# IDENTIFICATION AND ANALYSIS OF GENES REGULATING EARLY CONCEPTUS DEVELOPMENT, MATERNAL RECOGNITION OF PREGNANCY AND IMPLANTATION IN THE PIG

By

**JASON WAYNE ROSS**

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Iowa State University

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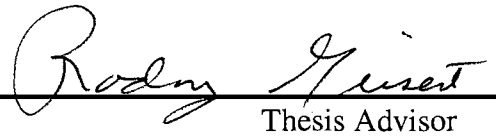

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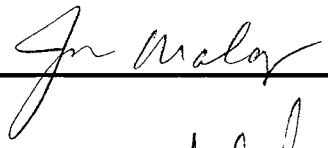

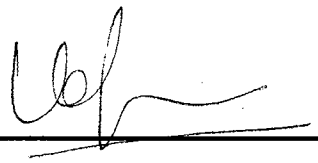

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# Chapter I:

## Introduction

Despite intensive genetic selection and improved management practices, the number of pigs born per litter in the United States has not dramatically changed during the last 4 decades. Geisert and Schmitt [2000] suggested that potential litter size of pigs could be greater than 14 pigs per litter if all ovulated ova produced a live offspring. The prolificacy of US and European breeds has been scrupulously compared to the more reproductively prolific Meishan pigs of China, which consistently farrow larger litters than US and European breeds. Litter size difference between the breeds of pigs is thought to result from uniform trophoblastic elongation among littermates of Meishan sows [Ford, 1997].

Similar to many other mammalian species, early porcine conceptus development is characterized by fertilization, cleavage, compaction, blastulation, hatching from the zona pellucida and implantation. However, porcine conceptuses also experience a unique morphological rearrangement of their trophectoderm on day 11 to 12 of gestation [Geisert and Yelich, 1997]. This process, termed rapid trophoblastic elongation, occurs concurrently with the conceptus synthesis and release of estrogen, the maternal recognition signal. Trophoblastic elongation serves to establish a maternal-fetal interface with which the conceptus can initiate implantation and placentation. The uniqueness of this phenomena lies in the rapid rate (40 mm/h) and to what extent (> 1 meter) porcine conceptuses elongate. During this transformation, individual conceptuses acquire uterine space to ensure nutrient exchange with the dam and future survival. However, this short time period of conceptus development in the pig represents the majority of a 25-46%

embryonic mortality rate [Pope, 1994]. Among US and European pigs, the initiation of trophoblastic elongation in conceptuses can be variable among littermates, possibly leading to unequal allocation of uterine space and causing the demise of slightly less developed physiologically normal conceptuses.

Trophoblastic elongation represents one of the most important stages of early development in swine subsequently having impacts on litter size, one of the most economically important traits affecting the pig industry. Therefore, the purpose of the following review of literature is to describe early conceptus development in the pig and assess the current knowledge of genes that may regulate trophoblastic elongation, maternal recognition and implantation.

# Chapter II:

# Literature Review

## Porcine Embryonic Development

Heuser and Streeter [1929] were the first to describe the morphological changes that occur during peri-implantation conceptus development in the pig. Cellular cleavage of the isolecithal ovum in mammals is a process of cell division in which several equal holoblastic divisions occur because of stored lipid sources within the cytoplasm. With each cleavage division there is a reduction in cell size. The first two cleavage divisions occur rapidly in pig zygotes as the embryo reaches the 4-cell stage of development within 24 h after fertilization. Following the initial divisions, cleavage rate becomes slower although still occurring approximately every 24-26 h until the embryos reach the 8-16 cell stage of development. During the early cleavage stages of development, particularly at the 4-cell stage of development, the blastomeres readily take up uridine and incorporate it into RNA [Tomanek et al., 1989]. These data indicate that maternal mRNA stored in the oocyte is depleted and the activation of the embryonic genome has commenced. Following the transition from the 4 to 8-cell stage, the embryos leave the oviduct and enter into the uterus. By day six following fertilization the embryos have undergone blastulation resulting in a defined trophectodermal cell layer and a thickening inner cell mass within the prominent blastocoele. On day 7 to 8 of gestation the blastocyst hatches from the zona pellucida and begins to expand in diameter with differentiation of the inner cell mass and active trophoblast proliferation. The conceptus, consisting of the embryo proper and its extraembyonic membranes, reaches a spherical diameter of 2-3 mm by day 10 of gestation. Heuser and Streeter [1929] were the first to

describe the variation in conceptus morphologies that were present between days 11 to 12 of gestation. Their classical paper presented photographs of spherical, tubular and filamentous conceptuses in the pig uterus. Following formation of filamentous conceptuses, the initial attachment of the conceptus trophectoderm to the uterine epithelium occurs on day 13 of gestation [Perry et al., 1981; Dantzer, 1985]. The allantois expands from the embryonic hindgut on day 14 and is as long as the embryo by day 17 [Friess et al., 1980]. The allantois continues to expand, completing full contact with the meter long chorion by day 19 of gestation. The chorion is completely vascularized by allantoic blood vessels by day 30 of gestation [Wislocky and Dempsey, 1946]. In essence, between days 12 and 30 of gestation, porcine conceptuses expand throughout the uterine lumen and establish an absorptive surface for nutrient exchange with the dam. The extent to which conceptuses elongate on day 11 to 12 of gestation has profound effects on the subsequent length of their placenta. Generally, conceptuses developing the largest placenta acquire the greatest uterine surface contact and have the highest chances of survival to term.

During late peri-implantation development, on approximately day 10 of gestation, a 3 mm spherical porcine conceptus will grow in diameter at a rate of 1 mm/4h through cellular hyperplasia [Geisert et al., 1982a]. Over the next 24-36 h, the conceptus will expand until it reaches an approximate 9-10 mm diameter [Geisert et al., 1982a; Pusateri et al., 1990]. On day 11 to 12 of gestation, the 9-10 mm spherical conceptuses undergo a rapid morphological transformation, termed trophoblastic elongation [Geisert et al, 1982a]. Trophoblastic elongation is initiated concurrently with conceptus synthesis and release of the maternal recognition signal, estrogen [Geisert et al., 1982a; Bazer et al.,

1986]. The unique phenomena of trophoblastic elongation in the pig is characterized by a 10 mm spherical conceptus transforming first into a tubular morphology (15-25 mm), then rapidly elongating (40 mm/h) until the conceptus becomes a thin filamentous form greater than 150 mm in length [Geisert et al., 1982a]. The transformation of the conceptus from a spherical to filamentous form occurs within 2 to 4 h. The elongation process is the result of cellular migration and remodeling of trophectodermal and endodermal cell layers, not cellular hyperplasia [Geisert et al., 1982a; Mattson et al., 1990; Pusateri et al., 1990]. Mattson et al. [1990] proposed that the cytoskeletal rearrangement of filamentous actin (f-actin) was responsible for the dramatic and rapid change in morphology. In 1981, Perry likened the rapid formation of a filamentous conceptus to rolling a ball of plasticene between two hands, forcing expansion of the ends while reducing the diameter. Expansion of the conceptus within the length of the uterine horns is a critical process necessary for individual porcine littermates to establish ample placental surface area required for adequate nutrient exchange throughout gestation [Stroband and Van der Lende, 1990; Geisert and Yelich, 1997]. Trophoblastic elongation also serves to deliver conceptus estrogen secretion throughout the length of the uterine horn preventing luteolysis. The need for elongation to cover the uterine horns and deliver estrogen is noted by the demonstration that at least two viable conceptuses per uterine horn are required at the time of maternal recognition to prevent luteolysis during pregnancy [Polge et al., 1966; Dziuk, 1968]. Although little information is available regarding stimuli responsible for the initiation of rapid trophoblastic elongation, it has been established that trophoblastic elongation is regulated by the conceptus itself and is not a direct response to signals of maternal origin. This hypothesis was confirmed by

Morgan et al. [1987a] when they demonstrated that spherical conceptuses in uteri prematurely stimulated with exogenous estrogen do not undergo trophoblastic elongation until reaching 10 mm in diameter, when trophoblastic elongation normally commences. Furthermore, it is not unusual to recover viable spherical, tubular and filamentous conceptuses within the same litter [Anderson, 1978; Geisert et al., 1982a]. The presence of multiple morphological stages of conceptuses within a litter suggests that the maternal system does not trigger uniform elongation among all of the developing embryos.

**Embryonic Mortality in the Pig**

In 1923, Corner was the first to determine that the number of corpora lutea present on the ovaries is an accurate estimate of ovulation rate and could be utilized to determine the rate of embryonic mortality at various stages of gestation. Using this methodology, embryonic mortality has been determined to range from 20 to 46% by term [Pope, 1994].

Numerous factors contribute to the loss of developing conceptuses in the pig throughout the 114 days of gestation. Broadly, there are two time periods in which embryonic mortality occurs; peri-implantation, between days 10 and 18; and post-implantation, between days 18 and 114 days of gestation. The majority of embryonic mortality occurs during peri-implantation development [Stroband and Van der Lende, 1990] in which the conceptuses commence a rapid morphological rearrangement of the trophoblast in an effort to acquire ample uterine lumenal surface area for development to term. Numerous factors are involved with the ability of the conceptus to undergo trophoblastic elongation and prepare for successful implantation.

Following fertilization and oviductal transport to the uterus, the first barrier to conceptus survival is the incompatibility of the uterine environment with regards to conceptus development. Conceptus-uterine asynchrony greater than 36 h has been demonstrated to cause conceptus death as early as day 8 of gestation [Geisert et al., 1991]. Pope et al. [1990] have shown that transfer of additional day 6 embryos to a day 7 pregnant uterus showed greater mortality and morphological variation of the day 6 embryos compared to additional day 7 embryos transferred to day 6 pregnant uteri. This indicates that conceptuses lagging in development with respect to the uterine environment are less competitive to more advanced embryos and endure increased mortality rates. In addition to conceptus-uterine asynchrony, asynchronous development among littermates during peri-implantation development correlates to embryonic mortality of the less developed conceptuses. Pope et al. [1990] also indicated that both day 5 and day 7 conceptuses transferred to a uterus corresponding to day 6 of gestation could survive until at least day 60 of gestation. However, when both day 5 and 7 conceptuses were transferred to the same uteri, day 7 conceptuses exhibited greater survival [Pope et al., 1990] indicating that through some mechanism the more advanced conceptuses govern the survival of less developed conceptuses. Collectively, these asynchrony studies indicate the ability of more developed conceptuses to produce factors that could alter endometrial secretions, producing a uterine environment that is unsuitable for appropriate growth of conceptuses lagging in development.

Estrogen, the maternal recognition signal in the pig, is likely the conceptus factor that alters the uterine environment and causes the demise of less developed littermates. Treatment of gilts containing transferred embryos lagging 24 h in conceptus-uterine

synchrony with estrogen on day 11 resulted in halted conceptus development. However embryos synchronous to the uterus in gilts receiving the same treatment elongated and appeared phenotypically normal [Morgan et al., 1987b]. These data indicate survival advantage possessed by more advanced conceptuses occurs through earlier production of estrogen that alters the uterine environment making it is unsuitable for the progression of less developed conceptuses. It is likely that conceptuses more advanced in development on day 11 can stimulate biochemical changes in the uterus via their own surge in estrogen production giving them a competitive advantage for the accumulation of uterine space and subsequent survival [Pope et al., 1990].

