2003).

At the time of maternal recognition of pregnancy, when IFNT is released by the developing conceptus, antiviral activity (assay for IFN) is detectable in the uterine vein. Through this mechanism, it is proposed that ISGs are upregulated in the extrauterine tissues, such as the CL and peripheral blood leukocytes (PBL) (Yankey et al., 2001; Gifford et al., 2007; Oliveira et al., 2007). For example, ISG-15 is expressed in CL of cyclic and pregnant sheep and
cattle and, more importantly, expression is upregulated during early pregnancy (Spencer et al., 1999; Yang et al., 2009). This is evidence for conceptus-induced ISG expression outside of the uterus, even though it was previously accepted that the effects of IFNT were confined to the uterus (Bazer et al., 1997). Correspondingly, in PBL, MX1 mRNA expression was found to increase in early pregnant sheep between Days 15 and 30 after mating (Yankey et al., 2001). Similarly, it was demonstrated that pregnancy could be identified at Day 18 to 20 in dairy cows by measuring steady-state concentrations of the mRNA for ISG15, MX1 and MX2 (Gifford et al., 2007). In this study, MX1 and ISG15 expression in blood samples of pregnant cows were increased compared to bred, nonpregnant cows (Gifford et al., 2007). Likewise, it was also demonstrated that MX2, ISG-15, and 2’-5’ oligoadenylate synthetase 1 (Oas1) are upregulated in pregnant compared to open cows (Stevenson et al., 2007; Green et al., 2010). These results are valuable because induced ISGs in extrauterine tissues suggest an approach for the early identification of failed inseminations, which would allow for a reduction in the interservice interval.

**MX proteins belong to the dynamin superfamily**

Myxovirus resistance proteins are a distinct subclass of the dynamin superfamily of high molecular weight guanosine 5’ triphosphatases (GTPases). Dynamins were first characterized associated with microtubules in the brain and were present in nerve terminals (Obar et al., 1990; Shpetner and Vallee, 1992). The dynamin superfamily of large GTPases is known to participate in diverse cellular processes including endocytosis, where they pinch off the endocytic vesicle, and in cell migration/invasion, where they modify actin and potentially regulate focal adhesion assembly (Kruchten and McNiven, 2006). Members of the dynamin superfamily also
oligomerize, or self-assemble into rings or helical stacks (Praefcke and McManon, 2004) which appears to be critical for their function and distinguishes them from small GTPases that act more like switches (Kruchten and McNiven, 2006). This assembling occurs when dynamin is coupled to GTP and it uses GTP hydrolysis, respectively, to drive scission of endocytic vesicles and plasma membranes (Hinshaw and Schmid, 1995; Sever, 2000). Upon nucleotide hydrolysis the dynamin polymer constricts and through this constriction dynamin can deform membranes (Danino et al., 2004) and, when associated with other proteins, dynamin can serve as a “pinchase” releasing vesicles from donor membrane compartments (Itoh et al., 2005).

The dynamin family members include classic dynamins, guanylate-binding proteins (GBPs) in animals, Vps1 in yeast, and MX proteins (Praefcke and McManon, 2004). All of the members of the dynamin superfamily share three conserved features which include the GTPase domain (300 aa), the middle or “assembly” region (220 aa), and the GTPase effector domain (120 aa) (Danino and Hinshaw, 2001). These domains likely define the enzymatic and self-assembly properties of this family of large GTPases (Danino and Hinshaw, 2001). The effector domain in MX proteins is referred to as the leucine zipper domain, and this domain interacts with the central interactive domain (CID) to form oligomers (Haller and Kochs, 2002).

The MX GTPases have a unique 25-90 amino acid extension at the amino terminus which is believed to mediate the specific targeting or functional interactions of MX proteins (Haller et al., 2007). The MX GTPases lack the pleckstrin homology domain (PH) and the proline/arginine rich domain (PRD), that dynamins contain. The PH domain of classical dynamins is responsible for interaction with negatively charged lipid membranes. Even though MX has no PH domain, the human MXA protein is localized to the endoplasmic reticulum and was shown to bind and tubulate lipids (Accola et al., 2002).
Antiviral activity of MX proteins

Myxovirus resistance proteins share structural and functional properties with dynamin, such as self-assembly and involvement on or with intracellular membranes. Yet, they are unique among this superfamily of proteins in their antiviral activity against multiple RNA viruses, which they inhibit at the early stage of the viruses’ life cycle. The MX1 gene is an interferon responsive gene (ISG) and is regulated by type I and type III IFN (Haller et al., 1980; Holzinger et al., 2007). The only known function of MX1 is in the antiviral response (Horisberger et al., 1983) and MX1 has antiviral activity in most vertebrates, including fish, birds, and invertebrates such as the disk abalone (Haller et al., 2007). The MX gene was first described in an inbred lab mouse strain, A2G, which contrary to most inbred strains, was found to carry the functional MX1 gene after surviving lethal doses of influenza virus (Staeheli et al., 1986). It was realized that MX1 is required for mice to recover from infection of influenza virus because it blocks the virus multiplication cycle, and the virus cannot transcribe and replicate its genome. The resistance to the virus was revealed to be caused by a single gene, MX1 (Lindenmann, 1962) localized on chromosome 16 (Reeves et al., 1988). Standard inbred laboratory mice carry defective alleles at the MX1 gene locus and this MX negativity is most likely due to a founder effect, also described for IFN regulated Oas1b gene (Mashimo et al., 2003).

The monoclonal antibodies to murine MX1 cross-reacted with an interferon induced protein in humans and the two human MX genes were identified; 76kDa MXA and 73kDa MXB (Aebi et al., 1989). The human MX genes share approximately 63% sequence identity (Aebi et al., 1989), and, interestingly, by introducing MXA into MX1-negative mice, resistance to influenza virus can be restored (Grimm et al., 2007).
Different isoforms of MX have different cellular localization and antiviral specificities depending on the species (Lee and Vidal, 2002). Mouse MX1 and sheep MX2 are localized in and around the nucleus, respectively (Haller and Kochs, 2002; Assiri et al., 2006) and human MXA, MXB, sheep MX1, and mouse MX2 are localized in the cytoplasm (Haller and Kochs, 2002). The cellular location of the MX proteins in rodents, both the nucleus and the cytoplasm, controls viral replication at two different replication sites (Haller and Kochs, 2002). In the cytoplasm, MX proteins inhibit a range of RNA viruses, such as Bunyaviruses that replicate in the cytoplasm (Haller et al., 1998). Correspondingly, nuclear MX proteins inhibit viruses that replicate in the nucleus by blocking primary transcription of viral RNA (Krug et al., 1985). These viruses include the orthomyxoviruses, influenza A (FLUAV) and Thogoto virus (THOV) for example (Horisberger et al., 1983; Haller et al., 1998).

In humans, MXA is the only MX protein with detectable antiviral activity and it blocks viral replication regardless of intracellular location (Haller and Kochs, 2002). The antiviral specificity of MXA is very large and includes Bunyaviruses, orthomyxoviruses, paramyxoviruses, rhabdoviruses, and picornaviruses (Haller and Kochs, 2002). Myxovirus protein A interferes with the transport of the Thogoto virus nucleocapsid protein, which is transported to the nucleus, the site of viral transcription and replication. Similarly MXA blocks transport of the Bunyavirus nucleocapsid to the Golgi where this virus assembles (Haller and Kochs, 2002).

Antiviral activity is a novel function of a GTPase, and the MX protein’s structural interaction with itself plays an important role in its antiviral activity. As previously stated, through the central interactive domain (CID) and the effector domain (leucine zipper) MX proteins can form into oligomers in uninduced cells (Haller and Kochs, 2002). These oligomers are thought to be intracellular stores prepared to act to fight a viral infection (Janzen et al., 2000). The GTPase
activity was revealed to be required for antiviral activity when Pitossi and associates (1993) discovered that MX mutants that failed to hydrolyze/bind GTP failed to mediate virus resistance (Pitossi et al., 1993). Point mutations in human MXA and in rat MX2 revealed that the GED in the carboxyl terminus was important for viral target recognition (Zurcher et al., 1992; Johannes et al., 1997), and similarly, Kochs and associates (1999) also showed that a monoclonal antibody directed against the C-terminus of MXA neutralized its antiviral activity (Kochs et al., 1999). Furthermore, antibodies targeted to the central interactive region reduced GTPase activity approximately 5-fold and abolished the ability of MXA to associate with viral targets, effectively neutralizing its antiviral activity (Flohr et al., 1999). This indicates that a central interactive region is also important for MXA function (Flohr et al., 1999).

Myxovirus resistance proteins are highly conserved among vertebrates, however they are only known for their antiviral activity and lack a developmental or a constant/active function within cells (Lee and Vidal, 2002). Interestingly, not all MX proteins have been shown to participate in the antiviral response. For example, the human MXA protein is a key component of the antiviral response and is induced by type I IFNs whereas the human MXB protein has no detectable antiviral activity. Myxovirus resistance protein B is expressed at significant levels in the absence of interferon stimulation and is localized to the cytoplasmic face of nuclear pores where it is hypothesized to play a role in their regulation (King et al., 2004). By expressing GTPase defective MXB mutants, nuclear import is blocked and cell-cycle progression is delayed in HeLa cells (King et al., 2004). The conclusion made from this study was that MXB and possibly other MX proteins could have a regulatory role in nucleocytoplasmic trafficking (King et al., 2004).
Cellular functions for MX proteins

Most isoforms of MX lack any detectable antiviral function (Thomas, 2006). From this information it is becoming increasingly clear that important roles in normal cellular function may be the property of some MX proteins.

The function of MX in the absence of virus or IFN is an area where little research has been done. However, MX proteins exhibit many characteristics that would indicate a role in basic cellular function. For example, MX proteins are structurally similar to dynamins and belong to the superfamily of GTP binding proteins (Praefcke and McManon, 2004). As previously mentioned, the dynamin superfamily of large GTPases participates in diverse cellular processes, including endocytosis and cell migration/invasion. Human MXA has been shown to self-assemble into high-order oligomers like dynamin and was also shown to bind to lipid vesicles in vitro and transform them into tubules like classical dynamins (Accola et al., 2002). The same research also showed that MXA colocalizes with subcompartments of the smooth endoplasmic reticulum and this is an area thought to have no association with viral replication (Accola et al., 2002). These reflections could describe the mechanism in which MX1 fights virus but they are also consistent with MXA possibly mediating normal cellular function outside of its antiviral function in the cell.

Recently, human MX proteins have been demonstrated to have roles in endocytic trafficking and calcium signaling. For example, overexpressing MXA in Chinese Hamster Ovary (CHO) cells disturbs trafficking in the endocytic pathway and this could be caused by a possible interaction with dynamin (Jatiani and Mittal, 2004). Also, MXA was identified as a regulatory protein involved in calcium signaling dependent on GTP binding, but not hydrolysis (Lussier et
al., 2005). This suggests that MXA could function in its monomeric form after GTP binding (Lussier et al., 2005).

A study done by Chang and associates (1990) discovered that the expression of MX proteins were stimulated in organs such as the brain, lung, spleen, liver, kidney, skeletal muscle, and uterus, in the mouse when induced with either human natural interferon, human recombinant interferon, or double stranded poly(I):(C) (Chang et al., 1990). Most intriguing from this study however, was that the MX1 protein was found to be constitutively expressed in the mucosal epithelial lining of the uterus and duodenum suggesting direct gene activation independent of interferon in the blood (Chang et al., 1990). Similarly, work from Ott and associates demonstrated that MX1 was constitutively expressed in the sheep endometrium, where it appeared to be regulated by steroid hormones (Ott et al., 1998).

Additional work from Ott and associates provided evidence for a role of oMX1 outside of its role as an antiviral protein and recently, has provided evidence for constitutive expression of the oMX1 protein. Expression of MX1 protein was detected in an ovine glandular epithelial (oGE) cell line in the absence of exogenous virus or IFN (Racicot and Ott, 2010). It was determined that IFN-beta, but not IFN-alpha, mRNA was present in oGE cells and, therefore, endogenous IFN expression could be responsible for the low level expression of oMX1 in uninduced cells (Racicot and Ott, 2010). However, another classical interferon-stimulated gene, ISG15, was not detected in these same samples and therefore this could indicate that other factors regulate MX1 (Racicot and Ott, 2010). This same research also demonstrated that oMX1 interacts with tubulin beta (TUBB) in oGE cells (Racicot and Ott, 2010). Immunofluorescence analysis revealed that MX1 in the cytoplasm of uninduced cells was dispersed in punctate patterns, not associated with microtubules, but with IFN treatment it became dispersed along microtubules during interphase
The networks of microtubules in a cell serve as highways for transport of molecules and vesicles in addition to providing structural support and it is hypothesized that MX1 participates in transport of proteins or vesicles along these microtubule networks (Racicot and Ott, 2010). Additionally, during mitosis, oMX1 was highly colocalized with TUBB in the spindle fibers (Racicot and Ott, 2010) and it is possible that oMX1 may have a role in transporting proteins or vesicles along spindle fibers that are vital to mitosis or cytokinesis (Racicot and Ott, 2010).

**MX1: role in reproduction**

There is evidence that MX proteins have roles outside of their antiviral properties and following the identification that the ruminant signal for maternal recognition of pregnancy was a type I IFN (Imakawa et al., 1987; Vallet et al., 1988), a number of investigators began to examine if known ISGs were also a part of the MRP process. Charleston and Stewart (1993) were the first to show that MX1 mRNA was expressed in the ovine uterus and induced by pregnancy and IFNT (Charleston and Stewart, 1993). Later, the temporal and spatial expression of MX1 mRNA and protein during the estrous cycle and early pregnancy was characterized in sheep (Ott et al., 1998). In this study, during early pregnancy in the sheep uterus, there was a cascade response of MX1 induction across the uterine wall, with expression in the LE increasing first, and then the GE, stroma, and finally the myometrium (Ott et al., 1998). As previously mentioned, this pattern suggested that MX1 was regulated in the ovine uterus by multiple induction systems. This is because other ISGs are only expressed in the stroma and deep GE due to the expression of IRF2, a transcriptional repressor of ISGs, in the LE and shallow GE of pregnant ewes (Choi et al., 2001). In cyclic ewes, MX1 expression was greatest in LE and
shallow glands when \( P_4 \) receptors were lowest and \( P_4 \) concentrations were at their highest (Ott et al., 1998). Interestingly, if \( P_4 \) action on the endometrium was blocked using a \( P_4 \) receptor antagonist, IFNT was unable to induce MX1 expression in the endometrium (Ott et al., 1999). Results from this study showed that MX1 was expressed and regulated in the endometrial epithelium of cyclic ewes and strongly induced during early pregnancy (Ott et al., 1998). These results also clearly linked progesterone and IFNT in regulating uterine MX1 expression, which suggests that these proteins may play a role in early pregnancy beyond their antiviral functions.

Myxovirus resistance gene-1 is not only expressed in the uterine endometrium of sheep (Ott et al., 1998), but it is also expressed in the uterus of cattle, pigs, horses (Hicks et al., 2001), rodents (Chang et al., 1990) and primates (Ott, unpublished observations) with different mechanisms for pregnancy recognition signaling. In cows, MX1 mRNA increased 10 fold above that observed in cyclic cows on days 15-18 and, as in ewes, MX1 was strongly expressed in the LE, stroma, and the myometrium by day 18 (Hicks et al., 2001). It is hypothesized that MX1 functions as a conceptus induced component of the antiluteolytic mechanism and/or a regulator of endometrial secretion during early pregnancy (Hicks et al., 2003).

**MX1: role in secretion**

“Conventional” (also referred to as “classical”) secretion occurs through the ER/Golgi pathway where proteins destined for secretion are transported from the ER to the Golgi apparatus concomitant with post-translational modifications. At the trans-Golgi network (TGN) the proteins are sorted to secretory vesicles and are transported to the plasma membrane where they fuse and release their contents out into the extracellular space (Palade, 1975). This type of secretion is regulated by intracellular signals, or it can be completely unregulated. For example,
specialized signals in secretory cells can tightly regulate the release of vesicle contents or some secretory proteins can be continuously released without any signal (Tooze and Huttner, 1990).

In contrast to conventional secretion, which is the mechanism for transporting the majority of proteins out of cells, some proteins lack the signal peptide necessary for classical secretion and are not secreted through this endoplasmic reticulum/Golgi –dependent pathway. These proteins can be secreted in the absence of an intact ER/Golgi transport system, are not glycosylated, and they lack a typical hydrophobic secretion signal sequence (Kuchler and Thorner, 1992; Nickel, 2005). These proteins are identified as being secreted “unconventionally” and their secretion is insensitive to chemicals like monensin and brefeldin A (BFA) that inhibit conventional secretion (Prudovsky et al., 2002). Unconventional secretion has been recognized for over ten years, and although the exact mechanisms are unknown, four possible mechanisms have been identified. These mechanisms include direct translocation through the plasma membrane via resident transporters (Cleves and Kelly, 1996), fusion of lysosomes with the plasma membrane (Clark and Griffith, 2003), membrane shedding (Martinex et al., 2005) and secretion through vesicle intermediates (Denzer et al., 2000; Nickel, 2005).

It is hypothesized that MX1 is involved in the endometrial secretory process during early pregnancy by regulating secretion in uterine epithelial cells (Toyokawa et al., 2007a; 2007b). This hypothesis is supported by work which showed that MX1 is secreted into the uterine lumen and may play a role in regulating proteins secreted via an unconventional secretory mechanism (Toyokawa et al., 2007a). First, it was discovered that the abundance of MX1 increased in uterine flushings from Day 17 pregnant sheep compared to Day 15 cyclic and pregnant ewes. Next, oMX1 was detected in oGE cell conditioned media; these experiments together proved MX1 is a secreted protein (Toyokawa et al., 2007a). Then, oGE cells were treated with
brefeldin A (BFA), a blocker of conventional secretion, and oMX1 secretion increased in a dose dependent manner. From these experiments it was determined that oMX1 is an unconventionally secreted protein (Toyokawa et al., 2007a). Beta-2microglobulin, a conventionally secreted protein was reduced in this study while other unconventionally secreted proteins, galectin-1 (LGALS 1) and ISG15 were not, supporting the hypothesis that MX1, like these proteins, is secreted unconventionally (Toyokawa et al., 2007a). Finally, small interfering RNAs (siRNAs) were used to knockdown MX1 expression in oGE cells, and ISG15, another unconventionally secreted protein, was also reduced in the oGE cell conditioned media (Toyokawa et al. 2007b). Yet, there was no reduction in the amount of B2M, a conventionally secreted protein (Toyokawa et al. 2007b). Because, ISG15 is also an unconventionally secreted protein these results led to the hypothesis that MX1 may be a regulator in a pathway of unconventional secretion (Toyokawa et al. 2007b). These results were consistent with the roles of members of the dynamin superfamily in intracellular transport and also supported the hypothesis that MX1 is important for cellular functions independent of its antiviral function.

**Conventionally and unconventionally secreted proteins**

Many proteins believed to be important during early pregnancy are known to be secreted via the conventional pathway. For example, Cathepsin L (CSTL) and Cystatin C (CST3) are both conventionally secreted proteins that are believed to be involved in regulating conceptus growth and placentation. In the ewe, both of these proteins are detectable in uterine flushings, and in cyclic ewes, CSTL and CST3 gene expression are both upregulated in the LE at Day 12 and are maintained to Day 14 (Song et al., 2005; 2006b). However, by Day 16 these genes are no longer expressed unless further stimulated by IFNT. Cathepsin L is a peptidase that can
degrade extracellular matrix and catabolize intracellular proteins, and is hypothesized to regulate uterine receptivity for implantation and trophoblast invasion (Salamonsen, 1999). Cystatin C, an inhibitor of CSTL, is hypothesized to regulate CSTL protease action in the endometrial LE and in the trophoblast during the adhesion phases of implantation (Carson et al., 2000). Beta-2 microglobulin is another conventionally secreted protein that is induced in cattle and sheep by pregnancy due to P4 and/or IFNT (Vallet et al., 1991; Choi et al., 2003; Gray et al., 2006).

Uterine histotrophe is composed of a number of unconventionally secreted proteins. Uterine epithelial cells and oGE cells secrete LGALS1, galectin-15 (LGALS15), and fibroblast growth factor -2 (FGF2), all of which are known to be unconventionally secreted (Nickel, 2005) and are hypothesized to affect trophoblast growth and function (Gray et al., 2004; Song et al., 2005; Michael et al., 2006). In the ovine uterus, LGALS15 is expressed by the luminal epithelium and superficial glandular epithelium (Gray et al., 2005). It is critical to blastocyst elongation and implantation because it stimulates trophectoderm cell migration and attachment via integrin binding and activation (Farmer et al., 2008). Fibroblast growth factor -2 is detectable in the uterine lumen during early pregnancy and is hypothesized to increase IFNT production by the trophectoderm (Michael et al., 2006).

Other unconventionally secreted proteins include ISG15, MX1 (Toyokawa et al. 2007a; 2007b), and recently MX2 (Ott et al., unpublished). Preliminary results suggest that MX2, like MX1, is secreted unconventionally. In a recent study, monensin which is an inhibitor of the classical secretory pathway was used to block conventional secretion and MX2 levels in oGE-conditioned culture medium were not reduced, but were actually increased along with MX1 and ISG-15. Interferon stimulated gene -15, like MX1, is a critical gene in the host response to viral infection (Lenschow et al., 2007), but its role in pregnancy is not clear. Interferon stimulated
gene-15 is upregulated during early pregnancy in ruminants, primates, pigs and mice where it covalently attaches to intracellular proteins (ISGylation) and regulates numerous intracellular responses (Ashley et al., 2010). In rodents, when the ISG-15 gene was deleted, 50% of fetuses were resorbed after 7.5 dpc (Ashley et al., 2010). This protein was detected in epithelial and stromal cells in the nucleus, perinuclear space, cytosol, mitochondria, rough endoplasmic reticulum, and the cell membrane through day 50 of pregnancy (Austin et al., 2004).

**Summary**

For pregnancy to be established in all viviparous mammals, a biochemical dialogue must be established between the uterine endometrium and the conceptus to rescue corpus luteum function. Failure of this communication results in regression of the CL and failure of the pregnancy. In ruminants, the conceptus produces the antiluteolysin, IFNT, to save the CL. In addition, IFNT alters uterine secretory function (Barros et al., 1991; Ott et al., 1992; Bazer et al., 1997; Spencer et al., 1999; Mann and Lanning, 2001; Binelli et al., 2001; Spencer and Bazer, 2002). Prior to formation of a functional placenta, conceptuses are dependent on uterine secretion (histotrophe) for survival. Our working hypothesis is that MX1 is a regulator of secretion from uterine epithelial cells and that conceptus IFNT regulates the secretory process to ensure provision of the necessary uterine secretory components for its survival. Previous work showed that MX1 regulated the unconventionally secreted protein, ISG15, and possibly an unconventional secretory pathway. Therefore the objective for this thesis was to further characterize the role of MX1 as a regulator of unconventional secretion.
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Chapter 2

DEVELOPMENT OF A QUANTITATIVE ASSAY FOR MX1 PROTEIN

Abstract

The myxovirus resistance protein -1, MX1, is an interferon-stimulated gene that is present in both the endometrium and immune cells of ruminants in response to IFN-T and progesterone. Methods for its quantification are only semiquantitative, consisting of protein immunoblotting/ Western blotting, which can be quite variable from blot to blot. We therefore developed a Meso Scale Discovery (MSD) assay for ovine MX1 (oMX1) that is sensitive and rapid. The assay uses a monoclonal IgM MX1 antibody and an amino terminal rabbit polyclonal ovine MX1 antiserum which binds both native and recombinant oMX1. The MSD assay accurately detected known amounts of roMX1 added to Mammalian Protein Extraction Reagent (MPER), phenol/serol free Dulbecco’s Modified Eagle’s Medium with 5% antibiotic antimycotic (ABAM), and distilled water over the range of 2.4-10,000 ng/mL MX1. Additionally, the assay accurately detected known amounts of roMX1 added to bovine plasma over the range of 2.4-40,000 ng/mL MX1. In summary, we have developed a sensitive, rapid assay to quantify ovine MX1 in different diluents and plasma that does not involve biohazardous or radioactive materials.

Introduction

The myxovirus resistance-1 (MX1) protein is an antiviral protein belonging to the dynamin superfamily of large GTPases that functions as a component in the antiviral response. The MX proteins have been described in species ranging from humans to an invertebrate, the disk abalone (De Zoysa et al., 2007). Expression of MX1 protein increases in response to type I
interferon (IFN) making it a member of the large IFN-stimulated gene (ISG) family (Charleston and Stewart, 1993; Ott et al., 1998; Haller et al., 2007). In ruminants, the maternal recognition of pregnancy signal is a unique type I interferon, IFNT, and MX1 is upregulated in the endometrium of ruminants in response to IFNT and progesterone (P₄) during early pregnancy (Charleston and Stewart, 1993; Ott et al., 1998). At least two MX genes have been confirmed in cattle (Ellinwood et al., 1998; GeneBank accession # NM_173941) and sheep (Ott et al., 1998; Ott and Assiri, unpublished). The temporal and spatial mRNA expression in the endometrium of pregnant and nonpregnant sheep showed that MX1 mRNA was greatest in cyclic ewes when progesterone concentrations were at their maximal (Days 11-13 after estrus), and pregnant animals had the highest concentration of MX1 mRNA at Day 17 (Ott et al., 1998). Regulation of MX1 proteins by IFNT and progesterone suggest that they may play a role outside of their antiviral function, and may play a role in early pregnancy. Recently it was shown that MX1 is a component of uterine secretions and was increased in the uterine secretions of early pregnant ewes (Toyokawa et al., 2007a). Our working hypothesis is that MX1 is involved in endometrial secretory processes during early pregnancy and may be a conceptus-induced regulator of secretion by uterine epithelial cells (Toyokawa et al., 2007a; 2007b).

An assay for measuring oMX1 is essential for further studying its function. The only currently available method, immunoblotting, is only semiquantitative and can be quite variable from blot to blot. Here, we describe development and validation of a Meso Scale Discovery (MSD) assay. The MSD products are based on MULTI-ARRAY™ technology. Both electrochemiluminescence detection and patterned arrays are used in this assay. In this assay, background signals are minimized because the assay is stimulated by electricity, which is decoupled from the MSD SULFO-TAG™ labels that emit the electrochemiluminescence (light).
that is measured. We developed this assay for measuring oMX1 in Mammalian Protein Extraction Reagent (MPER), phenol/serum-free Dulbecco’s Modified Eagle’s Medium with 5% antibiotic antimycotic (ABAM), distilled water, and bovine plasma with the goal of creating a quantitative assay for MX1 protein. Additionally, the percentage of MX1 recovered from cellular lysates was calculated to check for components that may interfere with this assay.

Materials and methods

Ovine MX1

Recombinant MX1 was produced in One Shot® TOP10 Chemically Competent E. coli (Invitrogen) as described by Racicot and Ott (Racicot and Ott, 2010). Concentrations of purified MX1 were determined by the bicinchonic acid assay (Pierce, Rockford, IL), according to the manufacturer’s instructions using bovine serum albumin (BSA) as the standard. The purity of rMX1 was determined by one-dimensional SDS-PAGE and coomassie blue staining.

MSD Assay

The entire procedure for the MX1 assay was performed in a 96 well standard bind plate (Meso Scale Discovery, Gaithersburg, MD). The plate was first rinsed 3 times with 150 uL/well of 1X PBS (10X: 80 g NaCl/ 2 g KCl/ 14.4 g Na2HPO4 /2.4 g KH2PO4/ pH to 7.4/ add dH2O to 1.0 L) because the plate is hydrophobic and this creates a challenge in the initial coating (the solution tends to be pushed to the outer edges, away from the electrode surface). Each well of the plate was then coated with 25 uL of 150 ng (diluted in PBS) of the monoclonal IgM MX1 antibody (Hybridoma and Cell Culture Lab, Penn State University, mAB clone-29C). The plate was then covered and placed at 4°C overnight. The following morning the plate was removed, decanted of the MX1 monoclonal antibody and rinsed 3 times with 150 uL/well of 1X PBST (10X: 80 g NaCl/ 2 g KCl/ 14.4 g Na2HPO4 /2.4 g KH2PO4/ pH to 7.4/ add dH2O to 1.0 L/
0.05% Tween 20). Nonspecific binding sites were blocked with 3% non-fat milk (NFM) in PBST at 150 uL/well for 1 hour on a plate shaker at room temperature. After 3 rinses with PBST at 150 uL/well the rMX1 standard(s) and/or unknown samples were added and the volume adjusted to 25 uL/well. The plate was again sealed and placed on a plate shaker for 1 hour at room temperature. At the end of the hour, the samples were decanted from the plate and the plate was rinsed 3 times with 150 uL/well of PBST. An amino terminal rabbit polyclonal ovine MX1 antiserum (#90618 bleed number 3, prepared by Multiple Peptide Systems, San Diego, CA) was added to each well at a 1:1000 dilution prepared in 1% NFM at 25 uL/well. The plate was again sealed and placed on a plate shaker for 1 hour at room temperature. At the end of the incubation the plate was decanted and washed 3 times with 150 uL/well of PBST. The MSD SULFO-TAG™ labeled anti-rabbit antibody (MSD, Gaithersburg, Maryland) was added at 1 ug/mL and at a total of 25 uL/well. This antibody was also prepared in 1% NFM, and the plate was again sealed and placed on a plate shaker for 1 hour at room temperature. Finally, at the end of the 1 hour incubation, the plate was rinsed 3 times with 150 uL/well of PBST and then 150 uL/ well of a 1:4 dilution of MSD Read Buffer T (1X) with surfactant (MSD, Gaithersburg, Maryland) and distilled water was added and the plate was read on the MSD SECTOR™ Imager 2400 (SI2400) SI2400A (MSD, Gaithersburg, Maryland). This machine detects electrochemiluminescence from the MSD SULFO TAG™ labels that emit light when electrochemically stimulated and converts them to electrochemiluminescence (ECL) units. The instrument collects and quantitatively measures the light emitted from the plates over multiple cycles. All samples were assayed in triplicate.
Results/Discussion

Methods for oMX1 quantification are only semiquantitative and consist of protein immunoblotting/western blotting. We therefore developed a Meso Scale Discovery (MSD) assay for ovine MX1 (oMX1) that is sensitive and rapid. Standard curves were established using highly purified rMX1 in Mammalian Protein Extraction Reagent (MPER; Pierce, Rockford, IL; Figure 2-2), phenol/serum free Dulbecco’s Modified Eagle’s Medium (15.63 g/L DMEM, Sigma, St Louis, MO) with 5% antibiotic antimycotic (ABAM; Gibco; Figure 2-3), and distilled water (Figure 2-4) over the range of 2.4-10,000 ng/mL MX1. Additionally, the assay accurately detected known amounts of rMX1 in bovine plasma (Figure 2-5) over the range of 2.4-40,000 ng/mL MX1. Known amounts of roMX1 were added to ovine glandular epithelial (oGE) cell lysates to analyze the amount that could be recovered. Approximately 90.2% of roMX1 was recovered calculated based on the appropriate standard curve (MPER) at concentrations ranging from 4.8 – 624 ng/mL (Figure 2-6), indicating there was nothing in the lysates at these concentrations that interferes with the assay. Each sample was assayed in triplicate and all assays were repeated at least three times. The sensitivity of the assay was lower when it was applied to bovine plasma (Figure 2-5), however, we did not test the percentage of MX1 recovered from plasma, or if serial diluting the plasma sample would improve the sensitivity of the assay. Additionally, to complete the validation of this assay, cross-reactivity of the antibodies used in this assay should be tested, and the intrassay coefficients of variations (CVs) from the data collected need to be calculated. To conclude, we have developed a sensitive, rapid assay to quantify ovine MX1 in different diluents and plasma that does not involve biohazardous or radioactive materials.
Figure 2-1

Figure 2-1. Schematic of MX1 MSD assay. Each well of a standard bind plate is first coated with the monoclonal IgM MX1 antibody. When sample is added, the MX1 in that sample will bind to the IgM MX1 antibody through its specific epitope. The rabbit polyclonal MX1 antibody is then added to the plate and will bind to different epitopes of the MX1 that is already bound to the monoclonal IgM MX1 antibody. Finally, an MSD Sulfo-Tag™ labeled anti-rabbit IgG antibody is added to the plate which will recognize the rabbit polyclonal antibody through epitopes. This MSD Sulfo-Tag™ emits light that the machine detects when electrochemically stimulated, and the light measured is converted to electrochemiluminescence (ECL) units.
Figure 2-2

Figure 2-2. Linear range of sensitivity of the oMX1 MSD assay in mammalian protein extraction reagent (MPER). Standard curves were created from the mean of 2 observations at each dose of roMX1. Purified roMX1 was diluted in MPER from 2.4-10,000 ng/mL.
Figure 2-3. Linear range of sensitivity of the oMX1 MSD assay in distilled water. Standard curves were created from the mean of 2 observations at each dose of roMX1. Purified roMX1 was diluted in distilled water from 2.4-10,000 ng/mL.
Figure 2-4. Linear range of sensitivity of the oMX1 MSD assay in phenol/serum free medium. Standard curves were created from the mean of 2 observations at each dose of roMX1. Purified roMX1 was diluted in phenol/serum free media from 2.4-10,000 ng/mL.
Figure 2-5. Linear range of sensitivity of the oMX1 MSD assay in bovine plasma. Standard curves were created from the mean of 2 observations at each dose of roMX1. Purified roMX1 was diluted in bovine plasma from 2.4-40,000 ng/mL.
Figure 2-6

Recovery of MX1 from oGE Cell Lysates

<table>
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<tr>
<th>Amount MX1 added ng/25ul</th>
<th>Measured</th>
<th>% Recovery</th>
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<tbody>
<tr>
<td>15.6</td>
<td>15.38</td>
<td>98%</td>
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<tr>
<td>7.8</td>
<td>7.21</td>
<td>92%</td>
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<td>3.9</td>
<td>4.09</td>
<td>105%</td>
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<tr>
<td>1.95</td>
<td>1.51</td>
<td>77%</td>
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<td>0.95</td>
<td>0.67</td>
<td>70%</td>
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<td>0.48</td>
<td>0.36</td>
<td>75%</td>
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<td>0.24</td>
<td>0.31</td>
<td>130%</td>
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<tr>
<td>0.12</td>
<td>0.09</td>
<td>75%</td>
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<td>0.06</td>
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Average % Recovered: 90.2%

Figure 2-6. Amounts of roMX1 added and recovered from ovine glandular epithelial (oGE) cell lysates. The amounts of roMX1 recovered were calculated based on the appropriate standard curve. The assay was performed in triplicate as described in Materials and Methods.
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Chapter 3

EFFECTS OF INHIBITION OF MX1 EXPRESSION ON INTERFERON-TAU STIMULATED SECRETION BY OVINE GLANDULAR EPITHELIAL CELLS

Abstract

The myxovirus resistance, or MX, proteins are members of the dynamin superfamily of large GTPases. This superfamily is known to participate in diverse cellular processes including endocytosis and cell migration/invasion. Myxovirus resistance protein-1, MX1, shares structural and functional properties with dynamins, such as self-assembly and interaction, on or with, intracellular membranes. These proteins are unique among this superfamily of proteins in their antiviral activity against multiple RNA viruses; however, there is increasing evidence that MX proteins have cellular roles in addition to their antiviral activity. To characterize the function of MX1 in uterine epithelial cells, and test the hypothesis that MX1 is a regulator of unconventional secretion, morpholino antisense oligonucleotides (MAO; Gene Tools Inc.) were used to induce transient “knock-down” of the MX1 protein. Treatment with the MX1-specific MAO resulted in robust knockdown of the MX1 protein in total protein lysates and in total protein secretions. Additionally, we were able to use a quantitative assay for the MX1 protein to validate the inhibition of MX1 in the oGE cell lysates. Proteins representing both the conventional (secreted through the ER/Golgi pathway) and the unconventional (secreted in the absence of an intact ER/Golgi transport system) secretory pathways were examined for changes in response to inhibition of the MX1 protein. In secretions, the MX1-specific MAO reduced the secretion of interferon stimulated gene-15 (ISG15) and galectin-1 (LGALS1), while treatment increased the secretion of galectin-15 (LGALS15) compared to the mismatch MAO and the IFNT only controls. One of the unconventionally secreted proteins analyzed did not show an effect to MX1 inhibition, myxovirus resistance-2 (MX2), and another unconventionally secreted protein,
fibroblast growth factor-2, (FGF2) was inconclusive due to the low amount of secreted protein. Interestingly, MX1-specific MAO treatment increased secretion of the conventionally secreted 5-kDa active form subunit of cathepsin L (CSTL) compared to the mismatch MAO and the IFNT only controls. There was no change in the other conventionally secreted proteins examined; cystatin C (CST3) and beta-2 microglobulin (B2M). Results presented here do not support the hypothesis that MX1 is a general regulator of unconventional secretion, but MX1 may regulate a specific pathway in unconventional secretion. Overall, these results point to a more complex role for MX1, because proteins of each class were affected.

Introduction

In cattle, substantial embryonic loss occurs during the peri-implantation period (25-40%), resulting in losses approaching half a billion dollars annually (Sreenan and Diskin, 1986; Humblot, 2001; Moore et al., 2005). The peri-implantation period is the time during early pregnancy that, regardless of species, a biochemical dialogue between the developing conceptus (embryo and associated membranes) and the maternal uterus must be established. For successful establishment of pregnancy to occur, the conceptus must signal its presence to the maternal system to prevent luteal regression. This communication between the conceptus and the maternal system is referred to as maternal recognition of pregnancy and in ruminants. The first key signal is a type I interferon, interferon tau (IFNT). Interferon tau is secreted by the mononuclear cells of the trophectoderm between Days 10 and 21 to 25 in sheep, with maximal production falling between Day 14 and Day 16 (Bazer, 1992; Roberts et al., 1999). Rescue of the CL in ruminants occurs during this time because IFNT binds type I IFN receptors on the endometrial epithelium and alters the luteolytic pulses of PGF$_{2\alpha}$, saving the CL from destruction.
(Spencer et al., 1996; Roberts et al., 1999; Spencer and Bazer, 2002). When IFNs bind to their receptors they activate the JAK-STAT signaling pathway which leads to the upregulation of a large number of interferon-stimulated genes in the uterus, CL, and peripheral blood immune cells (Vallet et al., 1991; Mirando et al., 1991; Ott et al., 1998; Spencer et al., 1998; Hansen et al., 1999; Johnson et al., 1999a; 1999b; 1999c; 2000; 2001; Stewart et al., 2001; 2002; Gray et al., 2002; Choi et al., 2003; Kim et al., 2003; Gray et al., 2004; Song et al., 2005; Gray et al., 2006; Song et al., 2006; 2007; Bazer et al., 2010). These genes are thought to play crucial roles in supporting conceptus growth, differentiation, and embryo implantation. Although we know the ruminant conceptus alters synthesis and secretion of many endometrial proteins, the mechanisms by which the conceptus regulates endometrial secretion remain poorly described. The experiments presented in this thesis focus on the myxovirus resistance or MX proteins.