While it is apparent that estrogen is needed as a maternal signal, timing and extent of estrogen exposure can have dramatic effects on continued conceptus development and survival. Inappropriate timing of estrogen stimulation can result in total litter loss as occurs with estrogenic alfatoxins, such as zearalenone, found in moldy corn [Long and Diekman, 1984]. While it doesn't inhibit conceptus elongation, exogenous estrogen, given on days 9 and 10 of gestation (48 to 72 h prior to normal conceptus estrogen release) alters uterine glandular secretion and disrupts the glycocalyx covering the endometrial epithelial cell surface. Disruption of the uterine surface glycocalyx is closely associated with embryonic mortality on day 15 to 18 of gestation [Morgan et al., 1987a; Blair et al., 1991; Geisert et al., 1991]. The uterine glycocalyx (UG) generally thickens during attachment and early placentation in the pig, which occurs between days 13 and 18 of gestation [Perry et al., 1981; Dantzer, 1985; Geisert et al., 1991]. However, the UG becomes somewhat reduced in areas of placentation compared to inter-attachment sites [Blair et al., 1991]. Premature administration of estrogen (days 9 and 10) impairs the

thickening of the UG, actually causing it to completely slough off by day 16 of gestation. The loss of the UG prevents cell-cell interactions necessary for conceptus attachment resulting in conceptus mortality [Blair et al., 1991].

Another component that affects the outcome of litter size at term is uterine capacity. Uterine capacity is defined as the maximum number of fetuses the uterus can support throughout the length of gestation when potentially viable conceptuses are not limiting [Christenson et al., 1987]. In an effort to increase litter size, early studies focused on increasing the viable number of conceptuses at the onset of pregnancy by superovulating gilts. Superovulated gilts contain significantly more intact conceptuses at day 30 of gestation [Dziuk, 1968], however this advantage in litter size is lost by term, indicating that the limitations of uterine capacity occur some time after day 30 of gestation. The Meishan breed of pigs that originated from China are known for their prolific reproductive capabilities. Meishan pigs have a similar ovulation rate and number of viable conceptuses at day 12 of gestation compared to US and European breeds. However, by term, Meishans farrow at least 3 piglets more than US and European breeds [Ford, 1997]. The greater litter size at term has largely been attributed to the ability of Meishan conceptuses to develop smaller, more vascular placentas that enable them to acquire ample nutrient exchange while minimizing uterine space [Ford, 1997]. Placental efficiency (fetal weight/placental weight) is highly variable between and even within litters, however placental efficiency may provide an effective selection tool for litter size [Wilson and Ford, 2001]. Wilson et al. [1999] indicated that selecting individual littermates based on placental efficiency (fetal weight/placental weight) significantly increased litter size after only one generation of selection albeit the number of animals

involved in the study was relatively small. In a larger commercial study, Vonnahme et al. [2002] demonstrated that a significant positive correlation exists between ovulation rate and viable conceptus at day 25 but not at days 36 or 44 of gestation. The opposite relationship exists for uterine length, which is not significantly correlated to viable conceptuses at day 25 of gestation but is positively correlated by days 36 and 44. These data suggest that the limitation of uterine capacity begins to negatively effect litter size sometime between day 25 and 36 of gestation. Furthermore, Vonnahme et al. [2002] indicate that placental efficiency is positively correlated to viable conceptus at all days of gestation evaluated (days 25, 36 and 44). These data lend confirmation to the smaller study done previously by Wilson et al. [1999] suggesting that placental efficiency may be involved in the regulation of uterine capacity. These data further indicate the limitation of embryo survival is related to development of the placenta, which is initially established during conceptus elongation on day 12 of gestation.

**Maternal Recognition of Pregnancy**

Maternal recognition of pregnancy is defined as conceptus release of a chemical signal that functions to prolong the lifespan of the corpus luteum (CL) beyond the length of a normal estrous cycle [Geisert et al., 1990]. Maintenance of the CL sustains progesterone production and subsequently provides a uterine environment favorable for conceptus development to term. In the pig, the presence of corpora lutea on the ovaries is a required source of progesterone throughout gestation as ovariectomy at any stage of pregnancy results in abortion [Nara et al., 1981]. Perry et al. [1973, 1976] were the first to show that porcine conceptuses are physiologically capable of producing estrogens

from the metabolism of steroid precursor molecules. It was later established that in the pig, conceptus synthesis and release of estrogen is in fact the signal for maternal recognition [Bazer et al., 1982]. Synthesis and release of estrogen by the developing pig conceptus is biphasic, transiently peaking during trophoblastic elongation on day 11-12 followed by a more sustained release of estrogen initiated on day 15-16 of gestation [Geisert et al., 1990]. Kidder et al. [1955] demonstrated that a single injection of estrogen to gilts after day 9 of the estrous cycle prolonged the lifespan of the corpora lutea. Gardner et al. [1963] lengthened the interestrous interval by administering exogenous estrogen on day 11-12 of the estrous cycle. Similar results were revealed by Geisert et al. [1987] who demonstrated an additional 7 days was added to the CL lifespan in response to exogenous estrogen given on day 12 of the estrous cycle. Ford et al. [1982a] extended CL survival when infusing estradiol valerate directly into the uterine lumen between days 11 and 15 of the estrous cycle. Similarly, King and Rajamahendran [1988] demonstrated implanting silastic beads impregnated with estrogen into the uterine lumen on day 10 was capable of inducing psuedopregnancy. Frank et al. [1977] induced psuedopregnancy in cyclic gilts by an average of 146 days (although highly variable) by injecting 5 mg of estradiol valerate daily during days 11 to 15 of the estrous cycle. Dhindsa and Dziuk [1968] showed that flushing conceptuses from the uterus before day 18 would not extend the psuedopregnancy beyond day 30 as efficiently as flushing the conceptuses on or after day 18, indicating that important conceptus signals are secreted near day 18 of gestation. In an effort to more closely mimic physiological conceptus release of estrogen described by Geisert et al. [1982b], an additional 2$^{nd}$ dose of estrogen administered between days 14-16 following the initial induction of exogenous estrogen

on day 11 to 12 of the estrous cycle prolongs the life of the corpora lutea greater than 60 days [Geisert et al., 1987]. These data confirmed conceptus biphasic secretion of estrogen was necessary to establish pregnancy as prolonged psuedopregnancy (>60 days) could only be induced when estrogen was administered at both day 11 and days 14 through 17, while a single injection at either time frame was incapable of inducing psuedopregnancy greater than 35 days [Geisert et al., 1987]. Because of its ability to induce psuedopregnancy when administered exogenously to cyclic gilts at time points temporally associated with normal conceptus estrogen release, estrogen has been largely accepted as the maternal recognition signal in the pig [Bazer et al., 1982].

The augmentation of uterine blood flow, necessary for conceptus survival, has been associated with increased estrogen content in the uterine lumen [Ford and Christenson, 1979; Ford et al., 1982b]. In addition to estrogen, prostaglandin $E_2$ ($PGE_2$), a vasodilator, also dramatically increases in uterine flushings during the time of conceptus elongation and maternal recognition of pregnancy [Geisert et al., 1982b]. Davis and coworkers [1983] demonstrated that porcine conceptuses have a significant increase in phospholipase A2 activity and prostaglandin production between day 7 and 14 of gestation, particularly PGE's. $PGE_2$ is known to accentuate the survival of the corpus luteum as indicated by its ability to increase both CL weight and progesterone production [Ford and Christenson, 1991]. It has also been suggested that the biphasic release of estrogen by porcine conceptuses regulates the movement of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in the uterine lumen [Bazer and Thatcher, 1977]. $PGF_{2\alpha}$, a vasoconstrictor synthesized and released by the uterine endometrium, is the luteolytic agent responsible for demise of the corpora lutea on day 15 of the estrous cycle in the pig [Moeljono et al., 1976].

The endocrine/exocrine theory suggested by Bazer and Thatcher [1977] describes the regulation of $PGF_{2\alpha}$ movement and is the proposed mechanism of estrogen action during porcine maternal recognition of pregnancy. During the estrous cycle and normal CL regression, the uterine endometrium functions as an endocrine tissue capable of releasing $PGF_{2\alpha}$ into the uterine venous blood return. Release of $PGF_{2\alpha}$ into the systemic vasculature permits delivery of the luteolysin to the CL via counter-current exchange with the ovarian artery and/or systemic delivery through the lungs resulting in luteolysis. The endocrine/exocrine theory states that estrogen of conceptus origin is responsible for redirecting the endometrial release of $PGF_{2\alpha}$ from an endocrine to an exocrine secretion thereby sequestering $PGF_{2\alpha}$ in the uterine lumen and eliminating its ability to function as a luteolysin. In most species, the lungs are capable of reducing the majority of $PGF_{2\alpha}$ to its inactive metabolite, 15 keto-13,14 dihydro-prostaglandin F2$\alpha$. However, due to the lowered enzymatic efficiency of porcine lungs to metabolize $PGF_{2\alpha}$, only 18% is reduced to the non-luteolytic metabolite following one passage through the lungs [Davis et al., 1979], indicating both systemic and local luteolytic pathways exist in the pig. Because the porcine lungs are so inefficient in reducing $PGF_{2\alpha}$ to an inactive form, the importance of preventing the endocrine release of $PGF_{2\alpha}$ is paramount. In non-pregnant animals, those not subjected to conceptus estrogen release, there is a sharp increase in plasma $PGF_{2\alpha}$ between days 12 and 18 while total uterine $PGF_{2\alpha}$ increases slightly during the same time frame [see review, Geisert et al., 1990]. In contrast, pregnant gilts that have been exposed to conceptus estrogen secretion, have lower plasma concentrations of $PGF_{2\alpha}$ and much greater total uterine $PGF_{2\alpha}$ content compared non-pregnant gilts. Ford et al. [1982] infused 375 ng of estradiol-17$\beta$ directly into the uterine horns of cyclic gilts

every 6 hours on days 11 to 15 and reported lower concentrations of $PGF_{2\alpha}$ in the utero-ovarian vein of gilts receiving estrogen treatment compared to controls. This confirmed earlier work done by Frank et al. [1977] who injected i.m. 5 mg of estradiol valerate per day and demonstrated reduced $PGF_2$ in the utero-ovarian veins. Collectively, these data support the endocrine/exocrine theory suggesting that indeed $PGF_{2\alpha}$ is sequestered in the uterine lumen and prevented from reaching peripheral plasma during pregnancy. Furthermore, these data suggest this phenomena occurs by the mechanistic actions of conceptus estrogen secretion.

Because a systemic pathway for prostaglandin delivery to the corpora lutea exists, the inhibition of $PGF_{2\alpha}$ endocrine release throughout the majority of the uterine horns is paramount. The extensive length of the uterine horns and the necessity for estrogen signaling throughout the uterus requires at least two conceptuses in each uterine horn to maintain a viable pregnancy [Polge et al., 1966; Dziuk, 1968]. This requirement is likely due to the inability of fewer conceptuses to biologically inhibit the entire uterus from prostaglandin endocrine release. Also, the fact both uterine horns must contain conceptuses indicates the potency of systemic delivery of $PGF_{2\alpha}$ from one uterine horn to the contralateral CL. Trophoblastic elongation is the mechanism allowing pig conceptuses to adequately deliver estrogen throughout the entire length of the uterine horns promoting the survival of the CL.

**Apposition and Attachment**

After conceptus elongation and release of estrogen for the maternal recognition of pregnancy signal, placental attachment in the pig is initiated between days 13 and 18 of gestation [Perry et al., 1981; Dantzer, 1985]. Porcine conceptuses are non-invasive *in vivo* and develop a diffuse, epitheliochorial type of placenta [King et al., 1982; Keys and King, 1990]. Integrins are thought to be an essential component contributing to successful apposition and attachment of the trophectoderm to the uterine epithelium. Surveyor et al. [1995] reported that the cell surface mucin, MUC-1, likely regulates uterine receptivity to conceptus attachment in mice via the prevention of integrin binding between the trophectoderm and uterine epithelium. Interestingly, uterine MUC-1 expression is similar between the mouse [Braga and Gendler, 1993] and pig [Bowen et al., 1996] with respect to its appearance during the estrous cycle and steroid hormone regulation. MUC-1 expression in the pig is greatest between day 0 and 4 of the estrous cycle. Ovarian hormones regulate expression of MUC-1 on the uterine epithelium, as it is virtually undetectable by day 10 in both cyclic and pregnant gilts [Bowen et al., 1996]. Both *in vivo* and *in vitro* MUC-1 expression is reduced in the uterine epithelium in response to progesterone stimulation [Bowen et al., 1996, 1997]. It appears that down-regulation of progesterone receptor in the epithelium on day 10 [Geisert et al., 1994] is associated with depletion of MUC-1 expression. Loss of MUC-1 in the uterine epithelium on day 10 opens the window for uterine receptivity for initiation of implantation during porcine pregnancy.

Muc-4, another cell surface expressed mucin, is also decreased on the uterine epithelium during implantation in rodents [McNeer et al., 1998; Carraway and Idris,

2001]. However, unlike MUC-1, the expression of MUC-4 differs in the pig uterine epithelium compared to rodents. MUC-4 expression increases during the period of implantation and placental expansion in the pig [Ferrell et al., 2003]. Placentation in the pig is non-invasive while the conceptuses themselves are highly invasive *ex utero*. The coordinated increase in MUC-4 expression, which is temporally associated with the down-regulation of MUC-1, may assist in controlling the proteolytic activity of porcine conceptuses and prevent invasive implantation in the pig. In contrast, invasive implantation in rodents may require the down-regulation of both MUC-1 and MUC-4.

Once the uterine environment is receptive to conceptus attachment following the down-regulation of MUC-1 on day 10 of gestation in the pig, successful apposition and attachment requires the appropriate expression of both ligands and receptors between the trophectoderm and the uterine epithelium. The primary uterine receptors responsible for trophectodermal binding are integrins. Integrins are cell surface glycoproteins [Hynes, 1992] that consist of numerous $\alpha$ and $\beta$ subunits, which play critical roles regulating conceptus attachment in the pig [see reviews Jaeger et al., 2001; Burghart et al., 1997]. The formation of an $\alpha\beta$ heterodimer results in a membrane bound receptor with the ligand specificity depending upon the specific subunit combination making the dimer. At least five $\alpha$ (1, 3, 4, 5 and v) and three $\beta$ (1, 3 and 5) subunits exist in the uterine epithelium of the pig [Bowen et al., 1996]. Of those, expression of $\alpha$1 and $\alpha$3 is low, $\alpha$v and $\beta$3 are constitutively expressed, and expression of $\alpha$4, $\alpha$5 and $\beta$1 is greatest during the time of estrogen signaling and initial conceptus attachment [Bowen et al., 1996]. Bowen et al. [1996, 1997] demonstrated that *in vitro* and *in vivo* expression of $\alpha$4, $\alpha$5 and $\beta$1 subunits is under progesterone regulation. Porcine conceptuses also express integrin

subunits α1, α4, α5, β1 and β3 [Bowen et al., 1996]. The formation of integrin heterodimers during the time of apposition and attachment in the pig indicates the necessity for specific ligands. Two primary ligands for integrins are fibronectin and vitronectin. Fibronectin has been identified on the trophectoderm while vitronectin is expressed on both the trophectoderm and the uterine epithelium [Bowen et al., 1996]. Tuo and Bazer [1996] identified oncofetal fibronectin in the porcine trophectoderm from days 12 to 15. Osteopontin and the transforming growth factor β latency-associated peptide are also likely to be involved in pig conceptus attachment as they both have the capability of binding integrin heterodimers [review Jaeger et al., 2001]. Osteopontin has been detected in the lumenal epithelium as early as day 15 of gestation, however it is expressed at much greater levels after day 25 of gestation [Garlow et al., 2002]. These data indicate that while osteopontin may be involved in initial attachment, it is much more likely to be involved in maintaining the intricate attachment between the conceptus and endometrium throughout gestation.

**Porcine Conceptus Gene Expression**

Efforts to identify genes regulating specific developmental events during early pig embryogenesis has resulted in the analysis of numerous messenger RNA transcripts theorized to be of critical developmental importance. While gene expression occurs during the earliest developmental stages, it is predominately under maternal regulation until the transition from a 4- to 8-cell embryo when major activation of the porcine embryonic genome occurs [Tomanek et al., 1989]. Numerous factors and genes are involved in the regulation of conceptus development prior to rapid trophoblastic

elongation [see review Maddox-Hyttel et al., 2001]. However, the intention of this review is to focus more specifically on genes affecting conceptus development during day 11 to 12 of gestation, the time of maternal recognition and rapid trophoblastic elongation.

Following the activation of the embryonic genome and initiation of early development, it is estimated that expression of approximately 10,000 genes is necessary for successful embryogenesis [Niemann and Wrenzycki, 2000]. Due to the developmental importance and the subsequent impact trophoblastic elongation has on placental attachment, uterine capacity and subsequent survival of porcine conceptuses, research efforts have focused on identification of conceptus genes that may regulate trophoblastic elongation. During this endeavor, research studies have predominately targeted the discovery of genes that are of potential developmental importance and associated with rapid trophoblastic elongation that occurs between days 10 and 12 of gestation [Geisert et al., 1982a].

Geisert and Yelich [1997] suggested that the programming for trophoblastic elongation may occur as much as 24 h prior to elongation as the activation of conceptus estrogen synthesis occurs concurrently with first signs of mesodermal differentiation in 5 mm spherical conceptuses. Mesodermal outgrowth and differentiation in the mouse embryo is dependent upon the appropriate expression of the transcription factor, brachyury [Herrman et al., 1990]. Porcine conceptus brachyury gene expression occurs simultaneous with the mesodermal outgrowth of 5 mm spherical conceptuses. Mesodermal outgrowth and brachyury expression is temporally associated with the gene

expression pattern of $P450_{arom}$ during porcine conceptus development [Yelich et al., 1997a].

Trophoblastic elongation is largely associated with a dramatic increase in estrogen synthesis and release by porcine conceptuses [Geisert et al., 1982b; Fischer et al., 1985]. Cytochromes P450 17α-hydroxylase ($P450_{17\alpha}$) and aromatase ($P450_{arom}$) are essential enzymes required for estrogen synthesis. Currently, there are three distinct $P450_{arom}$ isoforms in the pig, each of which is encoded by a unique gene [Graddy et al., 2000]. $P450_{arom}$ types I, II and III are specific to the porcine ovary, late gestation placental tissue [Corbin et al., 1995] and conceptus [Choi et al., 1996], respectively. Gene expression for both $P450_{17\alpha}$ and $P450_{arom}$ significantly increases during day 11 to 12 of gestation when filamentous conceptuses are forming [Yelich et al., 1997a]. The ability for conceptus release of estrogen to function in an autocine/paracrine fashion has recently been suggested by Kowalski et al. [2002] who isolated and demonstrated increasing expression of estrogen receptor β in elongating porcine conceptuses. Interestingly, Yelich et al. [1997a] could not detect estrogen receptor α gene expression in elongating conceptuses through RT-PCR. Moreover, immunocytochemical analysis indicated ERα expression in the uterine endometrium increases from day 0 until day 12 of the estrous cycle and pregnancy declining by day 15 [Geisert et al., 1993]. These data suggest that conceptus estrogen acts on both the conceptus and endometrium albeit through different receptor pathways. Thus, conceptus release of estrogen can execute separate and distinct autocrine and paracine functions that occur through different isoforms of ER within the conceptus and uterus.

Geisert et al. [1982b] demonstrated an increase in both $PGF_{2\alpha}$ and $PGE_2$ in uterine flushings was temporally associated with the increasing conceptus release of estrogen. Guthrie and Lewis [1986] indicated that during elongation, porcine conceptuses increase the synthesis and release of prostaglandins. Phospholipase A2 (PLA2) is responsible for the cleavage and release of free arachidonic acid, which is subsequently converted to prostaglandins via the actions of cyclooxygenase-2 (COX-2). As expected, the activity of PLA2 and expression of COX-2 both increase in filamentous conceptuses [Davis et al., 1983; Wilson et al., 2002]. The relationship between prostaglandin release and trophoblastic elongation is not known other than the temporal and spatial association between the two. However, prostaglandin production may be related to the remodeling of the trophoblast through the release of arachidonic acid, which permits membrane fluidity in the cell membrane phosholipid bilayer necessary for remodeling of the trophectoderm [Davis and Blair, 1993; Geisert and Yelich, 1997].

Insulin-like growth factors (IGFs), particularly IGF-I and IGF-II, which may have significant impact on the growth and development of the pig conceptus, have been well characterized throughout early gestation. Endometrial secretion of IGF-I is elevated during the time of trophoblastic elongation and declines shortly thereafter [Simmen et al., 1992] while conceptus gene expression for IGF-I increases steadily during pre-elongation stages peaking at day 12 of gestation [Letcher et al., 1989]. IGF-I receptor gene expression is present throughout peri-implantation conceptus development, however there is no variation in expression throughout early development [Green et al., 1995]. The enhanced release of IGF-I by the endometrium is correlated with an increase in conceptus $P450_{arom}$ gene expression [Ko et al., 1994; Green et al., 1995] and has been suggested to

regulate conceptus ability to produce estrogen [Hofig et al., 1991]. Uterine IGF-I secretion, which is significantly greater in the uterine lumen of pregnant gilts on day 12 of gestation compared to day 12 of the estrous cycle, may act in an autocrine/paracrine fashion to regulate uterine changes [Geisert et al., 2001]. Uterine receptivity to IGF-I occurs through the IGF-1 receptor that is copiously expressed in the uterine endometrium [Simmen et al., 1992]. Pig conceptuses also express IGF-II receptor [Chastant et al., 1994] and its stimulation is likely associated with growth and development before and during the time of conceptus elongation. Endometrial release of IGF-II into the uterine lumen significantly increases from day 10 to 12 of gestation and is much greater on day 12 of pregnancy compared to the equivalent day of the estrous cycle [Geisert et al., 2001]. Both IGF-I and -II can possibly stimulate conceptus growth and development by acting through the IGF- II receptor in the conceptus [Czech, 1986]. IGF binding proteins (IGFBP) function to bind IGF thereby regulating the degree to which IGF is capable of biologically stimulating a target cell [Rechler, 1993]. Lee et al. [1998] demonstrated that IGFBPs were present in the pig uterine lumen before day 11 of gestation and became absent in the lumen after day 11 of gestation. The loss of IGFBP-2 and -3 was not caused by a decrease in endometrial gene expression but rather through cleavage of IGFBP-2 and -3. Since the loss of IGFBPs correlated with the time of trophoblastic elongation, Lee et al [1998] suggested conceptus regulation of IGFBP cleavage. However, Geisert et al. [2001] have indicated that the cleavage of IGFBP-2 and -3 in the uterine lumen on day 10 and 12 occurs in both pregnant and cyclic gilts likely through the protease activity of kallikrein and/or matrix metalloproteinases. Cleavage of IGFBP-2 and -3 increases the

bioavailability of IGF-I and -II at a time period when conceptus growth and steroid production is peaking.