The MX proteins are antiviral proteins, and are strongly upregulated in the endometrium of ruminants during early pregnancy (Ott et al., 1998; Charleston and Stewart, 1993). At least two MX genes (MX1 & MX2) have been confirmed in cattle (Ellinwood et al., 1998; GeneBank accession # NM_173941) as well as in sheep (Ott et al., 1998; Ott and Assiri, unpublished). The temporal and spatial MX1 mRNA expression has been characterized in samples from the endometrium of pregnant and non-pregnant sheep (Ott et al., 1998). Cyclic animals were found to have highest MX1 expression when progesterone concentrations were at their maximal Days 11-13 after estrus, and pregnant animals had highest MX1 expression at Day 17 (Ott et al., 1998). Recent results have also shown that MX1 is a component of uterine flush and increased in the uterine flush of early pregnant ewes (Toyokawa et al., 2007a). Additionally, MX1 was shown to be constitutively expressed in an ovine glandular epithelial (oGE) cell line in the absence of exogenous virus or IFN (Racicot and Ott, 2010). Furthermore, when MX1 expression
was inhibited using small interfering RNA (siRNA) in oGE cells, secretion of the unconventionally secreted protein, ISG15, was also reduced but, interestingly, conventionally secreted proteins were unaffected. These results also indicated that MX1 itself is an unconventionally secreted protein (Toyokawa et al., 2007a). Brefeldin A (BFA) is an inhibitor of the classical secretory pathway and when it was used to treat oGE cells, B2M, a conventionally secreted protein was inhibited where MX1 actually increased in a dose dependent manner (Toyokawa et al., 2007a).

The regulation of MX proteins by IFNT and progesterone during this critical window of early pregnancy suggest that they may play a role in establishment and maintenance of pregnancy. Recently, it has been hypothesized that MX1 participates in regulating secretion, perhaps by mediating transport of proteins or vesicles along microtubule networks (Racicot and Ott, 2010). This hypothesis stemmed from the observation that MX1 interacts with tubulin beta (TUBB) in oGE cells (Racicot and Ott, 2010). Immunofluorescence analysis revealed that MX1 in the cytoplasm was dispersed in punctate patterns, not associated with microtubules in the absence of IFN but, with IFN treatment, it became dispersed along microtubules during interphase (Racicot and Ott, 2010). The networks of microtubules in a cell serve as highways for transport of molecules and vesicles in addition to providing structural support and it is hypothesized that MX1 participates in transport of proteins or vesicles along these microtubule networks (Racicot and Ott, 2010).

Collectively these studies support the hypothesis that MX1 is involved in the endometrial secretory process during early pregnancy by regulating secretion of uterine epithelial cells (Toyokawa et al., 2007a; 2007b). The objective of these experiments was to test the hypothesis that MX1 is a regulator of unconventional, but not conventional secretory pathways.
Materials and methods

Cell culture and treatments

Immortalized ovine glandular epithelial cells (oGE; Johnson et al., 1999) were cultured in T25 flasks in Dulbecco’s Modified Eagle’s Medium (15.63 g/L DMEM, Sigma, St Louis, MO) with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY) and 5% antibiotic antimycotic (ABAM; Gibco) under 5% CO2 at 38.5°C. When the cells were approximately 90% confluent, morpholino treatments were dissolved in nuclease-free water and were added to the cells at a 20 uM final concentration in fresh Dulbecco’s Modified Eagle’s Medium (15.63 g/L DMEM, Sigma, St Louis, MO) with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY) and 5% antibiotic antimycotic (ABAM; Gibco) along with endoporter reagent (6 ul/mL). The MX1 MAO has been designed by the technical support scientists at Gene Tools based on the following requirements/recommendations; a target sequence in the post-spliced mRNA in the region from the 5’cap to about 25 bases 3’ to the AUG translational start site, little or no self-complementarity, and less than 3 contiguous guanines and be 25 bases long. The lack of intron sequence available for the ovine MX1 pre-mRNA limits the use of morpholinos which block splicing events. The morpholinos were delivered to the cytoplasm of these cells using Endoporter reagent (Gene Tools Inc.).

The cells were incubated at 38.5°C for 6 hours. The media with MAO’s and endoporter was then removed. Fresh, phenol-free, DMEM (15.63 g/L DMEM, Sigma, St Louis, MO) with 5% antibiotic antimycotic (ABAM; Gibco), without FBS, containing IFNT (10,000 U/mL; provided by Fuller W. Bazer, Texas A&M University), 20 uM MAO , and endoporter (6 ul/mL) was added back to the cells. These cells were then incubated for 48 hours at 38.5°C. Conditioned media was collected at this point and an EDTA-free protease inhibitor cocktail
(Pierce, Rockford, IL) was added to the conditioned medium to a 1X final concentration. The conditioned medium was centrifuged at 2700 x g for 10 minutes to pellet cellular debris. The clarified media were then concentrated using Amicon Ultra 3k (Millipore Corporation, Billerica, MA) centrifuge tubes, washed twice with triple distilled water, and concentrated from 5 mL to approximately 250 uL. Cellular lysates were prepared using Mammalian Protein Extraction Reagent (MPER; Pierce, Rockford, IL) according to the manufacturer’s instructions. Protein concentrations were quantified using the bicinchonic acid assay (Pierce, Rockford, IL), according to the manufacturer’s instructions, using bovine serum albumin (BSA) as the standard. All MAO’s were purchased from Gene Tools Inc.

The treatments included a no treatment group (did not receive MAO’s, endoporter, or IFNT), an IFNT only group (did not receive MAO’s or endoporter, but did receive IFNT), a MX1-specific MAO group (received MX1-specific MAO’s, endoporter, and IFNT), and a mismatch MAO group (received nonsense MAO’s, endoporter, and IFNT). Within each experiment, treatments were replicated 3 times and the entire experiment was replicated three times.

**Western blot analysis**

Secreted proteins (25 ug) from each treatment were dissolved in 1X sample buffer (4X: 7.5 mL dH2O/ 760 mg Tris base/ 2 g sodium dodecyl sulfate (SDS)/ 10 mL glycerol/ pH to 6.5/ 5 mL 2-beta mercaptoethanol/ 300 uL bromphenol blue), boiled at 95°F for 5 minutes, and separated on a one dimensional SDS PAGE gel (6% stacking gel, 10-12% resolving gel) in 1X electrode buffer (10X: 30.3 g Tris base/ 144.2 g glycine/ 10 g SDS /pH to 8.3/ add dH2O to 1.0 L) at a constant current (70mA) for approximately 1.5 hr. Lysate protein (10 ug) was prepared and analyzed similarly.
Following separation by electrophoresis, the proteins were transferred to a nitrocellulose membrane (Invitrogen) using the iBlot® Dry Blotting System (Invitrogen) for approximately 8 minutes at 20 volts. Nonfat milk (5%) was used as a blocker for the MX1, MX2, LGALS15, CST3 proteins and bovine serum albumin (BSA) was used as a blocker for ISG15, LGALS1, FGF2, B2M, CSTL proteins. Blockers were dissolved in tris buffered saline tween-20 (TBST) (20 mL 1M Tris pH 7.5/ 137 mL 1 M NaCl/ 500 uL Tween 20/ dH2O to 1.0 L).

Immunoblotting was performed with a 1:1000 dilution of an amino terminal rabbit polyclonal ovine MX1 antiserum (90618 bleed number 3, prepared by Multiple Peptide Systems, San Diego, CA), a 1:1000 dilution of rabbit polyclonal human B2M antiserum (Abcam, Catalog # ab15976, Cambridge, MA), a 1:2000 dilution of mouse monoclonal bovine ISG15 (Provided by Dr. Thomas R. Hansen, Colorado State University), a 1:500 dilution of goat anti-human galectin-1 (R&D, Catalog # AF1152, MN), a 1:1000 dilution of rabbit polyclonal ovine galectin-15 antiserum (Provided by Tom Spencer, Texas A&M University), a 1:1000 dilution of rabbit polyclonal ovine MX2 antiserum (9708 bleed 3, Multiple Peptide Systems), a 1:500 dilution of rabbit anti-human/bovine FGF basic (R&D, Catalog # AB-33-NA, MN), a 1:1000 dilution of rabbit polyclonal human CST3 (Upstate, Catalog # 06-458, Temecula, CA), and a 1:500 dilution of rabbit anti-human/mouse/rat cathepsin L (BioVision, Catalog # 3192-100, Mountain View, CA) with 2% NFM/BSA rocking at 4°C overnight. Membranes were washed with TBST the following morning and a 1:200,000 dilution of goat anti-rabbit, goat anti-mouse, or rabbit anti-goat IgG-horseradish peroxidase conjugate (Pierce, Rockford, IL) was added. Membranes were then rocked at room temperature for an hour and washed with TBST again. Super Signal® West Femto Maximum Sensitivity Substrate chemiluminescence kit (Pierce) was added to the membranes for 10 minutes at room temperature to detect the immunoreactive proteins.
Detection of the chemiluminescence signal was performed using the Bio-Rad Chemidoc-XRS Multiimager system and Quantity One software (Bio-Rad, Hercules, CA).

**MSD Assay**

The entire procedure for the MX1 assay was performed in a 96 well standard bind plate (Meso Scale Discovery, Gaithersburg, MD). The plate was first rinsed 3 times with 150 uL/well of 1X PBS (10X: 80 g NaCl/ 2 g KCl/ 14.4 g Na2HPO4 /2.4 g KH2PO4/ pH to 7.4/ add dH2O to 1.0 L) because the plate is hydrophobic and this creates a challenge in the initial coating (the solution tends to be pushed to the outer edges, away from the electrode surface). Each well of the plate was then coated with 25 uL of 150 ng (diluted in PBS) of the monoclonal IgM MX1 antibody (Hybridoma and Cell Culture Lab, Penn State University, mAB clone-29C). The plate was then covered and placed at 4°C overnight. The following morning the plate was removed, decanted of the MX1 monoclonal antibody and rinsed 3 times with 150 uL/well of 1X PBST (10X: 80 g NaCl/ 2 g KCl/ 14.4 g Na2HPO4 /2.4 g KH2PO4/ pH to 7.4/ add dH2O to 1.0 L/ 0.05% Tween 20). Nonspecific binding sites were blocked with 3% nonfat milk (NFM) in PBST at 150 uL/well for 1 hour on a plate shaker at room temperature. After 3 rinses with PBST at 150 uL/well, the rMX1 standards (0.059 ng-125 ng) in mammalian protein extraction reagent (MPER) were added to the appropriate wells. Additionally, the oGE cell lysate samples (lysed using MPER) were added at 10 ug of lysate/well and the volume adjusted to 25 uL/well using additional MPER. The plate was again sealed and placed on a plate shaker for 1 hour at room temperature. At the end of the hour, the samples were decanted from the plate and the plate was rinsed 3 times with 150 uL/well of PBST. An amino terminal rabbit polyclonal ovine MX1 antiserum (#90618 bleed number 3, prepared by Multiple Peptide Systems, San Diego, CA) was added to each well at a 1:1000 dilution prepared in 1% NFM at 25 uL/well. The plate was again
sealed and placed on a plate shaker for 1 hour at room temperature. At the end of the incubation the plate was decanted and washed 3 times with 150 uL/well of PBST. The MSD SULFO- 
TAG™ labeled anti-rabbit antibody (MSD, Gaithersburg, Maryland) was added at 1 ug/mL and at a total of 25 uL/well. This antibody was also prepared in 1% NFM, and the plate was again sealed and placed on a plate shaker for 1 hour at room temperature. Finally, at the end of the 1 hour incubation, the plate was rinsed 3 times with 150 uL/well of PBST and then 150 uL/ well of a 1:4 dilution of MSD Read Buffer T (1X) with surfactant (MSD, Gaithersburg, Maryland) and distilled water was added and the plate was read on the MSD SECTOR™ Imager 2400 (SI2400) SI2400A (MSD, Gaithersburg, Maryland). This machine detects electrochemiluminescence from the MSD SULFO TAG™ labels that emit light when electrochemically stimulated and converts them to electrochemiluminescence (ECL) units. The instrument collects and quantitatively measures the light emitted from the plates over multiple cycles. All samples were assayed in triplicate. The results were analyzed using the proc ANOVA procedure in SAS (Version 9.2; SAS Institute, Cary, NC).

Results

Effects of inhibition of MX1 on unconventionally secreted proteins from ovine glandular epithelial cells

Morpholino antisense oligos (MAOs) were utilized to block the translation of MX1 mRNA in oGE cells. Treatment with the MX1-specific MAOs resulted in MX1 inhibition (approximately 50%) in both total cell lysates (Figure 3-1) and medium (Figure 3-2). Additionally, inhibition of MX1 protein in the lysates was quantified using the MX1 MSD assay for IFNT, nontreated, MX1-specific, and non specific morpholino antisense oligo (MAO )-treated oGE cells (Figure 3-3). The electrochemiluminescence (ECL) units were converted into
MX1 quantity (ng/mL) by interpolation from the rMX1 MPER standard curve. Analysis of variance revealed a main effect of treatment (p<0.001) on MX1 protein concentration. Further analysis using the Student Newman Keuls (SNK) multiple comparison test revealed that the IFNT treated group and the mismatch-MAO group were not different from each other, but differed from the Control (no treatment) and the MX1-specific MAO group which were not different. Overall treatment with MX1 MAO reduced MX1 protein concentrations in cell lysates by an average of 52%. ± 52 ng of MX1/mL compared to the IFNT only control. There was no effect of the nonspecific MAO MX1 concentrations (reduced MX1 protein concentrations in cell lysates by an average of 9% ± 47 ng of MX1/mL compared to the IFNT only control).

In the analysis of unconventionally secreted proteins, the inhibition of MX1 protein resulted in reduced secretion of ISG15 (Figure 3-4) and LGALS1 (Figure 3-5) in medium. Additionally, inhibition of MX1 also resulted in increased secretion of LGALS15 (Figure 3-6) in medium. One of the unconventionally secreted proteins analyzed did not show an effect to MX1 inhibition, MX2 (Figure 3-7), and another unconventionally secreted protein, FGF2 (Figure 3-8), was inconclusive due to the low amount of secreted protein.

**Effects of inhibition of MX1 on conventionally secreted proteins from ovine glandular epithelial cells**

In the analysis of conventionally secreted proteins, inhibition of MX1 protein resulted in increased secretion of the 5-kDa active form subunit of CSTL (Figure 3-9) in medium. The other two conventionally secreted proteins analyzed, B2M (Figure 3-10) and CST3 (Figure 3-11), were not inhibited by the knockdown of MX1.
Discussion

The objective of this study was to test the hypothesis that MX1 is a specific regulator of unconventional secretion. The approach taken was to examine several proteins representing both conventional and unconventional secretory pathways in response to selective inhibition of MX1. Results presented here do not support the hypothesis that MX1 is a general regulator of unconventional secretion. However, because some unconventionally secreted proteins but no conventionally secreted proteins were reduced when MX1 was inhibited, our hypothesis is partially supported and MX1 may regulate a specific pathway in unconventional secretion. Overall, these results point to a more complex role for MX1, because proteins of each class were affected. The MX1 protein is a member of the dynamin superfamily of large GTPases that were shown to be involved in the final release of vesicles entering the endosomal pathways, membrane deformation, and vesicle trafficking (Doo Song and Schmid, 2003). Previously, Ott and associates showed that suppressing MX1 mRNA using siRNAs resulted in reduction in the secretion of unconventionally secreted ISG15 and MX1, but not conventionally secreted B2M (Toyokawa et al., 2007a; 2007b). This led to the hypothesis that MX1 was a specific regulator of unconventional secretion.

Using MAO, we were able to induce a 52% average reduction in MX1 protein. This was confirmed using both Western blotting and a MX1 MSD assay for the oGE cell lysates, and by using Western blotting for the medium. In the analysis of unconventionally secreted proteins, inhibition of MX1 protein resulted in reduced expression of ISG15 (Figure 3-4) and LGALS (Figure 3-5) in medium. Interferon stimulated gene -15, like MX1 is critical in the host response to viral infection (Lenschow et al., 2007). It is hypothesized to modify signal transduction and cytoskeletal architecture because it covalently attaches to intracellular proteins (ISGylation) and
regulates numerous intracellular responses (Ashley et al., 2010). It is also upregulated during early pregnancy in ruminants, primates, pigs and mice, although the processes that ISG15 regulates in pregnancy are unknown (Ashley et al., 2010). Both ISG15 and LGALS1 are known to be unconventionally secreted proteins. Work from Ott and associates suggests that LGALS1 is a binding partner of MX1 and is also a component of uterine histotrophe (Welker, 2005; Toyokawa et al., 2007a; 2007b). The reduction in ISG15 and LGALS1 secretion induced by inhibition of MX1 protein in this experiment is consistent with our hypothesis that MX1 is a regulator of unconventional secretion. Previously, MX1 was shown to be associated with exosomes in oGE-conditioned culture medium (Racicot, 2009). This led to the hypothesis that MX1 could play a role in exosome formation and is then incorporated into exosomes in the process. In support of this, formation of multivesicular bodies and/or exosomes is one of the known unconventional secretory pathways (Racicot, 2009). In general, if MX1 is a regulator of a specific pathway of unconventional secretion, it may not have an effect on every unconventionally secreted protein. Accordingly, one of the other unconventionally secreted proteins, MX2, was not affected by the reduction of MX1 (Figure 3-7). The MX2 protein is a component of uterine histotrophe (Ott et al., unpublished), and is believed to play a role in basic cellular function based on results showing that human MXB possesses a regulatory role in nucleocytoplasmic trafficking (King et al., 2004).

Collectively, these results suggest that ISG15 and LGALS15 may be regulated by an unconventional pathway involving MX1, whereas MX2 is not. Both FGF-2 and galectin-1, two unconventionally secreted proteins, are secreted without requiring an external stimulus (Nickel, 2011). More recent work suggests that the secretion of FGF-2 does not involve membrane vesicles, but represents a direct protein translocation across the plasma membrane (Seelenmeyer
et al., 2008; Nickel, 2011). Additional work from this same group showed that FGF-2 secretion was not affected in the presence of Y-27632, an inhibitor of plasma membrane blebbing, and they also demonstrated that FGF-2 did not induce plasma membrane blebbing (Seelenmeyer et al., 2008). In this same study, which used Chinese hamster ovarian (CHO) cells, they failed to show an effect of LGALS-1 on plasma membrane blebbing as well. These results differ from Cooper and Barondes (1990) which proposed that LGALS-1 induced plasma membrane blebbing as a mechanism during the differentiation of myoblasts, resulting in myotubes. In this data, LGALS-1 appeared highly enriched in a subset of extracellular vesicles. The authors concluded that this was because LGALS1 has a specific subcellular distribution (Cooper and Barondes, 1990; Seelenmeyer et al., 2008). Even though Seelenmeyer et al. (2008) showed that LGALS1 was being efficiently exported, they concluded that its secretion was neither mediated by plasma membrane blebbing nor by the release of exosomes derived from multivesicular bodies. They concluded that the discrepancy between these observations may be due to differences in cell processing for imaging; Cooper and Barondes fixed their cells prior to analysis and the Nickel lab analyzed their cells via live cell imaging (Seelenmeyer et al., 2008). To date, the exact mechanism of secretion for galectin-1 is unknown.