Epidermal growth factor (EGF) and transforming growth factor-alpha (TGFα) are additional growth factors that can affect conceptus development. Interestingly, EGF and TGFα serve as ligands for the same receptor, EGF receptor (EGF-R) [Burgress, 1989]. Porcine conceptus TGFα gene expression is detected briefly during peri-implantation development from days 8 through 12 of gestation with maximal expression occurring on day 10. In contrast, conceptus EGF gene expression commences on day 15 of gestation and continues to increase into early organogenesis [Vaughan et al., 1992]. EGF-R is constitutively expressed in the conceptus from day 7 until at least day 22 of gestation [Vaughan et al., 1992]. While the gene expression profiles of TGFα and EGF differ drastically, it is likely that they both act via EGF-R and serve unique stimulatory roles affecting early conceptus development in the pig. In mouse conceptus development, TGFα stimulates fluid uptake thereby regulating blastocoele expansion [Dardik and Schultz, 1991]. Blastocyst formation in the pig occurs on day 8 of gestation and may be under the partial regulation of TGFα as the receptor is expressed as early as day 7. It is also highly possible that TGFα could illicit similar effects in porcine conceptuses regulating membrane fluidity, which is necessary for commencement of trophoblastic elongation [Geisert and Yelich, 1997]. However, EGF probably contributes most to early organ and placental development occurring from day 14 to 22 of gestation as indicated by its temporally associated gene expression.

Another family of growth factors that have been extensively investigated during conceptus-maternal interfacing that occurs between days 10 and 14 of gestation are the

three transforming growth factor β isoforms (TGFβ-1, -2 and -3). *In situ* hybridization analysis indicated gene expression for all three TGFβ isoforms tends to increase in the porcine conceptus trophectoderm and endoderm from days 10 to 14 of gestation while only TGFβ-2 increased in the embryonic ectoderm and mesoderm during days 12 to 14 [Gupta et al., 1998]. Gene expression for all three TGFβ isoforms increases in uterine lumenal epithelium on days 10 to 14 of gestation [Gupta et al., 1998], which coincides with increasing estrogen synthesis and release into the uterine lumen during conceptus elongation [Geisert et al., 1982b]. Yelich et al. [1997b] evaluated conceptus TGFβ-2 and -3 gene expression during trophoblastic elongation and reported that TGFβ-2 was not detectable through RT-PCR. However, Yelich and coworkers [1997b] confirmed that TGFβ-3 gene expression increased during the period of rapid morphological change in conceptus development during days 10 to 12 of gestation. Immunostaining for TGFβ receptors revealed both TGFβ receptor type I and II are expressed in the peri-implantation pig conceptus during days 10-14 of gestation [Gupta et al., 1996]. Presence of the TGFβ receptors indicates the ability of the conceptus to respond to TGFβ stimulation from conceptus or endometrial origin.

Throughout the last decade retinol, the common form of vitamin A, has received thorough investigation regarding the effects it may have on conceptus development in pigs, particularly during days 10-18 of gestation. Retinol likely plays integral roles orchestrating cell division, organogenesis and placental growth in all mammals [Roberts et al., 1993], however, when excessive, retinol can be embryotoxic [Thompson et al., 1964]. Retinol, retinal, and retinoic acid, collectively termed retinoids, induce biological actions via retinoic acid receptors (RAR) α, β, and γ. Yelich et al. [1997b] revealed that

all three RAR isoforms are expressed before, during and after rapid trophoblastic elongation. Both RARα and RARγ continue to be expressed in day 15 porcine conceptuses [Harney et al., 1994]. Roberts et al. [1993] reviewed the changes in uterine retinol and indicated its concentration in uterine flushings containing filamentous conceptuses during days 10-13 of gestation is 10-50 fold greater than uterine flushings during the same time frame only containing spherical conceptuses. Flushings containing spherical conceptuses had a concentration of retinol that was similar to that of uterine flushings from non-pregnant animals on day 11 to 12 of the estrous cycle. Vallet et al. [1996] published similar data indicating significantly higher RBP in the uterine lumen on day 13 compared to day 10 of gestation. Retinol transport from the uterine lumen to the conceptus is primarily under the regulation of retinol binding protein (RBP). RBP is a secretory product of the pig conceptus [Harney et al., 1990] whose gene expression increases steadily as conceptuses develop from 4 mm spheres into more advanced filamentous conceptuses [Yelich et al., 1997b]. Furthermore, gene expression of RBP by the uterine endometrium of day 12 pregnant gilts is highly dependent on morphological stage of conceptus development [Trout et al., 1992]. Endometrium from gilts bearing day 12 filamentous conceptuses had dramatically greater RBP gene expression than day 12 endometrium in the presence of spherical conceptuses. Synchronization and timing of the gene expression for both RAR's and RBP in the conceptus and endometrium suggests a significant dependency of conceptus development on retinol availability. Geisert and Yelich [1997] proposed that conceptus secreted estrogen stimulates the endometrial release of RBP resulting in the transport of retinol to the conceptus cytoplasm where it is converted to retinoic acid (RA). Available RA stimulates conceptus RAR's inducing

extracellular matrix (ECM) remodeling, needed for trophoblastic elongation to occur, both directly, and indirectly through downstream stimulation of morphogens such as TGFβ's.

During the past decade, cytokines are proving to be intimately involved with the regulation of conceptus development and the establishment of pregnancy in many species. Mathialagan et al. [1992] evaluated interleukin-6 (IL-6) gene expression in pig conceptuses and indicated that greatest expression occurred during the period of attachment and early placentation. A later study by Modric et al. [2000] reported that IL-6 gene expression in the preimplantation conceptus peaked in day 12 filamentous conceptuses but gene expression was not detectable in day 14 conceptuses. The ability of IL-6 to induce an acute phase pro-inflammatory response is imitated, albeit to a lesser degree, by interleukin-1 (IL-1) [Mantovani et al., 1998]. Using northern blotting, Tuo et al. [1996] demonstrated that IL-1β is transiently expressed by porcine conceptuses between 11 and 13 days of gestation. Peri-implantation IL-1β gene expression has also been documented to increase prior to initiation of blastocyst implantation in the mouse [Takacs and Kauma, 1996; Kruessel et al., 1997] and has been suggested as the initiator of conceptus-uterine cross-talk during early pregnancy in the human [Lindhard et al., 2002]. The importance of a conceptus induced acute phase inflammatory response in the pig uterus is not well understood although its occurrence has been described by Geisert and Yelich [1997]. Inflammation is generally associated with the recruitment of immune cells. During pregnancy in the pig, stromal leukocyte populations are significantly greater at attachment sites opposed to between attachment sites [Engelhardt et al., 2002]. The majority of these leukocytes morphologically resembled lymphocytes suggesting

they were predominately T, B and/or natural killer (NK) cells. This initial contact, coupled with embryonic signals, invokes an acute phase inflammatory response by the uterus and likely results in the differentiation of undifferentiated T-helper (Th0) cells to either type 1 (Th1) or type 2 (Th2) cells. Th1 mediated immunity is referred to as cell-mediated immunity and is generally associated with pregnancy failure [Raghupathy, 1997] while humoral immunity mediated by Th2 is required for the successful establishment of pregnancy [Wegmann et al., 1993]. The necessity for the appropriate maternal Th cell repertoire at the site of attachment is to regulate the immunological response of the maternal immune system, permissive to the presence of a conceptus expressing paternal alloantigens [Mellor and Munn, 2000]. Immunological stimulation of the uterine milieu is unavoidable, however, the induced inflammation is necessary and not necessarily devastating to the conceptus as it stimulates counter regulatory responses limiting induced damage while encouraging shifts in the maternal T cell repertoire more suitable for a successful pregnancy [Mellor and Munn, 2000].

Colony stimulating factor-1 (CSF-1) is an additional factor produced by the conceptus that is suspected to accentuate growth and differentiation. CSF-1 gene expression is present in conceptuses as early as days 10 to 12 of gestation. However, CSF-1 expression peaks at day 30 and continues to be expressed in fetal tissues throughout gestation [Tuo et al., 1995]. CSF-1 is thought to be responsible for the recruitment of macrophages to the site of implantation and involved in regulating placental development [Wood et al., 1997]. Osteopetrotic (*op/op*) mice lack the CSF-1 gene [Wiktor-Jedrzejczak et al., 1990], which is required for successful female fertility. Pollard and coworkers [1991] demonstrated that while *op/op* x *op/op* crosses resulted in

consistent infertility, pregnancies created by crossing heterozygous males (*+/op*) with *op/op* females were partially salvaged. This indicates that the necessity for conceptus produced CSF-1 to either compensate or attenuate the CSF-1 production in the uterine endometrium.

Leukaemia inhibitory factor (LIF) is a cytokine that has been proposed to regulate conceptus growth and development. Anegon et al. [1994] indicated that LIF is present in porcine uterine lumenal flushings on days 7-13 of the estrous cycle and peaks on day 12 of gestation. LIF likely has direct effects on the conceptus as both pre- and post-elongation conceptuses express LIF receptor β [Modric et al., 2000]. Pregnancy specific endometrial gene expression of LIF is detected in pregnant animals during the approximate time rapid trophoblastic elongation is initiated and could serve as a pathway for possible conceptus-uterine communication [Anegon et al., 1994]. LIF receptor is also expressed in the mouse uterus on day 4, the time of blastocyst implantation [Ni et al., 2002]. Interestingly, LIF receptor was not expressed in the uterus following progesterone priming although it was greatly increased following estrogen-mediated termination of delayed implantation [Ni et al., 2002]. Estrogen likely mediates similar effects in pigs as the transient increase in expression of LIF in the uterus occurs concurrently with increased conceptus estrogen production and LIF receptor expression.

Steroids are also likely involved in the regulation of porcine conceptus development. Kowalski et al. [2002] detected estrogen receptor β gene expression in filamentous conceptuses suggesting the increasing estrogen release during the time of trophoblastic elongation may have autocrine/paracrine affects on the conceptus. Luteal production of progesterone may also affect conceptus development. Plasma

concentration of progesterone peaks by day 10 of gestation or the estrous cycle. Conflicting evidence exists on the expression progesterone receptor (PR) mRNA by pig conceptuses. Ying et al. [2000] were not able to identify PR protein in day 6 conceptuses whereas two abstracts presented at annual research symposiums reported RT-PCR revealed PR gene expression in filamentous conceptuses [Dekaney et al., 1998; Kowalski et al., 2000]. However, Yelich et al. [1997a] were unable to amplify PR mRNA through RT-PCR in day 11 to 12 conceptuses ranging from 2 mm spheres to filamentous morphology. The occurrence of PR mRNA expression by the pig conceptus would suggest that maternal progesterone may induce responses by developing conceptuses resulting in the establishment of a communication pathway.

While the information presented in this section provides valuable information regarding gene expression during early porcine conceptus development. Genes controlling trophoblastic elongation remain largely unknown.

**Statement of the Problem**

The unique changes that occur during early porcine conceptus development and the competition that exists between littermates striving for sufficient uterine contact has been well documented and indicates the necessity for synchronous conceptus development. The overall goal of researchers investigating porcine conceptus development is to determine the developmental genes, growth factors, attachment factors and nutrients that could possibly be manipulated to reduce the strikingly high rate of embryonic mortality that occurs during early gestation. It is widely accepted that developmentally advanced conceptuses commencing rapid trophoblastic elongation prior

to the elongation of the less developed conceptuses is a major contributor to peri-implantation conceptus mortality. Advanced conceptuses cause embryonic loss primarily because trophoblastic elongation is associated with the dramatic increase in conceptus estrogen synthesis and release resulting in an advanced uterine environment unsuitable for the development of less developed conceptuses. Secondly, conceptuses that initiate trophoblastic elongation first attain the most uterine lumenal space available limiting placental growth and uterine surface contact to conceptuses whose trophoblastic elongation is slightly delayed.

Geisert and Schmitt [2000] suggested that artificially inhibiting trophoblastic elongation until all conceptuses within a litter are physiologically and developmentally capable of elongation will result in consistent placental space attained among littermates and also prevent uterine advancement beyond what all conceptuses can withstand. As well as their highly vascularized placentae, Ford [1997] indicated that the larger litter size in Chinese Meishan sows partially results from the uniform timing and length of trophoblast expansion that occurs in this breed of swine.

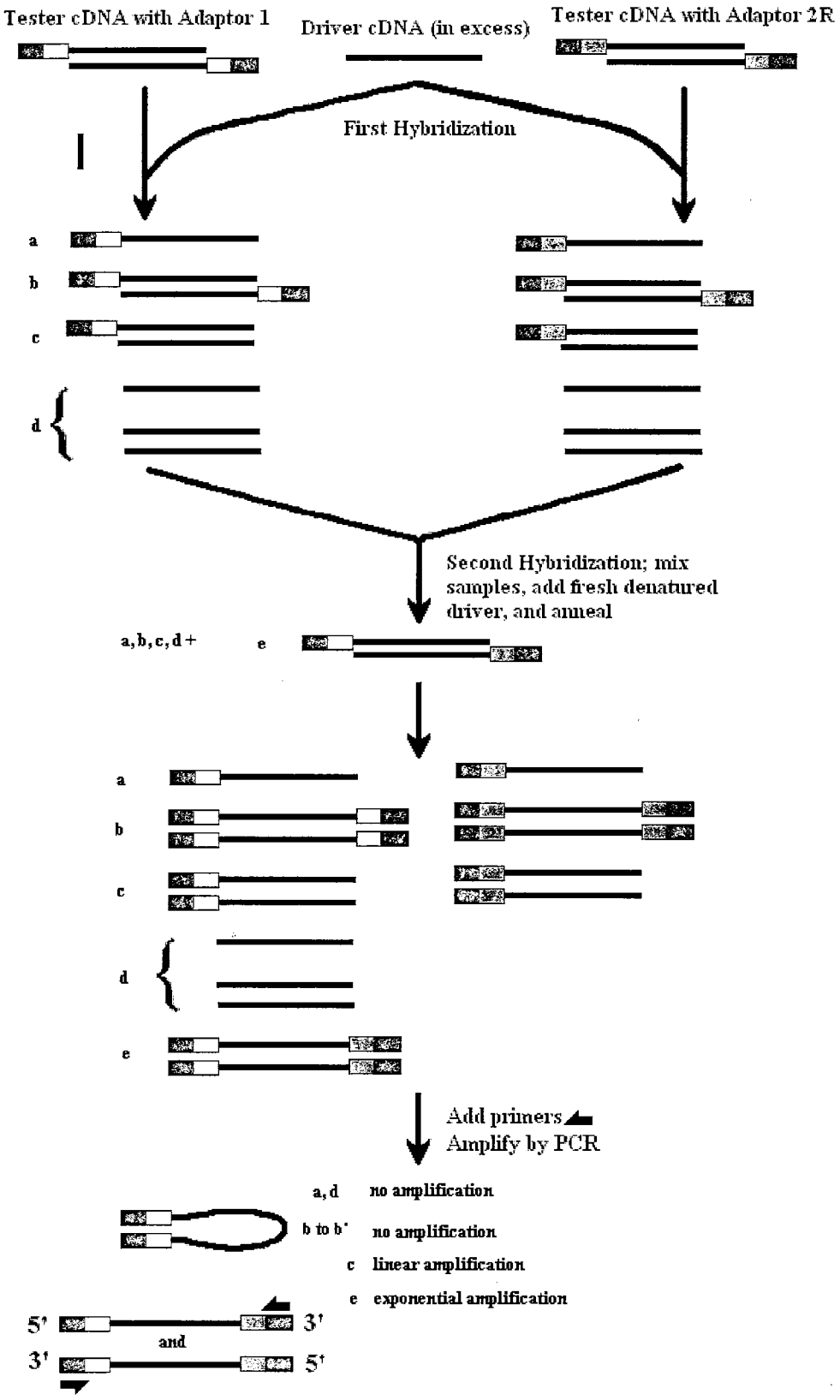
Currently, the activation of genes that initiate trophoblast elongation in the pig has not been resolved. It would be expected that genes involved specifically with trophoblastic elongation would have low expression prior to transformation to ovoid and tubular morphology, high expression during rapid elongation, followed by a dramatic decline post-elongation. Therefore, the intention of the present research is to determine genes that are morphologically specific or differentially expressed throughout the initiation and duration of rapid trophoblastic elongation.

**Approach**

With continued advancements in biotechnology, the ability to systematically characterize thousands of genes simultaneously is now available, particularly through the use of DNA microarrays. Unfortunately, at this time there is not currently a DNA microarray available for the pig conceptus. However, suppression subtractive hybridization (SSH) is a technique that enables the simultaneous analysis of thousands of genes facilitating the isolation and enrichment of differentially or uniquely expressed genes between different mRNA sources [Diatchenko et al., 1996]. SSH has previously been used to successfully characterize gene expression changes in the mouse oviduct in response to the presence of embryos [Lee et al., 2002], determine gene expression changes induced by specific factors in breast cancer cells [Atalay et al., 2002] and to identify differentially expressed genes during bovine blastocyst hatching [Mohan et al., 2002]. A schematic representation describing the molecular events occurring during the SSH procedure is depicted and described in figure 2.1.

Utilization of SSH to compare gene expression between spherical, tubular and filamentous porcine conceptuses will facilitate the isolation and enrichment of differentially expressed transcripts during trophoblastic elongation. It is likely that dramatic gene expression changes during the specific onset of trophoblastic elongation are involved in regulating the mechanisms by which this phenomena occurs. Identification and characterization of gene expression patterns during rapid trophoblastic elongation in the pig will provide a better understanding of the events required to initiate elongation and identify new targets that may be crucial factors affecting successful implantation and embryonic survival.

**Figure 2.1.** Synthesized cDNA from tester and driver mRNA populations are digested with RsaI yielding blunt ended fragments. The tester population is divided into two separate groups each ligated to a separate adaptor. Two hybridizations are performed. The first hybridization consists of adding excessive driver cDNA to each of the two tester cDNA populations resulting in type **a**, **b**, **c**, and **d** molecules. High and low abundance genes uniquely expressed in the tester population are equalized ($2^{nd}$ order of hybridization kinetics) producing type **a** molecules while abundant genes produce type **b** molecules. Type **c** molecules are produced from tester and driver cDNA sequences annealing while **d** molecules represent self-annealing templates due to excessive driver concentration. The second hybridization is conducted by combining the two primary hybridization samples in the presence of additional driver cDNA yielding the similar molecules as the primary hybridization (**a**, **b**, **c**, and **d**) with the addition of the new enriched type **e** hybrids. Type **e** molecules are double-stranded cDNA sequences unique to the tester population and containing both adaptors. Finally, all hybridization products are subjected to PCR. Molecules **a** and **d** cannot be amplified due to lacking primer annealing sites. Type **b** molecules have identical adaptors on both the 5' and 3' ends resulting in the formation of a hairpin structure preventing exponential amplification. Type c molecules contain adaptors only on one end and can therefore only be amplified linearly. Hybridization product **e** is the only molecule that can be exponentially amplified using PCR because it contains both adaptors. These cDNA sequences (type **e** molecules) represent the differentially expressed genes present in the tester population compared to the driver cDNA population.


Tester cDNA with Adaptor 1
Driver cDNA (in excess)
Tester cDNA with Adaptor 2R
First Hybridization
a
b
c
d
Second Hybridization; mix samples, add fresh denatured driver, and anneal
a, b, c, d +
e
a
b
c
d
e
Add primers
Amplify by PCR
a, d no amplification
b to b' no amplification
c linear amplification
e exponential amplification
5'
3'
and
3'
5'

## Chapter III.

# ANALYSIS AND CHARACTERIZATION OF DIFFERENTIAL GENE EXPRESSION DURING RAPID TROPHOBLASTIC ELONGATION IN THE PIG USING SUPPRESSION SUBTRACTIVE HYBRIDIZATION


### Abstract

During late peri-implantation development, porcine conceptuses undergo a rapid (2-3 hrs) morphological transformation from a 10 mm spherical morphology to a thin filamentous form greater than 150 mm in length. Elongation of the conceptuses is important for establishing adequate placental surface area needed for embryo and fetal survival throughout gestation. Genes involved with triggering this unique transition in conceptus development are not well defined. Objective of the present study was to utilize suppression subtractive hybridization (SSH) to characterize the change in gene expression during conceptus transformation from spherical (8-9 mm) to tubular (15-40 mm) to early filamentous (>150 mm) morphology. Spherical, tubular, and filamentous conceptuses were collected from pregnant gilts and subjected to SSH. Forward and reverse subtractions were performed to identify candidate genes differentially expressed during spherical to tubular and tubular to filamentous transition. A total of 384 transcripts were differentially screened to ensure unique expression. Of the transcripts screened, sequences were obtained for 142 that were confirmed to be differentially expressed among the various morphologies. Gene expression profiles during rapid trophoblastic elongation were generated for selected mRNAs using quantitative real-time RT-PCR. During the transition from tubular to early filamentous morphology, s-adenosylhomocysteine hydrolase and heat shock cognate 70 kDa expression were

significantly enhanced. A novel unknown gene was isolated and shown to be significantly up-regulated at the onset of rapid trophoblastic elongation and further enhanced in filamentous conceptuses.