Similarly, although LGALS15 is known to be secreted via an unconventional pathway, the exact mechanism of its secretion is unknown. Interestingly, LGALS15 was the only unconventionally secreted protein examined that was not reduce in response to MX1 inhibition, but actually increased in concentration (Figure 3-6) in medium. In the ovine uterus, LGALS15 is expressed by the luminal epithelium and superficial glandular epithelium (Gray et al., 2005). It is critical to blastocyst elongation and implantation because it stimulates trophectoderm cell migration and attachment via integrin binding and activation (Farmer et al., 2008). It is possible
that by blocking one of the pathways for unconventional secretion another pathway may be upregulated even though there is no evidence for this in the literature. There is evidence, as reported by Toyokawa (2007) however, that when the drugs that inhibit conventional secretion including brefeldin A (BFA) or monensin are used, secretion of unconventionally secreted proteins increases (Toyokawa et al., 2007a).

In the analysis of conventionally secreted proteins, inhibition of MX1 protein resulted in increased secretion of the 5-kDa active form subunit of CSTL (Figure 3-9) in medium. Cathepsin L is a cysteine proteinase (peptidase) which is a lysosomal enzyme that acts at acid pH to degrade extracellular matrix and degrade intracellular proteins. The likely substrates of CSTL include proteoglycan, collagen 1, denatured collagen, and fibronectin (Salamonsen, 1999). It is known to cleave the N-terminal peptides of collagen that contain the covalent cross-links within and between collagen molecules and CSTL is hypothesized to regulate uterine receptivity for implantation and trophoblast invasion (Salamonsen, 1999). Cystatin C, an inhibitor of CSTL, is hypothesized to regulate CSTL protease action in the endometrial LE and in the trophoblast during the adhesion phases of implantation (Carson et al., 2000). Interestingly, many metastatic cell types have an invasive phenotype that is linked with increased production of cysteine proteinases (CSTL) and an abnormal regulation of cystatin C (Salamonsen, 1999). In a study using differential display RT-PCR to identify genes differentially expressed between the human prostate carcinoma cell line (PC-3) and it’s highly metastatic derivative (PC-3M), MXA was shown to be absent in the metastatic prostate cancer cells (PC-3M) (Trepel et al., 2009). This same study found that by transfecting and causing exogenous, stabile MXA to be produced in PC-3M cells and highly metastatic LOX melanoma cells, the motility and invasiveness of these cells was inhibited. Additionally, MXA decreased the number of hepatic metastases when it was
stably transfected into PC-3M cells that were then injected into the spleens of SCID mice compared to the control plasmid transfected PC-3M cells (Trepel, 2009). With this information, it is interesting to note that we observed increased secretion of the 5-kDa active form subunit of CSTL when MX1 was inhibited. However, we did not identify any changes in CSTC, or the other conventionally secreted protein examined, B2M (Figure 3-10) and CST3 (Figure 3-11). Beta-2 microglobulin is another conventionally secreted protein that is induced in the uterus of cattle and sheep at pregnancy due to P4 and/or IFNT (Vallet et al., 1991; Choi et al., 2003; Gray et al., 2006).

The increased secretion of the conventionally secreted CSTL, but not CST3 or B2M, along with changes in some but not all of the unconventionally secreted proteins examined, do not support our hypothesis that MX1 is a general regulator of unconventional secretion. However, in partial support of our hypothesis, two proteins known to be secreted via the unconventional pathway were inhibited by the inhibition of MX1. It is possible that MX1 may regulate a specific pathway in unconventional secretion. These results point to a more complex role for MX1, because proteins of each class were affected. Overall, however, these results support the hypothesis that MX1 protein may have a basic cellular function in regulating secretion. Additional experiments will be crucial in defining the specific pathway and the specific proteins regulated by MX1. Clearly, the fact that MX1 is upregulated so quickly and strongly in response to conceptus IFNT suggests it plays an important role in establishment of pregnancy in ruminants.
Figure 3-1. Western blot analysis of MX1 in lysates of oGE cells treated with morpholino antisense oligos (MAOs). Ovine glandular epithelial cells were treated with IFNT (10,000 U/mL) and a MX1-specific MAO to induce a transient reduction in MX1 protein. Western blot analysis using antibody directed against MX1 showed MX1 inhibition in the MX1 MAO treated cells lysates compared to the mismatch MAO treated cells lysates. This Western blot illustrates 3 independent replicates with 5 ug of total cell lysate loaded. Negative control is a mismatch MAO. Other controls included IFNT (10,000 U/mL) only and no treatment.
Figure 3-2. Western blot analysis of MX1 in secreted proteins from oGE cells treated with morpholino antisense oligos (MAOs). Ovine glandular epithelial cells were treated with IFNT (10,000 U/mL) and a MX1-specific MAO to knockdown MX1 protein. Western blot analysis using antibody directed against MX1 showed MX1 inhibition in the MX1 MAO treated protein secretions compared to the mismatch MAO protein secretions. This western blot illustrates 3 independent replicates with 25 ug of total secreted protein loaded. Negative control is a mismatch MAO. Other controls included IFNT (10,000 U/mL) only and no treatment.
Figure 3-3. Graph of MX1 protein in 10 ug of oGE cell lysates quantified using the MX1 MSD assay in Control – NT, IFNT Only, MX1 KD morpholino antisense oligo (MAO) + IFNT, and Mismatch MAO + IFNT treated oGE cells. Bars represent MX1 protein in ng/mL calculated by the appropriate standard curve. Standard Error of the Mean (SEM) bars are represented; a and b indicate differences (P<.001). Negative control is a Mismatch MAO. Other controls included Control (no treatment) and IFNT only.
Figure 3-4. Western blot analysis of ISG15 in secreted proteins from oGE cells treated with morpholino antisense oligos (MAOs). Ovine glandular epithelial cells were treated with IFNT (10,000 U/mL) and a MX1-specific MAO to knockdown MX1 protein. Western blot analysis using antibody directed against ISG15 showed interferon stimulated gene-15 (ISG15) inhibition in the MX1 MAO treated protein secretions compared to the mismatch MAO protein secretions. This western blot illustrates 3 independent replicates with 25 ug of total secreted protein loaded. Negative control is a mismatch MAO. Other controls included IFNT (10,000 U/mL) only and no treatment.
Figure 3-5

Western blot analysis of LGALS1 in secreted proteins from oGE cells treated with morpholino antisense oligos (MAOs). Ovine glandular epithelial cells were treated with IFNT (10,000 U/mL) and a MX1-specific MAO to knockdown MX1 protein. Western blot analysis using antibody directed against galectin-1 (LGALS1) showed LGALS1 inhibition in the MX1 MAO treated protein secretions compared to the mismatch MAO protein secretions. This western blot illustrates 3 independent replicates with 25 ug of total secreted protein loaded. Negative control is a mismatch MAO. Other controls included IFNT (10,000 U/mL) only and no treatment.
Figure 3-6. Western blot analysis of LGALS15 in secreted proteins from oGE cells treated with morpholino antisense oligos (MAOs). Ovine glandular epithelial cells were treated with IFNT (10,000 U/mL) and a MX1-specific MAO to knockdown MX1 protein. Western blot analysis using antibody directed against galectin-15 (LGALS15) showed LGALS15 protein increased when MX1 was inhibited (MX1 MAO treated) compared to the mismatch MAO protein secretions. This western blot illustrates 3 independent replicates with 25 ug of total secreted protein loaded. Negative control is a mismatch MAO. Other controls included IFNT (10,000 U/mL) only and no treatment.
Figure 3-7. Western blot analysis of MX2 in secreted proteins from oGE cells treated with morpholino antisense oligos (MAOs). Ovine glandular epithelial cells were treated with IFNT (10,000 U/mL) and a MX1-specific MAO to knockdown MX1 protein. Western blot analysis using antibody directed against MX2 showed no change in MX2 between MX1 MAO treated protein secretions and the mismatch MAO protein secretions. This western blot illustrates 3 independent replicates with 25 ug of total secreted protein loaded. Negative control is a mismatch MAO. Other controls included IFNT (10,000 U/mL) only and no treatment.
Figure 3-8. Western blot analysis of FGF2 in secreted proteins from oGE cells treated with morpholino antisense oligos (MAOs). Ovine glandular epithelial cells were treated with IFNT (10,000 U/mL) and a MX1-specific MAO to knockdown MX1 protein. Western blot analysis using antibody directed against fibroblast growth factor-2 (FGF2) was inconclusive. This western blot illustrates 3 independent replicates with 25 ug of total secreted protein loaded. Negative control is a mismatch MAO. Other controls included IFNT (10,000 U/mL) only and no treatment.
Figure 3-9. Western blot analysis of CSTL in secreted proteins from oGE cells treated with morpholino antisense oligos (MAOs). Ovine glandular epithelial cells were treated with IFNT (10,000 U/mL) and a MX1-specific MAO to knockdown MX1 protein. Western blot analysis using antibody directed against cathepsin-L (CSTL) showed increased expression of the 5-kDa active form subunit in the MX1 MAO treated protein secretions compared to the mismatch MAO protein secretions. This western blot illustrates 3 independent replicates with 25 ug of total secreted protein loaded. Negative control is a mismatch MAO. Other controls included IFNT (10,000 U/mL) only and no treatment.
Figure 3-10. Western blot analysis of B2M in secreted proteins from oGE cells treated with morpholino antisense oligos (MAOs). Ovine glandular epithelial cells were treated with a MX1-specific MAO to knockdown MX1 protein. Western blot analysis using antibody directed against Beta-2 microglobulin (B2M) showed no change in B2M between MX1 MAO treated protein secretions and the mismatch MAO protein secretions. This western blot illustrates 3 independent replicates with 25 ug of total secreted protein loaded. Negative control is a mismatch MAO. Other controls included IFNT (10,000 U/mL) only and no treatment.
Figure 3-11. Western blot analysis of CST3 in secreted proteins from oGE cells treated with morpholino antisense oligos (MAOs). Ovine glandular epithelial cells were treated with IFNT (10,000 U/mL) and a MX1-specific MAO to knockdown MX1 protein. Western blot analysis using antibody directed against cystatin-C (CST3) showed no change in CST3 between MX1 MAO treated protein secretions and the mismatch MAO protein secretions. This western blot illustrates 3 independent replicates with 25 ug of total secreted protein loaded. Negative control is a mismatch MAO. Other controls included IFNT (10,000 U/mL) only and no treatment.
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Chapter 4

EFFECTS OF MX1 OVEREXPRESSION ON PROTEIN SECRETION BY OVINE GLANDULAR EPITHELIAL CELLS

Abstract

Myxovirus resistance protein -1, MX1, is a member of the dynamin superfamily of large GTPases, which are known to participate in diverse cellular processes including endocytosis and cell migration/invasion. They share structural and functional properties with dynamins, including self-assembly and involvement on or with intracellular membranes, but are unique among this superfamily of proteins in their antiviral activity against multiple RNA viruses. The MX1 protein is also an interferon-stimulated gene that is present in both the endometrium and immune cells of ruminants in response to IFNT and progesterone during early pregnancy. We hypothesize that the MX1 protein has cellular roles in addition to its antiviral response. To characterize the function of MX1 in uterine epithelial cells we conducted experiments to overexpress the MX1 protein in ovine glandular epithelial cells (oGE) and analyze the effects on secretion of specific proteins known to be present in the uterus during the peri-implantation period.

Introduction

Early embryonic loss (before Day 30) has been estimated at 75-80%, with the majority of early embryonic losses, up to 40%, occurring between Days 8 and 17 of pregnancy (Thatcher et al., 1990; 1994). Maternal recognition of pregnancy (MRP), a term coined by Roger Short in 1969, occurs during the peri-implantation period. In domestic ruminants, the conceptus produces interferon tau (IFNT) at this time, an antiluteolysin that acts on the endometrium to alter the
luteolytic pulses of prostaglandin F2 alpha (PGF2a) and thereby save the corpus luteum (CL) and maintain progesterone (P₄) production (Short, 1969; Barros et al., 1991; Ott et al., 1992; Bazer et al., 1997; Spencer et al., 1999; Mann and Lanning, 2001; Binelli et al., 2001; Spencer and Bazer, 2002). In the ewe, IFNT is secreted by the mononuclear cells of the trophectoderm between Days 10 and 21 to 25, with maximal production falling between Day 14 and Day 16 (Bazer, 1992; Roberts et al., 1999). In addition to blocking luteolysis, when IFNs bind to their receptors they activate the JAK-STAT signaling pathway, which leads to the upregulation of a large number of interferon stimulated genes in the uterus, CL and peripheral blood immune cells (Vallet et al., 1991; Mirando et al., 1991; Ott et al., 1998; Spencer et al., 1998; Hansen et al., 1999; Johnson et al., 1999a; 1999b; 1999c; 2000; 2001; Stewart et al., 2001a; 2002; Gray et al., 2002; Choi et al., 2003; Kim et al., 2003; Gray et al., 2004; Song et al., 2005; Gray et al., 2006; Song et al., 2006b; 2007; Bazer et al., 2010).

One of these genes, the myxovirus resistance-1, MX1, gene, is an integral part of the innate immune system, and is highly upregulated in response to type I IFN (Haller et al., 2007). In response to IFNT and progesterone, the MX1 protein is upregulated in the endometrium of ruminants (Charleston and Stewart, 1993; Ott et al., 1998), is a component of uterine secretions, and is increased in early pregnant ewes (Toyokawa et al., 2007a).

Outside of its role in the immune system, there is little known about the function of MX1, however MX proteins exhibit characteristics that would suggest they have a basic cellular function. Human MXB, for example, has been implicated in nuclear import and cell cycle progression (King et al., 2004) and recently, MX1 was shown to be constitutively expressed in an ovine glandular epithelial (oGE) cell line in the absence of exogenous virus or IFN (Racicot and Ott, 2010). Work has recently demonstrated that MX1 is an unconventionally secreted
protein (Toyokawa et al., 2007a; 2007b). Unconventionally secreted proteins lack a canonical secretion signal and are secreted even when conventional secretory pathways (ER-Golgi) are blocked (Hejine, 1985; Nickel, 2005).

Collectively these studies support the hypothesis that MX1 is involved in the endometrial secretory process during early pregnancy by regulating secretion in uterine epithelial cells (Toyokawa et al., 2007a; 2007b). The objective of these experiments was to identify proteins that may be regulated by MX1. Because MX1 is induced in the endometrial epithelium by the conceptus, we propose that MX1 may mediate the uterine response to the conceptus by increasing secretion of specific proteins that are necessary for conceptus growth and development. In this study the MX1 protein was selectively overexpressed in oGE cells to evaluate any changes in proteins secreted via the conventional and unconventional secretory pathways.

**Materials and methods**

**Cloning**

A full length MX1 cDNA (NCBI sequence # X66093) in the PCRII-TOPO vector was prepared using sequential restriction enzyme sequential digestion with SalI (NEB, Ipswich, MA) and KpnI (NEB, Ipswich, MA). These same enzymes were also used to cut the pCMV-Myc mammalian expression vector as well (Clontech, Mountain View, CA). The PCR products were gel purified using the QIAquick Gel Extraction Kit (50; Qiagen, Valencia, CA). The vector and clone was ligated together and then One Shot® TOP10 Chemically Competent E. coli (Invitrogen) were transformed and selected on LB agar plates containing ampicillin. Plasmid sequencing was conducted using the Big Dye system (Applied Biosystems, Foster City, CA) and
the ABI Hitachi 3730XL DNA Analyzer at the genomics core facility (The Huck Institutes of Life Sciences, Penn State). Sequence analysis was accomplished using the NCBI BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

**Cell culture and treatments**

Immortalized ovine glandular epithelial cells (oGE) cells (Johnson et al., 1999) were cultured in T25 flasks in Dulbecco’s Modified Eagle’s Medium (15.63 g/L DMEM, Sigma, St Louis, MO) with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY) and 5% antibiotic antymycotic (ABAM; Gibco) under 5% CO2 at 38.5°C. When the cells were approximately 90% confluent, they were transfected with either the pCMV-Myc mammalian expression vector or the pCMV-Myc mammalian expression vector containing the MX1 cDNA. There was also a no treatment control, which did not receive any transfection reagents or plasmid and an IFNT only control which received only IFNT. Transfection of the pCMV-Myc plasmid was accomplished using Lipofectamine™ (Invitrogen), due to its wide acceptance and reliability in transfecting plasmid DNA into cells and CombiMag reagent (Oz Biosciences), which is designed to be used with any other transfection reagent and uses magnetic nanoparticles for improving transfection reagent efficiency.

Lipofectamine™ 2000 Transfection Reagent (Invitrogen) (6 uL), CombiMag (Oz Biosciences) (3 uL), control plasmid (3 ug), or MX1 plasmid (3 ug) were each mixed with Opti-MEM Reduced Serum medium (Opti-MEM RS) (Invitrogen) to a total volume of 200uL and incubated at room temperature for 5 minutes. The plasmids were then mixed with the Lipofectamine, and immediately added to the CombiMag, mixed vigorously by repeated pipetting, and incubated at room temperature for 20 minutes. Culture medium was then aspirated, and the cells were washed twice with Opti-MEM RS. The controls, no treatment and
IFNT only, received 3 mL of Opti-MEM RS. The MX1 and control plasmid treatments received 2.4 mL of Opti-MEM RS and the plasmid/Lipofectamine/CombiMag mixture (600uL) was also added to the appropriate flasks to bring their total volume to 3mL. All of the flasks were incubated in 5% CO2 at 38.5°C on a magnet (Oz Biosciences) for 15 minutes. The magnet was then removed, and the flasks were left to incubate under 5% CO2 at 38.5°C for 6 hours. Opti-MEM RS media containing the plasmid/Lipofectamine/CombiMag mixture was removed and fresh phenol free Dulbecco’s Modified Eagle’s Medium (15.63 g/L DMEM, Sigma, St Louis, MO) with 5% antibiotic antimycotic (ABAM; Gibco), without ABAM or FBS was added to the flasks. Appropriate flasks were then treated with recombinant ovine IFNT (10,000U/mL; provided by Fuller W. Bazer, Texas A&M University) and all of the flasks were incubated under 5% CO2 at 38.5°C for 48 hours. At the end of 48 hours, conditioned media were collected and protease inhibitor cocktail without EDTA (Halt, Pierce, Rockford, IL) was added to the conditioned media to achieve a final concentration of 1X. The conditioned medium was centrifuged at 2700 x g for 10 minutes to remove cellular debris. Then, the medium was concentrated using Amicon Ultra 3k (Millipore Corporation, Billerica, MA) centrifuge tubes, washed 2 times with triple distilled water, and ultimately concentrated from 5 mL to approximately 250 uL. Lysates were collected using Mammalian Protein Extraction Reagent (MPER; Pierce, Rockford, IL) according to the manufacturer’s instructions. Protein concentrations were quantified with the bicinchonic acid assay (Pierce, Rockford, IL), according to the manufacturer’s instructions using bovine serum albumin (BSA) as the standard. All treatments were done in triplicate and the entire experiment was replicated at least 3 independent times.
Western blot analysis

Secreted total protein samples (20 ug) were dissolved in 1X sample buffer (4X: 7.5 mL dH2O/ 760 mg Tris base/ 2 g sodium dodecyl sulfate (SDS)/ 10 mL glycerol/ pH to 6.5/ 5 mL 2-Bmercaptoethanol/ 300 uL bromphenol blue), heated at 95°F for 5 minutes, and separated on a SDS PAGE gel (6% stacking, 10 and 12% resolving gels) in 1X electrode buffer (10X: 30.3 g Tris base/ 144.2 g glycine/ 10 g SDS /pH to 8.3/ add dH2O to 1.0 L) at a constant current (70mA) for approximately 1.5 hr. Lysate total protein (10 ug) was prepared similarly. Following electrophoresis proteins were transferred to nitrocellulose membranes (Invitrogen) using the iBlot® Dry Blotting System (Invitrogen) for approximately 8 minutes at 20 volts. Non-fat milk (5%) was used as a blocker for the MX1, C-MYC, MX2, LGALS15, CST3 proteins and bovine serum albumin (BSA) was used as a blocker for ISG15, LGALS1, FGF2, B2M, CSTL proteins. Blockers were dissolved in tris buffered saline tween-20 (TBST) (20 mL1M Tris pH 7.5/ 137 mL 1M NACL/ 500 uL Tween 20/ dH2O to 1.0 L).