## Introduction

As in the majority of mammalian species, successful embryonic development in the pig requires temporally and spatially specific gene expression essential to placental and embryonic differentiation during early gestation. The importance of expressing the appropriate developmental transcripts during development of pig conceptuses is evident with prenatal mortality ranging from 20% to 46% by term [Pope, 1994]. The majority of prenatal mortality in the pig occurs during peri-implantation conceptus development [Stroband and Van der Lende, 1990]. The peri-implantation period is the most critical stage of conceptus development as a rapid morphological transformation of the trophoblast occurs just prior to conceptus attachment to the uterine surface [Geisert et al., 1982a]. Rapid transformation of the trophoblast, termed trophoblastic elongation, occurs between days 11 to 12 of gestation. Trophoblastic elongation is initiated when a conceptus reaches a 9-10 mm spherical morphology and then rapidly transforms into a long filamentous thread greater than 150 mm in length within 2-3 hrs [Geisert et al., 1982a]. The process of trophoblastic elongation is characterized by four distinct morphological stages (spherical, ovoid, tubular and filamentous). Elongation of the conceptus is a short-lived phenomenon that results from cellular remodeling and migration, rather than through cellular hyperplasia [Geisert et al., 1982a]. Secretion of the conceptus produced maternal recognition signal, estrogen, occurs simultaneously with rapid elongation of the trophoblastic membrane [Geisert et al., 1990, Bazer and Thatcher,

1977]. Conceptus release of estrogen induces an acute phase response by the endometrium that alters the uterine environment, which may be unfavorable for less developed littermates [Pope, 1994; Geisert and Yelich, 1997]. Because the pig has a diffuse epitheliochorial type of placentation, rapid trophoblastic elongation provides an essential biological function to satisfy the conceptus' necessity for maximal placental-uterine contact to ensure adequate downstream nutrient exchange throughout gestation [Stroband and Van der Lende, 1990].

It has been estimated that approximately 10,000 genes must be appropriately expressed for successful pre-implantation and early fetal development [Niemann and Wrenzycki, 2000]. A number of mRNAs hypothesized to be involved with early porcine conceptus development have been evaluated. Yelich et al. [1997b] characterized gene expression for retinoic acid receptors (RAR) $\alpha$, $\beta$ and $\gamma$ as well as retinal binding proteins (RBP) during early porcine conceptus development and trophoblast elongation. Results indicate expression of RAR$\alpha$ and RBP increase during transition to the filamentous morphology. Estrogen receptor-$\beta$ has been localized in the porcine conceptus and its expression appears to be enhanced during trophoblastic elongation [Kowalski et al., 2002], which follows a pattern similar to aromatase expression [Yelich et al., 1997a].

At present, little information is available from screening the large number of genes that may be responsible for initiating rapid trophoblastic elongation. Due to the limited insight on the transcriptional regulation of this critical developmental process, the objective of the present investigation was to utilize suppression-subtractive hybridization (SSH) to characterize and analyze differentially expressed genes during rapid trophoblastic elongation in the pig. Identification and characterization of gene expression

patterns during rapid trophoblastic elongation in the pig will provide a better understanding of the events required for successful implantation and embryonic survival.

## Materials and Methods

### Conceptus Collection

Research was conducted in accordance with and approved by the Oklahoma State Institutional Animal Care and Use Committee. Twenty-five crossbred, cyclic gilts were checked for estrus twice daily in the presence of an intact boar and naturally mated at the onset of the second estrus and again 24 hrs later. Gilts were hysterectomized between day 11 and 12 of gestation as previously described for our laboratory [Gries et al., 1989]. After removal of the uterine horns, conceptuses from each uterine horn were flushed into a sterile petri dish with 20 mL of physiological saline. Due to the limited time frame when conceptuses are in tubular transitional development (2-3 hrs) and difficulty in determining when tubular conceptuses are in the uterus following mating, one uterine horn was removed on day 11.5 of gestation in a subset of gilts. Conceptuses were flushed into a sterile petri dish from the uterine horn and evaluated to determine an appropriate time-delayed removal of the second horn corresponding to the predicted time conceptuses would be in a tubular morphology as described by Geisert et al. [1982a]. Morphology of conceptuses collected following flushing from the uterine horns was recorded and conceptuses of identical morphologies were transferred to cryogenic vials, snap-frozen in liquid nitrogen, and stored at -80°C until RNA was extracted.

**RNA Isolation**

Total RNA was extracted from individual conceptuses and pools of conceptuses of identical morphologies as previously described in our laboratory [Yelich et al., 1997b]. Briefly, conceptuses were denatured for 15 min on ice using 500 µl of denaturing solution (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-β-mercaptoethanol), 500 µl phenol (pH 5.1), 70 µl 2M sodium acetate (pH 4.0), and 140 µl chloroform/iso-amyl-alcohol (49:1 fresh dilution). The aqueous phase was recovered following centrifugation at 14 000 rpm for 20 min at 4°C and added to a tube containing 500 µl of chloroform, and centrifuged at 10 000 rpm for 10 min at 4°C. The aqueous phase was recovered, placed in a sterile tube, and 7 µl of Rnaid binding matrix (BIO 101, LaJolla, CA) was added, vortexed briefly, and gently agitated for 25 min at 22-25°C. Following rotation, the suspension was centrifuged at 10 000 rpm for 2 min and the aqueous phase was discarded. The remaining pellet containing the glass beads bound to total RNA was washed three times using 250 µl of 50% RNA wash (BIO 101, LaJolla, CA) and 50% ethanol solution followed by centrifugation at 10 000 rpm for 2 min at 22-25°C. The pellet was dried at 22-25°C for 10 min and re-suspended in 50 µl of nuclease-free $H_2O$. The resuspended solution was heated at 56°C for 5 min and centrifuged at 10,000 rpm for 2 min. Approximately 40 µl of the aqueous phase containing the purified total RNA was transferred to a sterile tube and stored at -80°C. Isolated RNA concentrations were calculated based on absorbance at the 260 nm wavelength. Purity of RNA was determined from the 260:280 ratio.

**Suppression Subtractive Hybridization**

Suppression Subtractive Hybridization was performed using the Clontech PCR-Select cDNA Subtraction Kit (K-1804-1, Clontech Laboratories Inc., Palo Alto, CA) as described previously [Mohan et al., 2002]. Forward and reverse subtractions were conducted for two different comparisons during trophoblast elongation. The first comparison was made between spherical (8-9 mm) and tubular (15-40 mm) morphologies allowing the isolation of gene products differentially expressed during the onset of rapid trophoblastic elongation. The second comparison was made between tubular (15-40 mm) and filamentous (>150 mm) morphologies encompassing gene expression changes during the transitional completion when the conceptuses have began to cover the surface area of the uterine horns. Driver and tester cDNA was produced from 10 μg total RNA for each morphology of each comparison following the manufacturers' guidelines. Briefly, synthesized cDNA was digested with the restriction enzyme Rsa1 and the tester cDNA populations were divided into to two tubes and ligated to both adaptor 1 or adaptor 2R. Prior to ligation at 16° C overnight, 2 μl from each adaptor ligation for each tester population were combined to serve as an unsubtracted control and were diluted into 1 mL sterile water following ligation. The subtractive hybridization was performed by adding 1.5 μl driver cDNA to each tube, one containing 1.5 μl of adaptor 1 and the other containing 1.5 μl adapter 2R-ligated tester cDNA (tester cDNA was approximately 30 times less concentrated than driver cDNA) in 1 μl 4X hybridization buffer. Samples were denatured at 98° C for 1.5 min, then allowed to anneal at 68° C for 8 hours. Following the first hybridization, the two samples were combined simultaneously with the excess addition of 1 μl freshly denatured driver cDNA and hybridization was

continued at 68° C overnight. Products from the second hybridization were diluted in 200 µl of dilution buffer (20 mM HEPES, pH 8.3; 50 mM NaCl and 0.2 mM EDTA) and heated at 68°C for an additional 7 min and stored at -20°C.

**PCR Amplification of Subtracted Products**

Two PCR amplifications were performed on the subtracted and unsubtracted tester products. Primary PCR amplifications were conducted for each tester using diluted subtracted products following the second hybridization or the diluted unsubtracted cDNA. One microliter of sample was added to 24 µl PCR master mix prepared using the reagents supplied in the kit, and cycling conditions commenced as follows: 75°C for 5 min to extend the adaptors; 94°C for 25 sec; and 27 cycles at 94°C for 10 sec, 66°C for 30 sec, and 72°C for 1.5 min. Amplified products were diluted 10-fold in sterile water and 1 µl of diluted primary PCR products were added to 24 µl of secondary PCR master mix containing nested primers, 1 and 2R, to ensure specific amplification of double-stranded templates containing both adaptors. Secondary PCR was performed at 94°C for 10 sec, 68°C for 30 sec and 72°C for 1.5 min (cycle number varied between 12 and 27 cycles). Primary and secondary PCR products were analyzed on a 2% agarose gel (Figure 3.1).

**Cloning of Subtracted cDNA Templates**

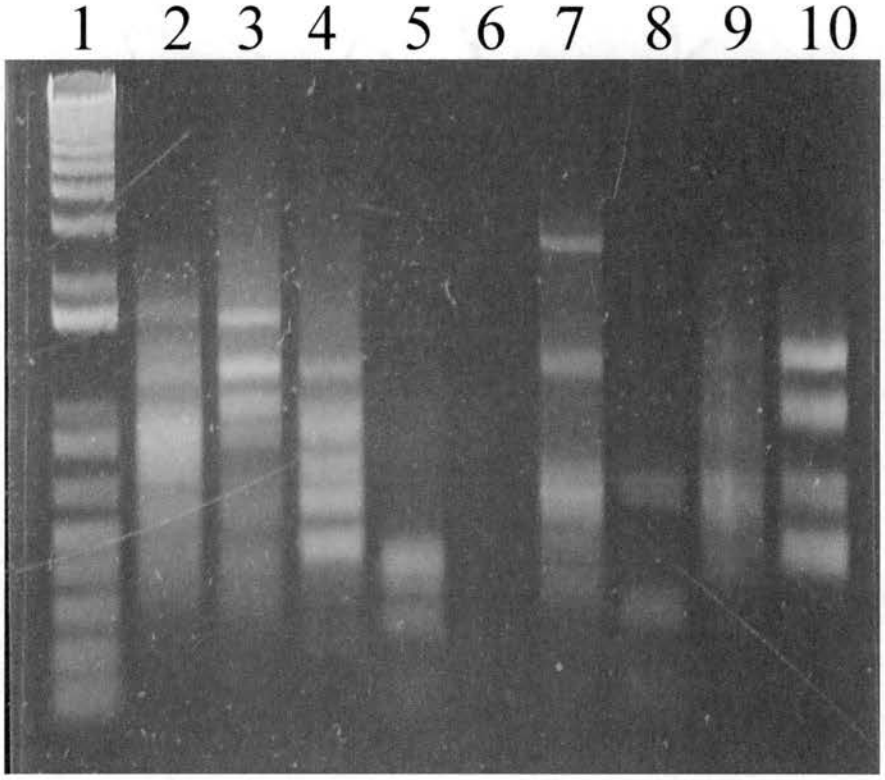
Following the secondary PCR amplification, subtracted products from each tester cDNA population were cloned into the PCR IV vector of the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and used in the transformation of competent DH5α *Escherichia coli* cells. Colonies were grown overnight at 37°C on Luria Broth (LB) agar

plates containing carbenicillin, X-gal (5-bromo 4-chloro 3-indoyl-β-D-galactopyranoside) and isopropyl-β-D-thio-galactopyranoside for blue/white colony screening. Ninety-six plasmids for each tester were randomly selected and plasmid DNA was extracted (Wizard SV96 Plasmid DNA Purification System, Promega Corporation, Madison, WI) and eluted into 100 µl sterile nuclease-free water.

**Differential Screening**

To confirm unique expression of the subtracted products all transcript clones were subjected to differential screening. Plasmid DNA was denatured by adding 250 µl denaturing solution (0.5 M NaOH, 1.5 M NaCl) and incubating for 10 min at 22-25°C. Equal amounts of denatured, subtracted cDNA from each tester were spotted on four separate, positively charged nylon membranes (Roche Applied Science, Indianapolis, IN) using the BioDot apparatus (BioRad, Hercules, CA) and neutralized with 200 µl neutralization solution (1M Tris-HCl, pH 8.0, 1.5M NaCl). Following DNA spotting and neutralization, the membranes were washed by placing on filter paper saturated in 2X SSC for 2 min, removed from filter paper, UV cross-linked and stored at 4°C. Digoxigenin (DIG) labeled probes were created from subtracted and unsubtracted secondary PCR products using the DIG High Prime DNA Labeling and Detection Starter Kit (Roche Applied Science, Indianapolis, IN). Prior to DIG-labeling, PCR products were digested with RsaI for 6 hours at 37°C to remove adaptors and purified using PCR purification columns (Qiagen, Valencia, CA). Denatured template DNA (1 µg) was labeled with DIG at 16°C for 20 h. Labeling efficiency was determined by comparison to known concentrations of herring sperm DIG labeled DNA. The four membranes for each

**Figure 3.1.** Subtracted and unsubtracted PCR products following SSH comparing differential gene expression between spherical and tubular conceptuses, Lane1) DNA Ladder, Lane2) Spherical unsubtracted, Lane3) Spherical subtracted, Lane4) Tubular unsubtracted; and tubular vs. filamentous conceptuses, Lane5) Tubular subtracted, Lane7) Tubular unsubtracted, Lane8) Tubular subtracted, Lane9) Filamentous subtracted, Lane10) Filamentous unsubtracted.

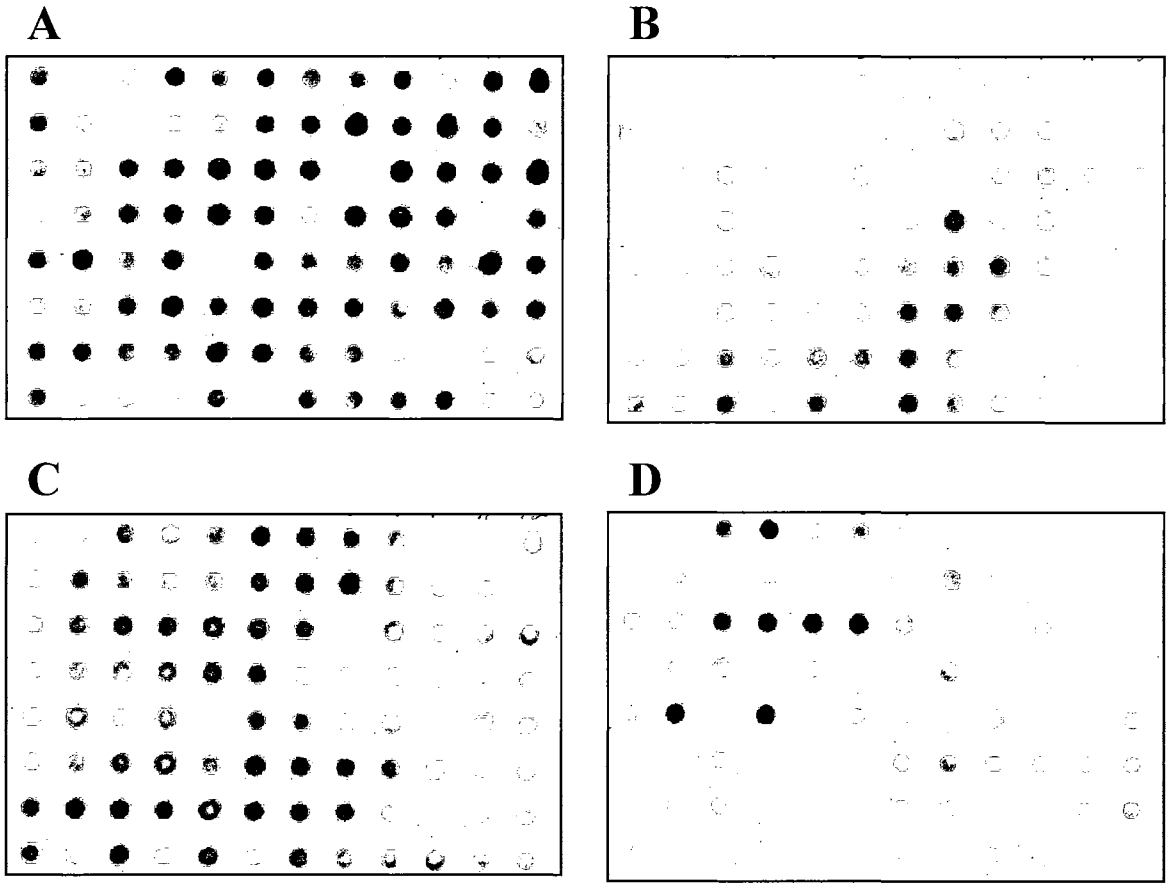

1 2 3 4 5 6 7 8 9 10

subtracted tester were hybridized overnight at 42°C with 125 ng DIG labeled DNA diluted in 5 mL DIG Easy Hyb (25 ng/mL) from subtracted and unsubtracted template products of morphologies for each comparison. Immunological detection of hybridization was conducted in accordance with manufacturers' protocol (Roche Applied Science, Indianapolis, IN) and membranes were exposed to X-ray film (X-Omat LS, Kodak, Rochester, NY) to visualize template-probe binding. Binding intensities were quantified using a densitometer and differential gene expression of cloned template was confirmed when binding intensity was 5X greater when probed with labeled subtracted tester cDNA compared to the intensity of subtracted driver cDNA and a greater binding intensity when hybridized with unsubtracted tester cDNA compared to unsubtracted driver cDNA (Figure 3.2). Plasmids containing confirmed differentially expressed templates were re-cultured and plasmid DNA was extracted (Wizard Plus Mini-prep DNA Purification System, Promega Corporation, Madison, WI) and subjected to dideoxy chain termination sequencing (Applied Biosystems, Model 373A Automated Sequencer, Oklahoma State University Recombinant DNA/Protein Resource Facility). Basic Local Alignment Search Tool (BLAST) [Altschul et al., 1990] was used to confirm sequence homology of each differentially expressed template.

**Quantitative One-Step RT-PCR**

Quantitative analysis of clones of interest, specifically, s-adenosylhomocysteine hydrolase (SAHH), heat shock cognate 70 KD protein (HSC70), and one unknown transcript, OSU-T1-50, were assayed using quantitative real-time RT-PCR [Hettinger et al., 2001]. Individual and small pools of conceptuses were evaluated at the four

**Figure 3.2.** Screening analysis confirming differential expression of 96 randomly selected tubular conceptus cDNA clones subtracted from a tubular vs. spherical hybridization. Each membrane is identically spotted and probed using DIG-labeled cDNA from the comparison: **A)** Tubular subtracted, **B)** Spherical subtracted, **C)** Tubular unsubtracted and **D)** Spherical unsubtracted.


A
B
C
D

morphologically distinct stages; spherical (n=8), ovoid (n=2), tubular (n=5) and early filamentous (n=6). The PCR amplification was conducted using the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). The unknown transcript was evaluated using a dual-labeled probe designed to have a 5' reporter dye (6-FAM) and a 3' quenching dye (TAMRA). SAHH and HSC70 were evaluated using the SYBR green reporter assay kit available from Qiagen (Valencia, CA). Sequences of the primer and probe set used for amplification of the unknown transcript and the primers for SAHH and HSC70 are presented in Table 3.1. Fifty nanograms of total RNA were assayed for each sample in duplicate. Thermal cycling conditions using the dual labeled probe were 48°C for 30 min and 95°C for 10 min, followed by 40 repetitive cycles of 95 °C for 15 sec and a combined annealing/extension stage, 60°C for 1 min. Cycling parameters using SYBR green detection were 50°C for 30 min followed by 95°C for 15 min then 40 repetitive cycles at 95 °C for 15 sec, 51 °C for 30 sec, 72 °C for 30 sec, and a fluorescent data acquisition step following a brief 15 sec incubation at 78.5 °C. Following PCR cycling with SYBR green a melting curve analysis was conducted using the following parameters: 95°C for 15 sec followed by 51°C for 30 sec to 95° C for 15 sec with a ramp time of 19 min 59 sec. Continuous fluorescent data acquisition was collected during final ramp enabling the generation of the melting curve graph to confirm that detectable fluorescence was strictly from amplified target cDNA. 18S ribosomal RNA was assayed as a normalization control to correct for loading discrepancies. Following RT-PCR, quantitation of gene amplification was made by determining the cycle threshold ($C_T$) based on the fluorescence detected within the geometric region of the semilog view of the amplification plot. Relative quantitation of target gene expression was evaluated using

**Table 3.1.** Primer and probe sequences used for real-time RT-PCR, reporting dye used for detection of amplified product and product size for s-adenosylhomocysteine hydrolase, heat shock cognate 70 kDa and the novel OSU-T1-50.

| Target | | Primer Sequence | Reporting Dye | Product Size(bp) |
|---|---|---|---|---|
| SAHH | Forward | 5'-TGTTGCTTTTATGTCTCTCTGG-3' | SYBR Green | 154 |
| | Reverse | 5'-GCTTGGCATTCTCTTAAACC-3' | | |
| HSC70 | Forward | 5'-GTCTTCCTTGCTCAAACG-3' | SYBR Green | 164 |
| | Reverse | 5'-AACTCACAGGCATACCTCC-3' | | |
| OSU-T1-50 | Forward | 5'-TCACGGTTAGTGTCGCATGA-3' | 6-FAM-CGTGTTTCCTACGTTGGGCGTCC-TAMRA | 72 |
| | Reverse | 5'-ACCCATGAACAGGTCCTGAA-3' | | |

the comparative $C_T$ method as previously described by Hettinger et al. [2001]. The $\Delta C_T$ value was determined by subtracting the target $C_T$ of each sample from its respective ribosomal 18S $C_T$ value. Calculation of $\Delta\Delta C_T$ involves using the highest sample $\Delta C_T$ value as an arbitrary constant to subtract from all other $\Delta C_T$ sample values (Table 3.4). Fold-changes in gene expression of the target gene are equivalent to $2^{-\Delta\Delta Ct}$.