Immunoblotting was performed with a 1:1000 dilution of an amino terminal rabbit polyclonal ovine MX1 antiserum (#90618, bleed number 3, prepared by Multiple Peptide Systems, San Diego, CA), a 1:3000 dilution of a monoclonal mouse C-MYC (Provided by Dr. Anthony Schmitt, Penn State University), 1:1000 dilution of rabbit polyclonal human B2M antiserum (Abcam, Catalog# ab15976, Cambridge, MA), a 1:2000 dilution of mouse monoclonal bovine ISG15 (Provided by Dr. Thomas R. Hansen, Colorado State University), a 1:500 dilution of goat anti-human galectin-1 (R&D, Catalog# AF1152, MN), a 1:1000 dilution of rabbit polyclonal ovine galectin-15 antiserum (Provided by Tom Spencer, Texas A&M University), a 1:1000 dilution of rabbit polyclonal ovine MX2 antiserum (#9708, bleed 3, MPS), a 1:500 dilution of
rabbit anti-human/bovine FGF basic (R&D, Catalog# AB-33-NA, MN), a 1:1000 dilution of rabbit polyclonal human CST3 (Upstate, Catalog# 06-458, Temecula, CA), or a 1:500 dilution of rabbit anti-human/mouse/rat cathepsin L (BioVision, Catalog # 3192-100, Mountain View, CA) with 2% NFM/BSA rocking at 4°C overnight. Membranes were washed with TBST the following morning and a 1:200,000 dilution of goat anti-rabbit, goat anti-mouse, or rabbit anti-goat IgG-horseradish peroxidase conjugate (Pierce, Rockford, IL) was added. Membranes were then rocked at room temperature for an hour and washed with TBST again. Super Signal® West Femto Maximum Sensitivity Substrate chemiluminescence kit (Pierce) was added to the membranes for 10 minutes at room temperature to detect the immunoreactive proteins. Detection of the chemiluminescence signal was performed using the Bio-Rad Chemidoc-XRS Multiimager system and Quantity One software (Bio-Rad, Hercules, CA).

Results

The pCMV-Myc mammalian expression vector containing a full-length MX1 cDNA was utilized to overexpress the MX1 protein in oGE cells. This plasmid causes the exogenous MX1 protein to be expressed fused with a c-myc epitope tag which allows for an assessment of the transfection/overexpression efficiency using a c-myc antibody. Transfection with the pCMV-Myc plasmid containing a full-length MX1 cDNA resulted in MX1 overexpression in both total cell lysates (Figure 4-1) and medium (Figure 4-3). The c-myc antibody was used to evaluate the exogenous MX1 that was overexpressed in the samples that received the pCMV-MYC plasmid containing the full-length MX1 cDNA (lysates; Figure 4-2 and the medium; Figure 4-4). Medium was then analyzed for the panel of conventionally and unconventionally secreted proteins to assess the effect of MX1 overexpression on secretion. Two of the unconventionally
secreted proteins analyzed were inconclusive due to the low amount of secreted protein, ISG15 (Figure 4-5), and FGF2 (Figure 4-6). There was no effect of MX1 overexpression on secretion of the unconventionally secreted proteins LGALS1 (Figure 4-7), LGALS15, (Figure 4-8), MX2 (Figure 4-9). Likewise there was no effect of treatment on conventionally secreted proteins CSTL (Figure 4-10), B2M (Figure 4-11), CST3 (Figure 4-12).

**Discussion**

The objective of this study was to determine if overexpression of the MX1 protein in oGE cells would affect secretion of unconventionally or conventionally secreted proteins. Our working hypothesis is that MX1 is a mediator of unconventional secretion in the uterus during early pregnancy. Previous work from showed that secretion of the unconventionally secreted ISG15, but not the conventionally secreted B2M was reduced when MX1 mRNA was blocked using siRNA (Toyokawa et al., 2007a; 2007b). This led to the hypothesis that MX1 is a regulator of unconventional secretion. However, those treatments included IFNT and it was not possible to determine the specific effects of MX1. Here our objective was to test this hypothesis by including a broader range of conventionally and unconventionally secreted proteins and inducing overexpression of rMX1 in the absence of IFNT. Using this approach, the effect of MX1 could be evaluated without other ISGs being upregulated and therefore complicating the interpretation of the results.

Western blot analysis using an antibody directed against MX1 showed greater MX1 protein expression in the MX1 plasmid treated lysates and secretions compared to vehicle treated lysates and secretions (Figures 4-1; 4-3). In these samples, the expression of the MX1 proteins was comparable to the IFNT treated sample. The MX1 protein overexpressed using this system
contained an amino terminal c-myc tag, and using an antibody directed against c-myc we were able to differentiate endogenous from exogenous MX1 (Figures 4-2; 4-4). Although we had conformation of MX1 overexpression through western blot analysis (Figures 4-1, 4-2, 4-3, 4-4), there was no effect on the unconventionally (Figure 4-7; 4-8; 4-9) or conventionally secreted proteins (Figure 4-10; 4-11; 4-12) examined. Additionally, two of the unconventionally secreted proteins analyzed were inconclusive due to the low amount of secreted protein (Figure 4-5; 4-6).

There are several explanations for these results. The simplest explanation is that MX1 is not involved in regulation of secretion. However, published results and experiments described in this thesis make this unlikely. What is more likely is that no effect was detected because the proteins regulated by MX1 would also need to be increased to be able to detect effects of overexpression of MX1. Therefore to evaluate whether MX1 can mediate the secretion of these proteins, MX1 will need to be overexpressed in combination with these unconventionally and conventionally secreted proteins. By doing this, the intracellular protein amount of any given protein can be expressed at a high level and in combination with MX1 overexpression, compared to low/endogenous MX1 expression, any changes in secretion can be determined. Future experiments should overexpress MX1 and the putative proteins whose secretion may be mediated by MX1. Through this approach, it could then be determined if MX1 alone has an effect on unconventionally secreted proteins. This approach also has weaknesses as well. If MX1 participates as part of a multi-protein complex or in a sequence of processes, then sole overexpression of MX1 might not yield any changes in protein expression.

It is also possible that no effect was detected due to presence of the myc tag on the rMX1. It is possible that this tag could interfere with the function of MX1. However, the myc-tagged rMX1 was secreted in high concentration suggesting that it was still traversing the same
physiological pathways as native MX1. In either case, one could overexpress MX1 without the tag to rule out this possibility.

Although the system for overexpressing MX1 was considered to be successful and we now have the ability to evaluate the effects of MX1 without other ISGs being upregulated, there was no effect on the unconventionally or conventionally secreted proteins examined. We suspect that no effect was detected because the proteins regulated by MX1 would also need to be increased to be able to detect any changes in secretion caused from overexpressing MX1. Our next step in this experiment will be to overexpress MX1 in combination with these proteins.
Figure 4-1. Western blot analysis of exogenous and endogenous MX1 in lysates of oGE cells transfected with the pCMV-Myc mammalian expression vector containing a full-length MX1 cDNA. Ovine glandular epithelial cells were transfected with the pCMV-Myc mammalian expression vector containing a full-length MX1 cDNA to overexpress the MX1 protein. Western blot analysis using an antibody directed against MX1 showed higher MX1 protein amounts compared to vehicle treated lysates. This Western blot illustrates 3 independent replicates with 10 ug of total lysates loaded. Negative control is vehicle only. Other controls included IFNT only and no treatment.
Figure 4-2. Western blot analysis of C-myc (exogenous MX1) in lysates of oGE cells transfected with the pCMV-Myc mammalian expression vector containing a full-length MX1 cDNA. Ovine glandular epithelial cells were transfected with the pCMV-Myc mammalian expression vector containing a full-length MX1 cDNA to overexpress the MX1 protein. Western blot analysis using an antibody directed against c-myc showed the c-myc epitope tag which fuses with MX1 protein expressed by the vector in the MX1 plasmid treated lysates. This Western blot illustrates 3 independent replicates with 10 ug of total lysates loaded. Negative control is vehicle only. Other controls included IFNT only and no treatment.
Figure 4-3

Figure 4-3. Western blot analysis of exogenous and endogenous MX1 in secreted protein from oGE cells transfected with the pCMV-Myc mammalian expression vector containing a full-length MX1 cDNA. Ovine glandular epithelial cells were transfected with the pCMV-Myc mammalian expression vector containing a full-length MX1 cDNA to overexpress the MX1 protein. Western blot analysis using an antibody directed against MX1 showed higher MX1 protein amounts compared to vehicle treated secreted protein. This Western blot illustrates 3 independent replicates with 20 ug of total secreted protein loaded. Negative control is vehicle only. Other controls included IFNT only and no treatment.
Figure 4-4. Western blot analysis of C-myc (exogenous MX1) in secreted protein from oGE cells transfected with the pCMV-Myc mammalian expression vector containing a full-length MX1 cDNA. Ovine glandular epithelial cells were transfected with the pCMV-Myc mammalian expression vector containing a full-length MX1 cDNA to overexpress the MX1 protein. Western blot analysis using an antibody directed against C-myc identified the C-myc epitope tag which fuses with MX1 protein expressed by the vector in the MX1 plasmid treated protein secretions only. This Western blot illustrates 3 independent replicates with 20 ug of total lysates loaded. Negative control is vehicle only. Other controls included IFNT only and no treatment.
Figure 4-5. Western blot analysis of ISG15 in secreted protein from oGE cells transfected with the pCMV-Myc mammalian expression vector containing a full-length MX1 cDNA. Ovine glandular epithelial cells were transfected with the pCMV-Myc mammalian expression vector containing a full-length MX1 cDNA to overexpress the MX1 protein. Western blot analysis using an antibody directed against interferon stimulated gene-15 (ISG15) showed ISG15 in the IFNT treated sample only. This Western blot illustrates 3 independent replicates with 20 ug of total secreted protein loaded. Negative control is vehicle only. Other controls included IFNT only and no treatment.
Figure 4-6. Western blot analysis of FGF2 in secreted protein from oGE cells transfected with the pCMV-Myc mammalian expression vector containing a full-length MX1 cDNA. Ovine glandular epithelial cells were transfected with the pCMV-Myc mammalian expression vector containing a full-length MX1 cDNA to overexpress the MX1 protein. Western blot analysis using antibody directed against fibroblast growth factor-2 (FGF2) showed no difference in FGF2 between the MX1 overexpression treated secreted protein and vehicle treated secreted protein. This Western blot illustrates 3 independent replicates with 20 ug of total secreted protein loaded. Negative control is vehicle only. Other controls included IFNT only and no treatment.
Figure 4-7.

**Figure 4-7. Western blot analysis of LGALS1 in secreted protein from oGE cells transfected with the pCMV-Myc mammalian expression vector containing a full-length MX1 cDNA.** Ovine glandular epithelial cells were transfected with the pCMV-Myc mammalian expression vector containing a full-length MX1 cDNA to overexpress the MX1 protein. Western blot analysis using an antibody directed against galectin-1 (LGALS1) showed no difference in LGALS1 between the MX1 overexpression treated secreted protein and vehicle treated secreted protein. This Western blot illustrates 3 independent replicates with 20 ug of total secreted protein loaded. Negative control is vehicle only. Other controls included IFNT only and no treatment.
Figure 4-8. Western blot analysis of LGALS15 in secreted protein from oGE cells transfected with the pCMV-Myc mammalian expression vector containing a full-length MX1 cDNA. Ovine glandular epithelial cells were transfected with the pCMV-Myc mammalian expression vector containing a full-length MX1 cDNA to overexpress the MX1 protein. Western blot analysis using an antibody directed against galectin-15 (LGALS15) showed no difference in LGALS15 between the MX1 overexpression treated secreted protein and vehicle treated secreted protein. This Western blot illustrates 3 independent replicates with 20 ug of total secreted protein loaded. Negative control is vehicle only. Other controls included IFNT only and no treatment.
Figure 4-9. Western blot analysis of MX2 in secreted protein from oGE cells transfected with the pCMV-Myc mammalian expression vector containing a full-length MX1 cDNA. Ovine glandular epithelial cells were transfected with the pCMV-Myc mammalian expression vector containing a full-length MX1 cDNA to overexpress the MX1 protein. Western blot analysis using an antibody directed against MX2 showed no difference in MX2 between the MX1 overexpression treated secreted protein and vehicle treated secreted protein. This Western blot illustrates 3 independent replicates with 20 ug of total secreted protein loaded. Negative control is vehicle only. Other controls included IFNT only and no treatment.
Figure 4-10. Western blot analysis of CSTL in secreted protein from oGE cells transfected with the pCMV-Myc mammalian expression vector containing a full-length MX1 cDNA. Ovine glandular epithelial cells were transfected with the pCMV-Myc mammalian expression vector containing a full-length MX1 cDNA to overexpress the MX1 protein. Western blot analysis using an antibody directed against cathepsin-L (CSTL) showed no difference in the 38- to 40-kDa latent pro-CTSL between the MX1 overexpression treated secreted protein and vehicle treated secreted protein. This Western blot illustrates 3 independent replicates with 20 ug of total secreted protein loaded. Negative control is vehicle only. Other controls included IFNT only and no treatment.
Figure 4-11

Figure 4-11. Western blot analysis of B2M in secreted protein from oGE cells transfected with the pCMV-Myc mammalian expression vector containing a full-length MX1 cDNA. Ovine glandular epithelial cells were transfected with the pCMV-Myc mammalian expression vector containing a full-length MX1 cDNA to overexpress the MX1 protein. Western blot analysis using antibody directed against beta-2 microglobulin (B2M) showed no difference in B2M between the MX1 overexpression treated secreted protein and vehicle treated secreted protein. This Western blot illustrates 3 independent replicates with 20 ug of total secreted protein loaded. Negative control is vehicle only. Other controls included IFNT only and no treatment.
Figure 4-12. Western blot analysis of CST3 in secreted protein from oGE cells transfected with the pCMV-Myc mammalian expression vector containing a full-length MX1 cDNA. Ovine glandular epithelial cells were transfected with the pCMV-Myc mammalian expression vector containing a full-length MX1 cDNA to overexpress the MX1 protein. Western blot analysis using antibody directed against cystatin-C (CST3) showed no difference in CST3 between the MX1 overexpression treated secreted protein and vehicle treated secreted protein. This Western blot illustrates 3 independent replicates with 20 ug of total secreted protein loaded. Negative control is vehicle only. Other controls included IFNT only and no treatment.
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Chapter 5

DETERMINATION OF THE EFFECT OF MX1 INHIBITION OR OVEREXPRESSION ON THE GLOBAL ARRAY OF PROTEINS SECRETED BY OGE CELLS

Abstract

The myxovirus resistance (MX) proteins are members of the dynamin superfamily of large GTPases, and members of this family are known to participate in diverse cellular processes including endocytosis and cell migration/invasion. The MX1 protein shares structural and functional properties with dynamins, such as self-assembly and involvement on or with intracellular membranes, but is unique among this superfamily of proteins in its antiviral activity against multiple RNA viruses. It is also an interferon-stimulated gene that is present in both the endometrium and immune cells of ruminants in response to interferon-tau (the maternal recognition of pregnancy signal from the conceptus) and progesterone. We hypothesize that MX1 functions in cellular processes that do not involve the antiviral response. The objective of this study was to identify cellular proteins whose patterns of secretion change when MX1 is altered.

Abundance of MX1 was modulated by transient knockdown and overexpression as described in Chapters 3 and 4, respectively. The objective of these experiments was to identify proteins regulated by MX1 by using knockdown and overexpression models and examining the secretory proteome using 2-dimensional gel electrophoresis followed by mass spectrometry of differentially expressed proteins.

Overall, the methods for modulating MX1 via transient knockdown and overexpression now give us the ability to identify cellular proteins whose patterns of secretion are altered by altering MX1. In the MX1 inhibitions gels, 8 proteins were potentially identified that were
inhibited when MX1 was inhibited, and 2 proteins were potentially identified in spots that increased when MX1 was inhibited. When MX1 was overexpressed, 4 proteins were potentially identified in spots that were increased as well. The percent of matched peptides between the potential proteins and the peptides recovered from the spots along with the quality score, varied greatly for each of the proteins. Further experiments with higher amounts of protein loaded, and from this new information will be the next steps to further these investigations.

**Introduction**

In cattle, substantial embryonic loss occurs during the peri-implantation period (25-40%), resulting in losses approaching half a billion dollars annually (Sreenan and Diskin, 1986; Humblot, 2001; Moore et al., 2005). During the peri-implantation period in domestic ruminants, the conceptus communicates to the maternal system by producing IFNT, which alters uterine gene expression, and prevents luteal regression by binding type I IFN receptors on the endometrial epithelium and altering the luteolytic pulses of PGF$_{2\alpha}$ (Spencer et al., 1996; Roberts et al., 1999; Spencer and Bazer, 2002). Interferons bind to Type I IFN receptors and activate JAK-STAT signaling leading to the upregulation of a large number of interferon stimulated genes not only in the uterus but in the corpus luteum (CL) and peripheral blood immune cells (Vallet et al., 1991; Mirando et al., 1991; Ott et al., 1998; Spencer et al., 1998; Hansen et al., 1999; Johnson et al., 1999a; 1999b; 1999c; 2000; 2001; Stewart et al., 2001; 2002; Gray et al., 2002; Choi et al., 2003; Kim et al., 2003; Gray et al., 2004; Song et al., 2005; Gray et al., 2006; Song et al., 2006; 2007; Bazer et al., 2010). The synthesis and secretion of a large number of endometrial proteins is thought to be important for both conceptus growth and placentation, both which are critical for embryonic-survival.
Work from our lab and others (Charleston and Stewart, 1993) determined that the myxovirus resistance proteins are upregulated in the endometrium of ruminants in response to IFNT and progesterone ($P_4$) and pregnancy (Charleston and Stewart, 1993; Ott et al., 1998). Expression of MX1 mRNA in the endometrium of non-pregnant sheep was highest when $P_4$ concentrations were at their maximal Days 11-13 after estrus. In pregnant ewes, MX1 expression was significantly greater and the highest expression levels coincided with maximal production of IFNT on Day 17 (Ott et al., 1998). Subsequently it was determined that MX1 was constitutively expressed in oGE cells and interacts with microtubules (Racicot and Ott, 2010) supporting that the MX1 protein may have a cellular function outside of its well-known antiviral activity.

The MX proteins belong to the dynamin superfamily of large GTPases. Members of this superfamily have cellular functions that include protein trafficking and endocytosis, leading us to hypothesize that MX proteins have similar roles. Recently, we showed that MX1 was a component of uterine secretions and MX1 concentrations in uterine secretions increased during early pregnancy in sheep (Toyokawa et al., 2007a). Furthermore, when MX1 mRNA expression was inhibited in oGE cells, secretion of the unconventionally secreted protein, ISG15, was also reduced. A conventionally secreted protein, beta-2 microglobulin, was unaffected in this study (Toyokawa et al., 2007a). Work from this same report also reported, for the first time, that MX1 was itself an unconventionally secreted.

Regulation of MX1 protein by IFNT and progesterone, along with the constitutive expression of this protein suggest that it may play role outside of its antiviral function, and may play a role in early pregnancy (Ott et al., 1998; Racicot and Ott, 2010). Our working hypothesis is that MX1 protein functions like other members of the dynamin superfamily, and is involved in the endometrial secretory process during early pregnancy (Toyokawa et al., 2007a; 2007b). The
objective of these experiments was to identify proteins regulated by MX1 by manipulating concentrations of MX1 using knockdown and overexpression models developed in Chapters 3 and 4, respectively, and examining the secretory proteome using 2-dimensional gel electrophoresis followed by mass spectrometry of differentially expressed proteins. The MX1 protein was selectively overexpressed by transfecting oGE cells with the pCMV-Myc mammalian expression vector (Clontech) containing a full-length MX1 cDNA. This approach offered a way to selectively upregulate MX1 protein without having to use IFNT which upregulates a large number of known and unknown ISGs (Vallet et al., 1991; Mirando et al., 1991; Ott et al., 1998; Spencer et al., 1998; Hansen et al., 1999; Johnson et al., 1999a; 1999b; 1999c; 2000; 2001; Stewart et al., 2001; 2002; Gray et al., 2002; Choi et al., 2003; Kim et al., 2003; Gray et al., 2004; Song et al., 2005; Gray et al., 2006; Song et al., 2006; 2007; Bazer et al., 2010). The pCMV-Myc mammalian expression vector allows a protein of interest to be expressed fused with the c-Myc epitope tag.

The MX1 protein was selectively inhibited by blocking the mRNA translation of MX1 in oGE cells using morpholino antisense oligos (MAOs). Morpholinos can block the translation of a protein or nuclear processing (splicing), depending on the sequence they target. The morpholino in this experiment has been designed to block the translation of MX1 protein.

**Materials and methods**

**Cell culture and treatments for inhibition of MX1**

Immortalized ovine glandular epithelial cells (oGE; Johnson et al., 1999) were cultured in T25 flasks in Dulbecco’s Modified Eagle’s Medium (15.63 g/L DMEM, Sigma, St Louis, MO) with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY) and 5% antibiotic
antimycotic (ABAM; Gibco) under 5% CO2 at 38.5°C. When the cells were approximately 90% confluent, morpholino treatments were dissolved in nuclease-free water and were added to the cells at a 20 uM final concentration in fresh Dulbecco’s Modified Eagle’s Medium (15.63 g/L DMEM, Sigma, St Louis, MO) with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY) and 5% antibiotic antimycotic (ABAM; Gibco) along with endoporter reagent (6 uL/mL). The MX1 MAO has been designed by the technical support scientists at Gene Tools based on the following requirements/recommendations; select a target sequence in the post-spliced mRNA in the region from the 5'cap to about 25 bases 3' to the AUG translational start site, ensure the selected sequence has little or no self-complementarity, and it needs to have less than 3 contiguous guanines and 25 bases long. The lack of intron sequence available for the ovine MX1 pre-mRNA limits the use of morpholinos which block splicing events. The morpholinos were delivered to the cytoplasm of these cells using Endo-porter reagent available for sale by Gene Tools.

The cells incubated at 38.5°C for 6 hours. The media with MAO’s and endoporter was then removed. Fresh, phenol-free, DMEM (15.63 g/L DMEM, Sigma, St Louis, MO) with 5% antibiotic antimycotic (ABAM; Gibco), without FBS containing IFNT (10,000 U/mL; provided by Fuller W. Bazer, Texas A&M University), 20 uM MAO, and endoporter (6 uL/mL) was added back to the cells. These cells were then incubated for 48 hours at 38.5°C. Conditioned medium was collected at this point and an EDTA-free protease inhibitor cocktail (Pierce, Rockford, IL) was added to the conditioned medium to a 1X final concentration. The conditioned medium was centrifuged at 2700 x g for 10 minutes to pellet cellular debris. The clarified media were then concentrated using Amicon Ultra 3k (Millipore Corporation, Billerica, MA) centrifuge tubes, washed twice with triple distilled water, and concentrated from 5 mL to
approximately 250 uL. Cellular lysates were prepared using Mammalian Protein Extraction Reagent (MPER; Pierce, Rockford, IL) according to the manufacturer’s instructions. Protein concentrations were quantified using the bicinchonic acid assay (Pierce, Rockford, IL), according to the manufacturer’s instructions using bovine serum albumin (BSA) as the standard. All MAO’s were purchased from Gene Tools Inc.

The treatments included an IFNT only group (did not receive MAO’s or endoporter, but did receive IFNT), a MX1-specific MAO group (received MX1-specific MAO’s, endoporter, and IFNT), and a mismatch MAO group (received nonsense MAO’s, endoporter, and IFNT).

**Cell culture and treatments for overexpression of MX1**

Immortalized ovine glandular epithelial cells (oGE) cells (Johnson et al., 1999) were cultured in T25 flasks in Dulbecco’s Modified Eagle’s Medium (15.63 g/L DMEM, Sigma, St Louis, MO) with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY) and 5% antibiotic antimycotic (ABAM; Gibco) under 5% CO2 at 38.5°C. When the cells were approximately 90% confluent, they were transfected with either the pCMV-Myc mammalian expression vector or the pCMV-Myc mammalian expression vector containing the MX1 cDNA. There was also a no treatment control, which did not receive any transfection reagents or plasmid. Transfection of the pCMV-Myc plasmid was accomplished using Lipofectamine™ (Invitrogen), due to its wide acceptance and reliability in transfecting plasmid DNA into cells and CombiMag reagent (Oz Biosciences), which is designed to be used with any other transfection reagent and uses magnetic nanoparticles for improving transfection reagent efficiency.

Lipofectamine™ 2000 Transfection Reagent (Invitrogen) (6 uL), CombiMag (Oz Biosciences) (3 uL), control plasmid (3 ug), or MX1 plasmid (3 ug) were each mixed with Opti-MEM Reduced Serum media (Opti-MEM RS) (Invitrogen) to a total volume of 200 uL and
incubated at room temperature for 5 minutes. The plasmids were then mixed with the Lipofectamine, and immediately added to the CombiMag, mixed vigorously by repeated pipetting, and incubated at room temperature for 20 minutes. Culture medium was then aspirated, and the cells were washed twice with Opti-MEM RS. The control, no treatment, received 3 mL of Opti-MEM RS. The MX1 and control plasmid treatments received 2.4 mL of Opti-MEM RS and the plasmid/Lipofectamine/CombiMag mixture (600 uL) was also added to the appropriate flasks to bring their total volume to 3 mL. All of the flasks were incubated in 5% CO2 at 38.5°C on a magnet (Oz Biosciences) for 15 minutes. The magnet was then removed, and the flasks were left to incubate under 5% CO2 at 38.5°C for 6 hours. Opti-MEM RS media containing the plasmid/Lipofectamine/CombiMag mixture was removed and fresh phenol free Dulbecco’s Modified Eagle’s Medium (15.63 g/L DMEM, Sigma, St Louis, MO) with 5% antibiotic antimycotic (ABAM; Gibco), without ABAM or FBS was added to the flasks. All of the flasks were incubated under 5% CO2 at 38.5°C for 48 hours. At the end of 48 hours, conditioned medium were collected and protease inhibitor cocktail without EDTA (Halt, Pierce, Rockford, IL) was added to the conditioned medium to achieve a final concentration of 1X. The conditioned medium was centrifuged at 2700 x g for 10 minutes to remove cellular debris. Then, the clarified medium was concentrated using Amicon Ultra 3k (Millipore Corporation, Billerica, MA) centrifuge tubes, washed 2 times with triple distilled water, and ultimately concentrated from 5 mL to approximately 250 uL. Lysates were collected using Mammalian Protein Extraction Reagent (MPER; Pierce, Rockford, IL) according to the manufacturer’s instructions. Protein concentrations were quantified with the bicinchoninic acid assay (Pierce, Rockford, IL), according to the manufacturer’s instructions using bovine serum albumin (BSA) as the standard.
Lyophylization of samples

A total of 400 ug of secreted proteins were used for the inhibition gels and included the three treatments stated above. For the overexpression gels, a total of 230 ug of secreted proteins were run and treatments consisted of the treatments stated above. These samples were covered with a parafilm seal and a sterile needle was used to ventilate the parafilm to allow for water vapor to escape during lyophilyzation. Samples were removed from -80°C and placed directly onto a shelf inside the freeze-dryer (VirTis, Gardiner, NY). The freeze-dryer was set at 100 millitorr and -56°C for approximately 24 hours. Samples were removed from the freeze-dryer and stored at -80°C until the first dimension of 2D gel electrophoresis.

Two dimensional gel electrophoresis

Rehydration buffer (BioRad, Hercules, CA) was prepared by adding 6.1 mL of nanopure water and mixing. The prepared rehydration buffer, 200 uL, was added to microcentrifuge tubes containing the lyophilized samples. Samples were sonicated at room temperature for 10 minutes and then vortexed at room temperature, medium speed, for 5 minutes to ensure full rehydration. The microcentrifuge tubes were then centrifuged at 15,000 RPM for 20 minutes at 4°C to eliminate interfering contaminants. At this point, the top 185 uL of sample were transferred to the back wall of an IEF focusing tray, leaving 1 cm on each end. An IPG strip (BioRad, Hercules, CA) was placed over the sample, gel side down with the positive end on the left side of the tray. The IPG strip was incubated for 1 hour at room temperature, and then was overlaid with 1.5 mL of mineral oil and allowed to incubate overnight at room temperature. The following morning the IPG strips were removed from the focus tray and transferred to the IEF cell with the positive end on the left side of the tray. The strips were again overlaid with 1.5 mL
of mineral oil and immediately the protocol for first dimension of 2D gel electrophoresis began and went as follows:

1- Ramp rapid to 250 V for 15 minutes
2- Ramp rapid to 8,000 V for 2.5 hours
3- Ramp rapid 8,000 Vhours for 20,000 Vhours
4- Hold at 500 V indefinitely

At the end of the run, IPG strips were removed from the IEF cell and the mineral oil was decanted. The strips were placed back into the focusing tray, gel side up, covered with plastic wrap and placed in -40 °C.

Equilibration buffer 1 (BioRad, Hercules, CA) was prepared by mixing the supplied substrate with 13.35 mL of 30% glycerol on a magnetic stirrer. IPG strips were removed from the freezer and allowed to thaw at room temperature for 10 minutes. Equilibration buffer 1 (1.5 mL/well) was added to each strip and incubated at room temperature for 10 minutes on a rocking platform. Equilibration buffer 2 (BioRad, Hercules, CA) was prepared by mixing the supplied substrate with 13.35 mL of 30% glycerol and 0.5 g iodacetamide. Once equilibration buffer 1 was removed, 1.5 mL of equilibration buffer 2 was added to each well and returned to the rocker for 10 minute incubation. Overlay agarose (Sharp, Mahwah, NJ) was prepared by heating in the microwave two times for 15 seconds. Equilibration buffer 2 was decanted from the IPG strips and the IPG strips were rinsed by dipping into a cylinder filled with 1X electrode buffer (10X: 30.3 g Tris base/ 144.2 g glycine/ 10 g SDS /pH to 8.3/ add dH2O to 1.0 L) for 10 seconds. The IPG strips were then transferred to the 2nd dimension gel tray with the gel side up and the positive side by the molecular weight marker. The strips were gently pushed to the bottom of the well with a pair of tweezers, and 7 uL of molecular weight marker (BioRad, Hercules, CA) was
added to the molecular weight marker well. Overlay agarose (1 mL) was added to the well of the molecular weight marker and used to cover the IPG strip. The agarose was allowed to incubate for 10 minutes at room temperature to allow it to solidify. Gels were then moved to the Criterion Dedeca Cell for SDS-PAGE (Biorad, Hercules, CA) where they ran at 70 V for 2.5 hours. Once the run was complete, gels were rinsed with distilled water 3 times for 5 minutes each. Gels were subsequently stained with coommassie blue R-250 (Biorad, Hercules, CA) overnight. Gels were then destained by adding destain for 1 hour on the rocker at room temperature and repeated 3 times. Gels were imaged using Quantity One software and Chemiimager (Biorad, Hercules, CA). The three gels within each experiment were analyzed for differences using PDQuest 2-D Analysis Software (Biorad, Hercules, CA), and eight spots within each experiment were picked for spot excision and digestion. Spot excision and digestion to prepare the samples for mass spectrometry was conducted by the Proteomics and Mass Spectrometry Core Facility (PSU, The Huck Institutes of Life Sciences). Mass spectrometry analysis was accomplished using the LC ESI MSe (LC-Q-Tof by Waters Q-Tof Premier, Milford, MA). Data was uploaded into Waters Protein Lynx Global Server software, version 2.3 to identify proteins using the sheep, Ovis aries, and the bovine, Bos taurus, specific databases available from NCBI.

Results

Effects of inhibition of MX1 on interferon tau-stimulated secretion by ovine glandular epithelial cells

Morpholino antisense oligos (MAOs) were utilized to block the translation of MX1 mRNA in oGE cells. The medium from MX1 knockdown, containing total secreted proteins (400 ug), was compared to oGE cells treated with IFNT only and a non-specific MAO to analyze
the entire oGE secreted proteome and identify proteins that may be altered when the MX1 protein is inhibited. Eight spots were excised from 2D PAGE gels for mass spectrometry analysis. Of the eight samples, 1 sample returned with no successful identification. After disregarding common laboratory contaminant proteins, the MX1 inhibition gels identified 8 proteins that were potentially inhibited when MX1 was inhibited. In spot 1, WD repeat-containing protein 38 (WDR38) was identified with a PLGS score of 121 and a percent coverage range from 7.1-14.2% (depending on protein ID) (Table 5-1). In spot 4, serpin peptidase inhibitor (SERPIN) was identified with a PLGS score of 490.3 and a percent coverage range from 10.8-15.4%, along with alpha-2-HS-glycoprotein (AHSG) with a PLGS score of 233.6 and a percent coverage range from 10-11.7% (Table 5-1). In spot 5, triosephosphate isomerase-1 (TPI1) was identified with a PLGS score of 295.9 and a percent coverage range from 49.8-54.2%, along with peroxiredoxin-6 (GPX6) with a PLGS score of 374.9 and 214.2 and a percent coverage range from 16.5-42.9% (Table 5-1). In spot 6, WDR38 was identified with a PLGS score of 83.8 and a percent coverage range from 7.1-14.2%, along with malate dehydrogenase 1B (MDH1B) with a PLGS score of 92.7 and a percent coverage of 3.6%, ankyrin repeat and IBR domain-containing 1 (ANKIB1) with a PLGS score of 105.8 and a percent coverage range from 2.3-4.6%, and lim domain 7 (LMO7) with a PLGS score of 105.7 and a percent coverage range from 1-3% (Table 5-1). Additionally, 2 proteins were potentially identified in spot 7 that increased when MX1 was inhibited, Cystatin E/M (CST6) with a PLGS score of 89.6 and a percent coverage range from 7.4-33.7%, and toll-like receptor 1 (TLR1) with a PLGS score of 141.2 and a percent coverage of 4.2% (Table 5-2).
Effects of overexpression of MX1 on secretion by ovine glandular epithelial cells

The pCMV-Myc mammalian expression vector containing a full-length MX1 cDNA was utilized to overexpress the MX1 protein in oGE cells. The medium from MX1 overexpression, containing total secreted proteins (230μg), was compared to oGE cells treated with vehicle and no treatment in order to analyze the entire oGE secreted proteome and identify proteins that may be altered by the MX1 protein. Eight spots were excised from 2D PAGE gels for mass spectrometry analysis. Of the eight samples, 5 samples returned with no successful identification. After disregarding common laboratory contaminant proteins, the MX1 overexpression gel identified 4 potential proteins in spots that were increased. In spot 4, SERPIN was identified with a PLGS score of 400.8 and 310.9 (depending on protein ID), and a percent coverage range of 7.7-10.1% (Table 5-3). In spot 6, glial fibrillary acidic protein (GFAP) was identified with a PLGS score of 138.8 and 138.6, and a percent coverage of 2.6% (Table 5-3). In spot 7, lactate dehydrogenase B (LDHB) was identified with a PLGS score of 219.3 and a percent coverage of 7.5%, along with MDH1B with a PLGS score of 106.8 and a percent coverage of 3.6% (Table 5-3).

Discussion

The objective of this study was to identify cellular proteins whose patterns of secretion are changed when MX1 is altered in a cell line that is a model for the secretory epithelium of the uterus. Previously we showed that suppression of MX1 mRNA resulted in a reduction in the secretion of unconventionally secreted ISG15 and MX1, but not conventionally secreted B2M (Toyokawa et al., 2007a; 2007b). This led to the hypothesis that MX1 is a regulator of unconventional secretion. To test this hypothesis, we examined the secretory proteome of oGE cells when MX1 was either inhibited or overexpressed using 2D SDS-PAGE. Differentially
expressed protein spots were excised from 2D SDS-PAGE gels for mass spectrometry analysis. For the inhibition assay, morpholino antisense oligos (MAOs) were used to cause inhibition of MX1, and this 2D gel was compared to secretions from oGE cells treated with IFNT only and a non-specific MAO in order to analyze the entire oGE secreted proteome and identify proteins that may be altered by the MX1 protein (Figure 5-1). For the overexpression assay, the pCMV-Myc mammalian expression vector containing a full-length MX1 cDNA was used to selectively overexpress MX1 alone, and this 2D gel was compared to oGE cell secretions treated with a vehicle control and a no treatment control in order to analyze the entire oGE secreted proteome and identify proteins that may be altered by the MX1 protein (Figure 5-2).

In the inhibition assay eight spots were excised from 2D PAGE gels for mass spectrometry analysis. Of the eight samples, 1 sample returned with no successful identification. A total of 8 proteins were identified that were potentially inhibited when MX1 was inhibited. To determine which proteins would be the most important for further investigations the percent coverage and the PLGS scores of the proteins were analyzed. The percent coverage is calculated based on matched peptides and expected peptide fragments between the sample and the protein identified. The ProteinLynx Global SERVER™ (PLGS) score, which is a score assigned by Waters Protein Lynx Global Server software, version 2.3, gives a quantitative measure of how likely the correct protein was identified. It does this by assigning the protein a score for high quality multiplexed LC-MS derived matches using an ion accounting algorithm. In this way the results were analyzed based on coverage and highest quality to determine which proteins were identified.

Of the 8 proteins identified, 3 proteins were disregarded due to their low PLGS score, percent coverage, and, additionally, molecular weights that were not consistent with the location
of the spot. These proteins included MDH1B, with a PLGS score of 92.7, a percent coverage of 3.6%, and an expected molecular weight of 53 kD, ANKIB1, with a PLGS score of 105.8, a percent coverage ranging between 2.3-4.6%, and an expected molecular weight of 122 kD, and LMO7, with a PLGS score of 105.7, a percent coverage ranging between 1-3%, and an expected molecular weight of 157 kD. The location of the spot that was excised from the gel has a molecular weight around 25 kD, and it is possible that these proteins were identified due to common functional motifs.

Of the remaining 5 proteins, TPI1, had the highest percent coverage (49.8-54.2%), the expected molecular weight of this protein is 26 kD which is consistent with where the spot was located (around 25 kD), and the PLGS score for this protein was 295.9. Triosephosphate isomerase-1 is a glycolytic enzyme that is responsible for regulating the equilibrium between dihydroxyacetone phosphate and glyceraldehyde-3-phosphate produced by aldolase in glycolysis (Orosz et al., 2006). Interestingly, the activity of this enzyme is critical for a cell; many severe diseases are associated with mutations that decrease TIM activity, and, additionally, the activity of this enzyme affects the pentosephosphate pathway and lipid metabolism, which are both connected to the glycolysis pathway (Fonvielle et al., 2005; Orosz et al., 2006; Wierenga et al., 2010). This protein was inhibited when MX1 was inhibited and could have severe implications due to the lethality of TIM deficiency (disease symptoms including hemolytic anemia and neurological disorders) (Wierenga et al., 2010). Peroxiredoxin-6 also had a high percent coverage (16.5-42.9%). The expected molecular weight of this protein is 25 kD which also consistent with where the spot was located (around 25 kD), and the PLGS score was 214.2 and 374.9, depending on the protein ID. Through alkyl and hydrogen peroxide reductase activities, this family of proteins (peroxiredoxins) is involved in antioxidant defenses and intracellular
signaling (Leyens et al., 2004). Interestingly, GPX6 has previously been described as becoming upregulated in bovine oocytes and cumulus cells during in vitro maturation and is mutually regulated by both cell types (Leyens et al., 2004).

The protein with the highest PLGS score (490.3) was SERPIN, which had a percent coverage range between 10.8-15.4%. The expected molecular weight of this protein is approximately 46 kD, however the location of the extracted spot, from which this protein was detected, was around 75 kD. This could be due to post translational modifications, which are known to change the pI and/or the molecular weight of a protein. For example, post translational modifications include: functional groups becoming attached to the protein (acetate, phosphate, lipids, carbohydrates), structural changes to the protein (formation of disulfide bonds), or even the removal of amino acids from the amino end or peptide chains being cut out by enzymes. Serpin peptidase inhibitor is known to inhibit a wide variety of proteases (Gettins, 2002). This protein is inhibited when MX1 is inhibited. The implications of this during early pregnancy would be very interesting to investigate because both proteases and protease inhibitors have been demonstrated to be expressed in the uterus during early pregnancy (Bazer et al., 2008).

The last 2 proteins that were inhibited when MX1 was inhibited were AHSG, and WDR38 (which was detected in 2 separate spots). Alpha-2-HS-glycoprotein (homologue is fetuin) was detected with a PLGS score of 233.6, and a coverage ranging between 10-11.7%. Although the expected weight of this protein, 38 kD, is below where the spot was extracted from (around 75 kD), this could again be due to post translational modifications. This protein is found at higher concentrations in plasma and tissues in developing animals compared to adults, and it belongs to class of plasma binding proteins. This protein contains a cystatin like domain, and cystatins are cysteine protease inhibitors. Interestingly this protein, which may inhibit cysteine
proteases, was inhibited when MX1 was knocked down and previous results in this thesis demonstrated that cathepsin L, a protease was upregulated when MX1 was inhibited. Further investigations involving this protein and its relationship with MX1 should be performed.

Finally, the last protein that was detected when MX1 was knocked down was WDR38 and it was detected in 2 separate spots. The molecular weight of this protein is around 32 kD and the two spots that were excised were both around 25 kD. Additionally, the coverage for both of these spots ranged between 7.1-14.2%, however, the PLGS score for spot 1 was 121 and for spot 6 it was only 83.8, lower than any of the other 4 potentially identified proteins. The proteins belonging to the WD protein family have a high degree of diversity in sequence and cellular function including adaptor/regulatory modules in signal transduction, cytoskeleton assembly, and pre-mRNA processing; however there is little information in the literature for WDR38 specifically (Yu et al., 2000).

Additionally, 2 proteins were potentially identified that increased when MX1 was inhibited, CST6 with a PLGS score of 89.6 and a percent coverage range from 7.4-33.7% and TLR1 with a PLGS score of 141.2 and a percent coverage of 4.2%. The spot these proteins were identified from was located at approximately 10-15 kD, which is consistent with the molecular weight for CST6. Toll-like receptor-1 has specificity for gram positive bacteria, and due to the well characterized role that MX1 has in antiviral activity against multiple RNA viruses; it is interesting to contemplate TLR1 becoming upregulated when MX1 is inhibited. However, the expected molecular weight of TLR1 is 83 kD and due to the low percent coverage of this protein it should not be considered a top priority of investigation in upcoming experiments. Interestingly, CST 6 is a cysteine protease inhibitor. Again, previous results in this thesis demonstrated that cathepsin L, a protease was upregulated when MX1 was inhibited. Further
investigations involving MX1 and cysteine proteases and cysteine protease inhibitors, both of which are important during early pregnancy would be interesting to study in further research involving the MX1 (Bazer et al., 2008).

Finally, when MX1 was overexpressed and secretory proteins examined by 2-D SDS-PAGE, visual inspection identified a number of differences. Eight of these were excised and submitted for mass spectrometry analysis. Of the eight samples, 5 samples returned with no successful identification. After disregarding common laboratory contaminant proteins, the MX1 overexpression gel identified 4 potential proteins in spots that were increased. Of the 4 proteins identified, 2 proteins were disregarded due to their low PLGS score and percent coverage. These proteins included MDH1B, with a PLGS score of 106.8, a percent coverage of 3.6%, and GFAP, with a PLGS score of 138.6/8, a percent coverage of 2.6%. The expected molecular weights of these proteins were close to 50 kD, which was relatively close to the location these spots were excised from (around 37 kD and 50-75 kD respectively). It is again possible that these proteins were identified due to common functional motifs, and should not be considered any further.

Lactate dehydrogenase B was identified as one of the 2 remaining proteins. The PLGS score for this protein was 219.3, the percent coverage was 7.5%, and the molecular weight corresponded with the spot location. This protein catalyzes pyruvate to lactate in glycolysis, and functions in binding, catalytic activity, oxidoreductase activity, and L-lactate dehydrogenase activity (NCBI # NP_776525). Most intriguing though, SERPIN was identified as the other protein that was increased when MX1 was overexpressed. This is most interesting because it was also noted as one of the proteins that were inhibited when MX1 was knocked down. The PLGS score was 310.9 and 400.8 depending on the protein ID, making SERPIN the highest quality protein in both the inhibition and overexpression gels, and the percent coverage ranged between 7.7-10.1%.
Again, the serpin peptidase inhibitor is known to inhibit a wide variety of proteases (Gettins, 2002). Further investigations involving the MX1 protein and proteases/protease inhibitors, especially SERPIN, should be evaluated in the next possible experiment, especially because of how important proteases/protease inhibitors are to early pregnancy.

Additionally, it should be noted that another protein was potentially inhibited when MX1 was inhibited and upregulated when MX1 was overexpressed. Malate dehydrogenase 1B was listed as a potential protein in both of the inhibition and overexpression results. This protein was discarded, however, as previously documented, due to its extremely low percent coverage, 3.6% in both cases, which was from 1 matched peptide of (17aa) out of the (451 aa) protein.

Although it would be premature to develop hypotheses for each of these proteins, we have potentially identified target proteins that are regulated by MX1 in the uterus, the goal of these experiments. Overall, the methods for modulating MX1 via transient knockdown and overexpression now give us the ability to identify cellular proteins whose patterns of secretion are altered by altering MX1. Although many potentially regulated proteins have now been identified, the most interesting proteins from these experiments may be the proteases and the protease inhibitors. Further investigations involving the MX1 protein and these proteins, especially SERPIN, should be the future focus, especially because of how important proteases/protease inhibitors are to early pregnancy. We realize that the amount of protein in the majority of these spots was low, and further experiments involving western blotting and 2D SDS-PAGE with higher amounts of protein loaded along with additional experiments involving the new information on proteases and protease inhibitors will be the next steps to further these investigations. Additionally, many more experiments will be crucial in defining more of the
specific proteins regulated by MX1 to aid in understanding the complex role MX1 possesses in regulating secretion.
Figure 5-1. Two-dimensional SDS-PAGE on the global array of secretory proteins from MX1 MAO-treated oGE cells. Molecular weight markers are shown on the left side in kilodaltons. Red squares indicate spots that were chosen for mass spectrometry analysis and are labeled 1-8. Total secreted proteins were collected from cells treated with IFNT, mismatch MAO, or the MX1-specific MAO.

Figure 5-2. Two-dimensional SDS-PAGE of the global array of secretory proteins from MX1 overexpressed-treated oGE cells. Molecular weight markers are shown on the left side in kilodaltons. Red squares indicate spots that were chosen for mass spectrometry analysis and are labeled 1-8. Total secreted proteins were collected from cells treated with no treatment, control plasmid, or the MX1 plasmid.
Figure 5-1

IFNT – Negative Control

Mismatch MAO – Negative Control

MX1 MAO
Figure 5-2

NT – Negative Control

Control Plasmid – Negative Control

MX1 Plasmid
Table 5.1. The summarized results of MS analysis of protein spots underrepresented in oGE cells when MX1 was inhibited. The spot number representing where the spot was selected from in the gel is listed first, followed by the molecular weight estimate of the chosen spot in column 2. The protein identification(s) is listed next in column 3, followed by the protein name in column 4. Column 5 indicates the database from which the protein was identified. Column 6 indicates the PLGS score assigned by Waters Protein Lynx Global Server software, version 2.3 and gives a quantitative measure of how likely the correct protein was identified. The percent coverage is in column 7, which explains the percent of the sequence from MS analysis that can be explained by the published sequence in the NCBI database. The molecular weight is listed in column 8, followed by the function and/or description of the protein in column 9. Column 10 lists the references for the description and/or function of the proteins.

Table 5.2. The summarized results of MS analysis of protein spots overrepresented in oGE cells when MX1 was inhibited. The spot number representing where the spot was selected from in the gel is listed first, followed by the molecular weight estimate of the chosen spot in column 2. The protein identification(s) is listed next in column 3, followed by the protein name in column 4. Column 5 indicates the database from which the protein was identified. Column 6 indicates the PLGS score assigned by Waters Protein Lynx Global Server software, version 2.3 and gives a quantitative measure of how likely the correct protein was identified. The percent coverage is in column 7, which explains the percent of the sequence from MS analysis that can be explained by the published sequence in the NCBI database. The molecular weight is listed in column 8, followed by the function and/or description of the protein in column 9. Column 10 lists the references for the description and/or function of the proteins.

Table 5.3. The summarized results of MS analysis of protein spots overrepresented in oGE cells when MX1 was overexpressed. The spot number representing where the spot was selected from in the gel is listed first, followed by the molecular weight estimate of the chosen spot in column 2. The protein identification(s) is listed next in column 3, followed by the protein name in column 4. Column 5 indicates the database from which the protein was identified. Column 6 indicates the PLGS score assigned by Waters Protein Lynx Global Server software, version 2.3 and gives a quantitative measure of how likely the correct protein was identified. The percent coverage is in column 7, which explains the percent of the sequence from MS analysis that can be explained by the published sequence in the NCBI database. The molecular weight is listed in column 8, followed by the function and/or description of the protein in column 9. Column 10 lists the references for the description and/or function of the proteins.
<table>
<thead>
<tr>
<th>Spot Number</th>
<th>MW estimate of Spot (kDa)</th>
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<th>Protein Name</th>
<th>Database</th>
<th>PLGS Score</th>
<th>Coverage</th>
<th>Av Mass(Da) (PI)</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20-25</td>
<td>43009, 53161, 75688</td>
<td>WD repeat-containing protein 38</td>
<td>Bos taurus</td>
<td>121</td>
<td>7.1-14.2%</td>
<td>32722 (9.4)</td>
<td>WD protein family: sequence repeat of about 40aa. High degree of diversity, including adaptor/regulatory modules in signal transduction, cytoskeleton assembly, and pre-mRNA processing.</td>
<td>NCBI# NP_001157418.1; Yu et al., 2000; Marchler-Bauer et al., 2011.</td>
</tr>
<tr>
<td>4</td>
<td>75</td>
<td>3831, 12927, 59913, 69681, 75647, 84448</td>
<td>Serpin peptidase inhibitor</td>
<td>Bos taurus</td>
<td>490.3</td>
<td>10.8-15.4%</td>
<td>46104 (6.1)</td>
<td>Inhibits a wide variety of proteases. Protective against enzymes of inflammatory cells, such as neutrophil elastase.</td>
<td>NCBI # NP_776307.1; Kushner, Mackiewics, 1993; Gettins, 2002.</td>
</tr>
<tr>
<td>4</td>
<td>75</td>
<td>4894, 7204, 44130, 83022, 83274, 89355</td>
<td>Alpha-2-HS-glycoprotein/ Fetuin</td>
<td>Bos taurus</td>
<td>233.6</td>
<td>10-11.7%</td>
<td>38418 (5.1)</td>
<td>Present at higher levels in plasma and tissues in developing animals compared to adults. Belongs to class of plasma binding proteins. Fetuin-A is a carrier of otherwise insoluble calcium phosphate. Cystatin like domain, and cystatins are cysteine protease inhibitors.</td>
<td>NCBI # NP_776344.1; Dziegielewska et al., 1998; Jahnen-Dechent, et al., 1999.</td>
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<td>1947, 15733, 48249, 73214, 80203,</td>
<td>Triosephosphate isomerase-1</td>
<td>Bos taurus</td>
<td>295.9</td>
<td>49.8-54.2%</td>
<td>26689 (6.5)</td>
<td>Isomerase and triose-phosphate isomerase activity. Regulates the equilibrium between dihydroxyacetone phosphate and glyceraldehyde-3-phosphate produced by aldolase in glycolysis.</td>
<td>NCBI # NP_001013607.1; Orosz et al., 2006.</td>
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<td>2371, 14556, 43644, 66403, 66418, 73311, 80261, 88051, 30822, 47020</td>
<td>Peroxiredoxin-6</td>
<td>Bos taurus</td>
<td>374.9/214.2</td>
<td>16.5-42.9%</td>
<td>25066/25100 (5.6) (6.0)</td>
<td>Hydrolase, oxidoreductase, peroxidase, and peroxiredoxin activity. Confer a protective role in cells. Upregulated in bovine oocytes and cumulus cells during in vitro maturation and is mutually regulated by both cell types.</td>
<td>NCBI # NP_777068.1; Leyens et al., 2004.</td>
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<td>32722 (9.4)</td>
<td>WD protein family: sequence repeat of about 40aa. High degree of diversity, including adaptor/regulatory modules in signal transduction, cytoskeleton assembly, and pre-mRNA processing.</td>
<td>NCBI# NP_001157418.1; Yu et al., 2000; Marchler-Bauer et al., 2011.</td>
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<td>Protein Name</td>
<td>Database</td>
<td>PLGS Score</td>
<td>Coverage</td>
<td>Av Mass(Da) (PI)</td>
<td>Function</td>
<td>Reference</td>
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</tr>
<tr>
<td>6</td>
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<td>7315, 17351, 44994, 77710</td>
<td>Malate dehydrogenase 1B, MDH1B</td>
<td>Bos taurus</td>
<td>92.7</td>
<td>3.6%</td>
<td>53088 (6.8)</td>
<td>Binding, malate dehydrogenase activity, oxidoreductase activity.</td>
<td>NCBI # NP_001076921.1, Marchler-Bauer et al., 2011.</td>
</tr>
<tr>
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<td>19551, 30998, 46646</td>
<td>Ankyrin repeat and IBR domain-containing 1</td>
<td>Bos taurus</td>
<td>105.8</td>
<td>2.3-4.6%</td>
<td>122050 (4.8)</td>
<td>Binding proteins that couple with ion channels and pumps, cell adhesion molecules, and calcium release channels via ANK repeats. Speculated to restrict sodium channels and cell adhesion molecules and to be involved with calcium homeostasis.</td>
<td>NCBI # XP_581455.4, Bennett and Chen, 2001.</td>
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<tr>
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<td>53633, 82083</td>
<td>Lim domain 7</td>
<td>Bos taurus</td>
<td>105.7</td>
<td>1-3%</td>
<td>157572 (7.8)</td>
<td>This protein contains domains that bind C-terminal peptides (PDZ domain) although binding to non-C-terminal peptides and lipids have also been demonstrated, and there is also a protein-protein interaction domain (LIM).</td>
<td>NCBI # NP_001103271.1, Marchler-Bauer et al., 2011.</td>
</tr>
<tr>
<td>Spot Number</td>
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<td>Protein Name</td>
<td>Database</td>
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<td>Reference</td>
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</tr>
<tr>
<td>7</td>
<td>10-15</td>
<td>22493, 63522, 42937, 70085</td>
<td>Cystatin E/M</td>
<td>Bos taurus</td>
<td>89.6</td>
<td>7.4-33.7%</td>
<td>10699/16355 (7.2) (7.7)</td>
<td>Cysteine protease inhibitors. The domains of Cystatins are also found within extracellular proteins kininogen, His-rich glycoprotein, and fetuin.</td>
<td>NCBI # NP_001012782.1; Marchler-Bauer et al., 2011.</td>
</tr>
<tr>
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<td>4259, 4282</td>
<td>Toll-like receptor 1</td>
<td>Ovis aries</td>
<td>141.2</td>
<td>4.2%</td>
<td>83509 (6.6)</td>
<td>Belongs to the toll-like receptor family, which are pattern recognition receptors for pathogen-specific molecular patterns in the innate immune system. TLR1 has specificity for gram positive bacteria.</td>
<td>Takeuchi et al., 2002.</td>
</tr>
<tr>
<td>Spot Number</td>
<td>MW estimate of Spot (kDa)</td>
<td>Protein ID</td>
<td>Protein Name</td>
<td>Database</td>
<td>PLGS Score</td>
<td>Coverage</td>
<td>Av Mass(Da) (PI)</td>
<td>Function</td>
<td>Reference</td>
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<td>4</td>
<td>75</td>
<td>12927, 59913, 69681, 75647, 84448, 3831</td>
<td>Serpin peptidase inhibitor</td>
<td>Bos taurus</td>
<td>400.8/310.9</td>
<td>7.7-10.1%</td>
<td>46104 (6.1)</td>
<td>Inhibits a wide variety of proteases. Protective against enzymes of inflammatory cells, such as neutrophil elastase. Upon acute inflammation blood levels can raise many folds.</td>
<td>NCBI # NP_776307.1; Kushner, Mackiewics, 1993; Gettins, 2002.</td>
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<td>87814, 70311, 13099, 16146, 58849, 84759</td>
<td>Glial fibrillary acidic protein</td>
<td>Bos taurus</td>
<td>138.8/138.6</td>
<td>2.6%</td>
<td>49452/47902/49511 (5.1) (5.2)</td>
<td>An Intermediate filament along with keratins, desmin, vimentin and neurofilament proteins, which are all evolutionarily and structurally related. Although the function of GFAP is still not understood, the other members of this family are involved in structure/function of the cell’s cytoskeleton.</td>
<td>Fuchs and Weber, 1994.</td>
</tr>
<tr>
<td>7</td>
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<td>1486, 10925, 48067, 73269, 83055, 83780, 86003, 88046</td>
<td>Lactate dehydrogenase B</td>
<td>Bos taurus</td>
<td>219.3</td>
<td>7.5%</td>
<td>26723/36624/36697 (5.6)(6.0)</td>
<td>Functions in binding, catalytic activity, oxidoreductase activity, and L-lactate dehydrogenase activity. Ferments pyruvate to lactate in glycolysis.</td>
<td>NCBI # NP_776525.:, Marchler-Bauer et al., 2011.</td>
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<tr>
<td>7</td>
<td>37</td>
<td>7315, 17351, 44994, 77710</td>
<td>Malate dehydrogenase 1B, MDH1B</td>
<td>Bos taurus</td>
<td>106.8</td>
<td>3.60%</td>
<td>53088 (6.8)</td>
<td>Binding, malate dehydrogenase activity, oxidoreductase activity.</td>
<td>NCBI # NP_001076921.1; Marchler-Bauer et al., 2011.</td>
</tr>
</tbody>
</table>
References

Accola, M., B. Huang, A. Al Masri, and M. A. McNivens. 2002. The antiviral dynamin family member, MxA tabulates lipids and localizes to the smooth endoplasmic reticulum. Journal of Biological Chemistry. 277(24); 21829-21835.


Humblot, P. 2001. Use of pregnancy specific proteins and progesterone assays to monitor pregnancy and determine the timing, frequencies and sources of embryonic mortality in ruminants. Theriogenology. 56(9); 1417-1433.


Johnson, G. A., A. M. Collins, W. J. Murdoch, and T. R. Hansen. 1999c. endometrial ISG17 mRNA and a related mRNA are induced by interferon-t and localized to glandular epithelial and stromal cells from pregnant cows. Endocrine. 10(3); 243-252.


oligoadenylate synthetase expression in the ovine uterus. Biology of Reproduction. 64(5); 1392-1399.


Roles of Stat1, Stat2, and interferon regulatory factor-9 (IRF-9) in interferon τ regulation of IRF-1. Biology of Reproduction. 66(2); 393-400.


Chapter 6

GENERAL DISCUSSION

Typical conception rates on commercial dairies average approximately 35%. This means that 65% of inseminations fail. Early embryonic loss (before Day 30) has been estimated at 75-80% of these losses, with a large portion (up to 40%) occurring between Days 8 and 17 of pregnancy (Thatcher et al., 1990; 1994). The long-term goal of research in our lab is to improve fertility by studying the critical stage of pregnancy when the early embryo interacts with the endometrium (the first 30 days of pregnancy) to establish pregnancy. During this time the conceptus produces IFNT, the signal from the conceptus to the maternal system to prevent luteal regression in ruminants. Interferon tau is thought to be critical for creating the appropriate embryotrophic uterine environment. However, besides IFNT secretion, nutrients such as amounts of glucose, amino acids, calcium, potassium, and glutathione are increased in uterine fluids 3-to-23 fold in pregnant compared to cyclic sheep (Day10-16) (Gao et al., 2009). Additionally, there are numerous other regulated substances in the uterus at this time, P₄-regulated growth factors and IGF binding proteins for example (Satterfield et al., 2008). It is also possible that other type I and/or type II IFNs expressed play important roles in establishing uterine receptivity to implantation due to their effect on the expression of many genes (Bazer, et al., 2008). This is accomplished by altering endometrial function through the synthesis and secretion of a large number of endometrial proteins which are critical in supporting the conceptus’s growth and development (Spencer et al., 2008).

When IFNs bind to their receptors they activate the JAK-STAT signaling pathway which leads to the upregulation of a large number of interferon stimulated genes in the uterus, CL and peripheral blood immune cells (Vallet et al., 1991; Mirando et al., 1991; Ott et al., 1998; Spencer
et al., 1998; Hansen et al., 1999; Johnson et al., 1999a; 1999b; 1999c; 2000; 2001; Stewart et al., 2001; 2002; Gray et al., 2002; Choi et al., 2003; Kim et al., 2003; Gray et al., 2004; Song et al., 2005; Gray et al., 2006; Song et al., 2006; 2007; Bazer et al., 2010). One of these proteins, MX1, is a member of the dynamin superfamily of large GTPases that has known anti-viral activity. Members of this superfamily have cellular functions that include protein trafficking and endocytosis. The similarity between MX1 and other proteins in this superfamily led to the hypothesis that MX1 may have basic cellular functions. More precisely, because of the number of domains it shares with dynamin, MX1 was further hypothesized to regulate secretion.

Recent work has showed that the MX1 protein is constitutively expressed in oGE cells in the absence of exogenous virus or IFN (Racicot and Ott, 2010). This supports the idea that the MX1 protein may have a basic cellular function in addition to its role as an induced antiviral protein. Additionally, using siRNAs to knockdown MX1, there was reduced secretion of unconventionally secreted ISG15, but not conventionally secreted B2M occurs (Toyokawa et al., 2007a; 2007b). Along with the similarity between MX1 and dynamin, these studies led to the hypothesis that MX1 is a regulator of unconventional secretion (Toyokawa et al., 2007a; 2007b).

To test our overall working hypothesis that the conceptus regulates uterine secretory function, an immortalized epithelial cell line (oGE; Johnson et al., 1999) derived from the uterine glands of sheep was used. The weakness of using is a cell line is that the complex system being studied is removed from the physiological whole animal (where the question being tested originated from). The benefit for using a cell line, however, is that a large idea can be broken down into simpler experiments which are performed in a cell line derived from tissue appropriate for studying the question. This allows for a practical way to begin understanding the complex system, which is in question, before moving to a live animal model.
The first objective in this thesis was to create an assay for quantitatively measuring MX1. Once the assay was successful, the studies described in this thesis were designed to test our hypothesis by determining the effects of MX1 on secretion by oGE cells using a combination of transient inhibition and overexpression assays. Finally, we aimed to identify any cellular proteins whose patterns of secretion change when MX1 is altered using 2D gel electrophoresis and mass spectrometry.

It was becoming increasingly clear that an assay for quantitatively measuring MX1 was essential for further studying its function. There is currently no assay for MX1 quantification. We demonstrate in this thesis an assay that can quantitatively measure oMX1 in cellular lysates. Standard curves were established using roMX1, and the percentage of roMX1 recovered from cellular lysates was conducted to check for interfering components. However, to completely validate this assay the percentage of roMX1 recovered from the other diluents and plasma needs to be conducted, we need to check for any cross-reactivity in the antibodies used in this assay, and finally we need to calculate the intrassay coefficients of variations (CVs) from the data we have collected.

A key finding described in this thesis is that when MX1 was inhibited, proteins secreted via conventional and unconventional secretory pathways were affected. Thus the overall working hypothesis that MX1 is a global mediator of unconventional secretion was not supported. However, in partial support of our hypothesis, two proteins known to be secreted via the unconventional pathway were inhibited by the inhibition of MX1. It is possible that MX1 may regulate a specific pathway in unconventional secretion, however, the fact that proteins in both the conventional and the unconventional pathways were affected also points to a more complex role of MX1 then we initially hypothesized. These results suggest that the MX1 protein
may have a basic cellular function in regulating secretion; however, additional experiments will further define the specific pathway and the specific proteins being regulated.

In addition to confirming the inhibition of MX1, the overexpression of MX1 in oGE cells was confirmed as well via Western blot analysis. Unfortunately, overexpression of MX1 did not affect any of the unconventionally/conventionally secreted proteins examined. It is possible that no effect was seen because the proteins in question would also need to be increased to be able to detect the effects MX1 may have on their secretion.

To truly evaluate whether MX1 can mediate the secretion of these proteins, MX1 will need to be overexpressed in combination with these unconventionally and conventionally secreted proteins, which would be the next step to take in this experiment. Future experiments should overexpress MX1 and the putative proteins whose secretion is mediated by MX1. Through this approach, it could then be determined if MX1 alone has an effect on secreted proteins. This approach also has weaknesses as well, however. If MX1 participates as part of a multi-protein complex or in a sequence of processes, then sole overexpression of MX1 might not yield any changes in protein expression.

It is also possible that no effect was detected due to presence of the myc tag on the rMX1, (which could interfere with the function of MX1). However, the myc-tagged rMX1 was secreted in high concentration suggesting that it was still traversing the same physiological pathways as native MX1. In either case, one could overexpress MX1 without the tag, or move the tag to the other end of the MX1 protein to rule out this possibility. Overall, the results from inhibiting MX1 and causing inhibition of ISG15 and LGALS1 seem to support the idea that MX1 has a role in mediating secretion, and is not just merely cargo in the secretion process.
In addition to examining specific proteins found in uterine flushes known to be secreted via conventional and unconventional secretory pathways, we also undertook analysis of the whole proteome to determine effects of MX1 knockdown and overexpression using 2D SDS PAGE and mass spectrometry. With analysis of these proteins in regard to quality and the percent of matched peptides and expected peptide fragments between the sample and the protein identified, 5 proteins TPI1, GPX6, SERPIN, AHSG, and WDR38 were possibly inhibited when MX1 was inhibited, CST6 was potentially increased when MX1 was inhibited, and SERPIN and LDHB were potentially increased when MX1 was overexpressed. Further investigations involving the MX1 protein and the proteases and the protease inhibitors, especially SERPIN, should be the future focus, specifically because of how important proteases/protease inhibitors are to early pregnancy (Bazer et al., 2008). We realize that the amount of protein in the majority of these spots was low, and further experiments involving western blotting and 2D SDS-PAGE with higher amounts of protein loaded, along with additional experiments involving the new information on proteases and protease inhibitors will be the next steps to further these investigations.

The results presented here have enhanced what we know about the possible basic cellular function of MX1 and its role as a regulator of secretion. They have led to rejection of the working hypothesis that MX1 is a global regulator of unconventional secretion. However, experiments were performed in only one cell line and they should be replicated in other cell lines before a definitive answer can be determined. These results combined with previous studies further define the role of MX1 in uterine epithelial cells and provide clues to the role of MX1 as a regulator of secretion. Overall, these results contribute to the knowledge of the biochemical communication between the conceptus and the maternal system. Understanding the factors
associated with conceptus survival, such as the mechanism of how the conceptus regulates endometrial secretion could lead to techniques to reduce early embryonic mortality, thereby improving fertility. Importantly, MX1 may play a direct role in regulating these functions by regulating endometrial secretory processes necessary for embryo survival until placentation.
References


Johnson, G. A., K. J. Austin, A. M. Collins, W. J. Murdoch, and T. R. Hansen. 1999c. Endometrial ISG17 mRNA and a related mRNA are induced by interferon-t and localized to glandular epithelial and stromal cells from pregnant cows. Endocrine. 10(3); 243-252.


Appendix A:

Antiviral assay on bovine uterine flush samples to determine pregnancy status

Using a convenient microtiter assay for interferons that is based on reduction of cytopathic effect (CPE) in host cells, we were able to determine pregnancy status in uterine flush samples collected from beef cattle (Rubinstein et al., 1981). Because the maternal recognition of pregnancy signal from the conceptus is an interferon, interferon tau (IFNT), which elicits antiviral activity, we were able to use this assay to test for the antiviral activity present in uterine flush samples due to the presence of IFNT.

First, propagation of the Indiana strain of vesicular stomatitis virus (VSV) (ATCC Manassas, VA) had to be conducted. The immortalized mouse fibroblast cell line (L929) cells (ATCC Manassas, VA) were cultured to confluence in Dulbecco’s Modified Eagle’s Medium (15.63 g/L DMEM, Sigma, St Louis, MO) with 2% fetal bovine serum (FBS; Gibco, Grand Island, NY) under 5% CO2 at 38.5°C and then treated with VSV until complete CPE was achieved. The cell culture medium from these cells was then harvested, aliquoted, and stored in -80°C. The virus was then tittered using Maden Darby Bovine Kidney (MDBK) cells, cells of epithelial origin provided by Jan Vilcek, and the correct dilution of virus to add to cells to achieve CPE (1:1000) was realized.

Ohio State University (OSU) sent 19 fresh uterine flush samples shipped at 4°C and, upon arrival, half of each flush sample was stored at 4°C and the other half of each flush sample was stored at -20°C. The fresh and frozen samples were both run in the assay to verify that the same cows would be determined as pregnant whether the uterine flush was stored at 4°C or frozen. Along with the original 19 fresh samples, OSU sent 10 additional frozen (-20°C) uterine flush samples to also evaluate. As a control, IFNT alone was used to treat the MDBK cells.
The assay was utilized by first plating MDBK cells in a 96 well culture plate and adding the uterine flush samples to the cells in a serial dilution across the plate. The cells incubated with the sample overnight, and were then challenged with VSV for approximately 24 hours. If there was no protection from the uterine flush, cells would not survive the VSV treatment, but if there was protection from the uterine flush the dilution at which the uterine flush protected the cells from death due to the VSV was evaluated. The same cows, 6 out of 19, were shown to be pregnant whether the uterine flush used in the assay was fresh or frozen; however the antiviral activity between fresh and frozen samples was slightly different (Table 1). Of the additional 10 frozen uterine flush sample, 8 of the 10 samples were found to be pregnant. The cows the assay identified as pregnant (Figures 1.1-1.8) were confirmed pregnant by OSU.
Table 1

<table>
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<th>Plate</th>
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<th>Antiviral Activity U/mL</th>
<th>Plate</th>
<th>Cow #</th>
<th>Antiviral Activity U/mL</th>
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<td>Plate 7</td>
<td>5339</td>
<td>162,000</td>
<td>Plate 7</td>
<td>5429</td>
<td>2,187,000</td>
</tr>
<tr>
<td>Plate 7</td>
<td>4118</td>
<td>243,000</td>
<td>Plate 8</td>
<td>334</td>
<td>729,000</td>
</tr>
</tbody>
</table>

Figure 1.1. Plate 1 Frozen Samples. Identification of cow # 212 as pregnant through the AVA. Uterine flush samples were run in duplicate in this assay.
Figure 1.2. Plate 2 Frozen Samples. Identification of cow # 271, #55 as pregnant through the AVA. Uterine flush samples were run in duplicate in this assay.

Figure 1.3. Plate 3 Frozen Samples. Identification of cow # 222, #287 as pregnant through the AVA. Uterine flush samples were run in duplicate in this assay.
Figure 1.4. Plate 4 Frozen Samples. Identification of cow # 308 as pregnant through the AVA. Uterine flush samples were run in duplicate in this assay.

Figure 1.5. Plate 5 Frozen Samples. IFNT control sample in the AVA. IFNT sample was run in duplicate in this assay.
Figure 1.6. Plate 6 Frozen Samples. Identification of cow # 2606, # 203, # 6512 as pregnant through the AVA. Uterine flush samples were run in duplicate in this assay.

Figure 1.7. Plate 7 Frozen Samples. Identification of cow # 6511, # 5339, # 5429, # 4118 as pregnant through the AVA. Uterine flush samples were run in duplicate in this assay.
Figure 1.8. Plate 8 Frozen Samples. Identification of cow # 334 as pregnant through the AVA. Uterine flush samples were run in duplicate in this assay.
Appendix B:

MX1 gene expression in pigs challenged with swine influenza A virus

A study was performed by The University of Minnesota to examine strategies to reduce aerosol spread of viral infections. Two separate groups of 3 pigs each were used in the study. One group of pigs was inoculated intranasally with swine influenza A virus. The other group of pigs served as a control. Although the study was not designed for gene expression analysis, we assayed for steady-state levels of MX1 mRNA and RPL19 mRNA (housekeeping gene) in the peripheral blood leukocytes of pigs challenged/not challenged with the influenza A virus at 0 and 48 hours after inoculation. Since time 0 was included in this study, each pig could serve as their own control. As described by (Gifford et al. 2006 & 2007) semi-quantitative PCR assays were optimized and validated. The primers used are listed in Table 1 and consisted of the MX1 forward and reverse primers along with RPL19 forward and reverse primers. The following times and temperatures were used for amplification: 95°C for 10 min, and 35 cycles of 95°C for 30 s, 62°C for 45 s, and 72°C for 1 min. Results demonstrate statistically significant increases in MX1 at 48 hours in the group of pigs challenged with virus compared to the control group of pigs (Table 1.2, Figure 1). It appears that out of the 3 pigs challenged, one of the pigs did not respond to the challenge with an increase in MX1, however, it is possible that the intranasal inoculations were not effective on this pig and may be the cause for this variability and lack of change in MX1.
Table 1. Gene, primer orientation, primer sequence (5’ to 3’), concentration, and amplicon size for primer sets used in semi-quantitative real-time PCR assays.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Conc. (nM)</th>
<th>Amplicon Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MX1</td>
<td>For.</td>
<td>TTGGGCTTTCAGATGCTTCG</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rev.</td>
<td>CGATGTCGTATGGCTGATTGC</td>
<td>300</td>
<td>191</td>
</tr>
<tr>
<td>RPL19</td>
<td>For.</td>
<td>CAGAGAGGAGGGGGAGAAGT</td>
<td>350</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rev.</td>
<td>GAGGCCGCAGGTCTAAG</td>
<td>300</td>
<td>142</td>
</tr>
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</table>

Table 1.2. Summarized results of MX1 and RPL19 mRNA expression in pigs challenged with swine influenza A virus.

<table>
<thead>
<tr>
<th>Pig #</th>
<th>Treatment</th>
<th>Time</th>
<th>MX1</th>
<th>RPL19</th>
<th>Delta CT</th>
<th>Delta/Delta CT</th>
<th>Relative</th>
<th>AVG</th>
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</thead>
<tbody>
<tr>
<td>35</td>
<td>Control</td>
<td>0</td>
<td>26.26</td>
<td>22.08</td>
<td>4.18</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>Control</td>
<td>48</td>
<td>25.78</td>
<td>23.20</td>
<td>2.58</td>
<td>-1.5</td>
<td>3.031433</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>0</td>
<td>28.64</td>
<td>22.47</td>
<td>6.17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>Control</td>
<td>48</td>
<td>26.29</td>
<td>22.24</td>
<td>4.05</td>
<td>-2.12</td>
<td>4.346939</td>
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</tr>
<tr>
<td>29</td>
<td>Control</td>
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<td>25.98</td>
<td>22.15</td>
<td>3.83</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Challenged</td>
<td>48</td>
<td>24.05</td>
<td>22.16</td>
<td>1.89</td>
<td>-1.935</td>
<td>3.823781</td>
<td>3.734051</td>
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<tr>
<td>20</td>
<td>Challenged</td>
<td>0</td>
<td>26.56</td>
<td>22.03</td>
<td>4.53</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Challenged</td>
<td>48</td>
<td>23.56</td>
<td>22.26</td>
<td>1.30</td>
<td>-3.23</td>
<td>9.38268</td>
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<tr>
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<td>29.26</td>
<td>24.45</td>
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<tr>
<td>19</td>
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<td>23.96</td>
<td>22.05</td>
<td>1.91</td>
<td>-2.89</td>
<td>7.412704</td>
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<tr>
<td>19</td>
<td>Challenged</td>
<td>0</td>
<td>26.52</td>
<td>22.19</td>
<td>4.33</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.2 The summarized results of MX1 and RPL19 mRNA expression in pigs challenged with swine influenza A virus. The pigs identification and/or number are listed first followed by the treatment group the pig was in. The time the blood was collected from the pig is listed in column 3, followed by the Ct value for MX1 in column 4 and the Ct value for RPL19 in column 5. The delta Ct value is in column 6, which is the difference between the Ct value of MX1 and RPL19 at the same time point. The delta/delta Ct value in column 7 is the difference in the Ct value of MX1 at time 0 and time 48 for each pig. The relative expression in column 8 is determined by raising 2 to the power of the negative value of delta-delta Ct for each sample. Column 9 is the average relative expression group calculated for each treatment group.
Figure 1

Change in relative amount of MX1 mRNA in PBLs following challenge with INFLUA virus in pigs.