### Statistical Analysis

Quantitative RT-PCR $\Delta C_T$ values were analyzed using the PROC MIXED of the Statistical Analysis System [SAS User's Guide, 1985]. Since uterine flushings of a few gilts contained multiple morphological stages, which were utilized in the analysis of gene expression, gilt was initially included in the statistical model. However, given that gilt, as a random effect, did not significantly alter the variation due to the model and not all gilts were represented across all stages of development, gilt was deleted from the model. The statistical model used in the analysis tested only the fixed effect of morphology (spherical, ovoid, tubular, and filamentous). Significance ($P < 0.05$) was determined by probability differences of least squares means between morphologies on conceptus gene expression of s-adenosylhomocysteine hydrolase, heat shock cognate 70 kDa protein and OSU-T1-50. Results are presented as arithmetic means ± SEM.

## Results

### Suppression Subtractive Hybridization

Following SSH, subtracted products were cloned and 96 template clones for each subtracted product of each comparison were randomly selected and differentially

screened. Screening indicated a total of 42 templates confirmed to be down-regulated and 18 up-regulated during the spherical to tubular transition. A total of 69 and 43 templates were confirmed up and down-regulated, respectively, during the tubular to filamentous transition. In all, 142 templates were subjected to dideoxy chain termination sequencing. Information regarding the identity and homology of morphologically specific genes, during spherical to tubular and tubular to filamentous is presented in Tables 3.2 and 3.3.

During the spherical to tubular transitions several genes were indicated using SSH to be down-regulated, such as thymosin-beta 4, mitochondrial cytochrome B, and F1Fo-ATP synthase complex mRNA. Also, numerous mitochondrial DNA template clones were down-regulated during this transition. Several ribosomal RNA transcripts were detected to be up-regulated during the onset of rapid trophoblastic elongation as well as a novel gene, which is referred to as OSU-T1-50. An approximate 200 base pair region of OSU-T1-50 had significant homology (87%) to a region on human chromosome 8 (GenBank accession #AC009682) but had no significant homology compared to over 5.8 million non-human and non-mouse expressed sequence tags.

Few genes, myeloid cell leukemia (Bcl-2 related protein) and several ribosomal genes were detected to be down-regulated during the tubular to filamentous transition. Numerous transcripts were up-regulated in the filamentous morphology including, pro-interleukin-1β, interleukin-1β (IL-1β), s-adenosylhomocysteine hydrolase (SAHH), heat shock cognate 70 kDa protein (HSC70), metallopanstimulin-1 (MPS-1) and elfin. The three transcripts selected to be investigated using quantitative one-step real time RT-PCR were OSU-T1-50, SAHH and HSC70.

**Table 3.2.** Identity, size and homology of porcine conceptus cDNA clones from spherical to tubular subtraction compared to known sequences in GenBank.

| Expression Pattern | Identity | Clone Number | Base Pairs submitted to BLAST | GenBank Accession Number | Homology |
|---|---|---|---|---|---|
| **Down-Regulated** | | | | | |
| | F1Fo-ATP synthase complex | OSU-S-6 | 313 | S70448 | Bovine 93% (205/220) |
| | | | | NM_006476 | Human 93% (204/219) |
| | | | | BC031384 | Mouse 92% (128/139) |
| | Similar to keratin 18 | OSU-S-14* | 324 | BC009754 | Human 87% (134/154) |
| | Thymosin Beta 4 | OSU-S-7* | 308 | BC022857 | Human 91% (158/173) |
| | | | | X16053 | Mouse 84% (140/167) |
| | | | | NM_031136 | Rat 85% (122/143) |
| | 16S Ribosomal RNA | OSU-S-31 | 427 | AY011178 | Pig 99% (374/375) |
| | Mitochondrial Cytochrome B | OSU-S-54 | 241 | AJ314556 | Pig 98% (182/185) |
| | Complete Mitochondrial DNA | OSU-S-11* | 679 | AF304203 | Pig 99% (678/679) |
| **Up-Regulated** | | | | | |
| | 28S Ribosomal RNA | OSU-T1-3* | 436 | X00525 | Mouse 100% (350/350) |
| | | | | M11167 | Human 100% (350/350) |
| | 16S Ribosomal RNA | OSU-T1-7* | 250 | AY011178 | Pig 99% (212/214) |
| | 18S Ribosomal RNA | OSU-T1-8* | 342 | AF102857 | Pig 99% (293/294) |
| | | | | X00686 | Mouse 99% (293/294) |
| | | | | X03205 | Human 99% (293/294) |
| | Unknown | OSU-T1-50 | 702 | AC009682 | Human 87% (179/206) |

* Indicates transcripts with multiple clones sequenced

**Table 3.3.** Identity, size and homology of porcine conceptus cDNA clones from tubular to filamentous subtraction compared to known sequences in GenBank.

| Expression Pattern | Identity | Clone Number | Base Pairs submitted to BLAST | GenBank Accession Number | Homology |
|---|---|---|---|---|---|
| **Down- Regulated** | | | | | |
| | 16S Ribosomal RNA | OSU-T2-19* | 242 | AY011178 | Pig 100% (182/182) |
| | Myeloid Cell Leukemia | OSU-T2-40* | 636 | AF144096 | Rat 96% (227/236) |
| | (Bcl-2Related Protein) | | | AJ307006 | Pig 93% (151/163) |
| | | | | BC005427 | Mouse 91% (465/510) |
| | | | | NM_021960 | Human 88% (175/199) |
| **Up-Regulated** | | | | | |
| | Pro-interleukin-1β | OSU-F-1* | 723 | X74568 | Pig 97% (319/328) |
| | Interleukin-1β | OSU-F-2* | 684 | M86725 | Pig 90% (623/686) |
| | | | | AB028216 | Dolphin 87% (427/492) |
| | | | | M37211 | Cow 84% (448/531) |
| | | | | X54796 | Sheep 83% (418/501) |
| | | | | U92481 | Horse 85% (298/349) |
| | | | | NM_000576 | Human 85% (280/329) |
| | S-adenosylhomocysteine hydrolase | OSU-F-3* | 452 | AJ422131 | Pig 99% (355/356) |
| | | | | BC010018 | Human 88% (238/272) |
| | | | | NM_016661 | Mouse 83% (66/79) |
| | Heat Shock Cognate Protein 70kD | OSU-F-7* | 650 | AF352832 | Human 91% (501/546) |
| | | | | X53335 | Cow 92% (482/522) |
| | | | | NM_024351 | Rat 90% (493/546) |
| | | | | M19141 | Mouse 89% (479/534) |
| | Ribosomal protein 8S | OSU-F-21 | 442 | NM_031706 | Rat 92% (260/281) |
| | | | | NM_001012 | Human 92 % (260/281) |
| | | | | NM_009098 | Mouse 91% (263/289) |
| | Metallopanstimulin-1 | OSU-F-30 | 382 | L19739 | Human 94% (244/259) |
| | Elfin | OSU-F-54* | 540 | NM_020992 | Human 85% (321/379) |
| | (PDZ and LIM domain 1) | | | | |

* Indicates transcripts with multiple clones sequenced

**Real Time RT-PCR Quantification**

Messenger RNA expression profiles of SAHH, HSC70 and OSU-T1-50 were generated using the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). Following amplification, $C_T$, $\Delta C_T$ and $\Delta\Delta C_T$ values were calculated as described previously in *Material and Methods* (Table 3.4).

Based on normalization with 18S ribosomal RNA, SAHH mRNA expression was greater ($P < 0.01$) in filamentous conceptuses compared to all other morphologies evaluated (Table 3.4). There was an approximately 7-fold increase of SAHH gene expression in filamentous conceptuses compared to spherical conceptuses (Figure 3.3). HSC70 gene expression was also significantly enhanced ($P < 0.001$) in filamentous conceptuses (Table 4), which displayed an approximate 10-fold increase in gene expression compared to spherical, ovoid and tubular morphologies (Figure 3.4). Gene expression for OSU-T1-50 was greater ($P < 0.004$) in tubular conceptuses compared to spherical while filamentous conceptus expression was greater ($P < 0.01$) compared to all other morphologies evaluated (Table 3.4). When compared to spherical conceptuses, gene expression for OSU-T1-50 was approximately 11 and 104-fold greater in tubular and filamentous morphologies, respectively (Figure 3.5).

**Table 3.4.** Quantitative RT-PCR analysis comparing gene expression across morphologies during rapid trophoblastic elongation for three of the transcripts identified using suppression subtractive hybridization: s-adenosylhomocysteine hydrolase, heat shock cognate 70 kDa, and an unknown transcript identified by its clone number, OSU-T1-50.

| Transcript | Morphology | Average Target $C_T$* | Average 18S rRNA $C_T$* | $\Delta C_T$‡¶ | $\Delta\Delta C_T$§ |
|---|---|---|---|---|---|
| SAHH | Spherical | 23.48 ± 0.23 | 17.47 ± 0.10 | 6.01 ± 0.21[a] | -0.20 |
| | Ovoid | 23.62 ± 0.82 | 17.78 ± 0.24 | 5.84 ± 0.58[a] | -0.37 |
| | Tubular | 23.20 ± 0.51 | 16.99 ± 0.14 | 6.21 ± 0.51[a] | 0.00 |
| | Filamentous | 20.72 ± 0.55 | 17.24 ± 0.12 | 3.47 ± 0.58[b] | -2.76 |
| HSC70kD | Spherical | 20.86 ± 0.28 | 17.47 ± 0.10 | 3.38 ± 0.25[c] | 0.00 |
| | Ovoid | 21.02 ± 0.80 | 17.78 ± 0.24 | 3.24 ± 0.57[c] | -0.15 |
| | Tubular | 20.17 ± 0.52 | 16.99 ± 0.14 | 3.18 ± 0.48[c] | -0.21 |
| | Filamentous | 17.35 ± 0.46 | 17.24 ± 0.12 | 0.11 ± 0.48[d] | -3.27 |
| OSU-T1-50 | Spherical | 33.22 ± 0.48 | 17.47 ± 0.10 | 15.75 ± 0.64[e] | 0.00 |
| | Ovoid | 32.60 ± 1.01 | 17.78 ± 0.24 | 14.82 ± 1.22[ef] | -0.93 |
| | Tubular | 29.21 ± 0.63 | 16.99 ± 0.14 | 12.22 ± 0.80[f] | -3.53 |
| | Filamentous | 26.30 ± 0.57 | 17.24 ± 0.12 | 9.05 ± 0.80[g] | -6.69 |

* $C_T$ = Cycle Threshold. Indicates cycle number in which amplification crosses the threshold set in the geometric portion of amplification curve.

‡ $\Delta C_T$ = Target transcript $C_T$ – 18S ribosomal $C_T$: Normalization of $C_T$ for target gene relative to ribosomal 18S RNA $C_T$.

¶ Statistical analysis of normalized expression levels between morphologies. Values with different superscripts for each of the target genes differ significantly: [ab]($P < 0.01$), [cd]($P < 0.001$), [ef]($P < 0.004$), [eg]($P < 0.001$), and [fg]($P < 0.01$).

§ $\Delta\Delta C_T$ = Mean $\Delta C_T$ – highest mean $\Delta C_T$ value: The mean value for the morphology with highest $\Delta C_T$ (lowest expression levels for target) was used as a calibrator to set the baseline for comparing mean differences in the $\Delta C_T$ values across all morphologies.

**Figure 3.3.** Fold difference in conceptus produced gene expression for s-adenosylhomocysteine hydrolase detected using one-step real-time RT-PCR (2-8 pools or individual conceptuses/morphology). The fold differences in gene expression were calculated as described in *Materials and Methods.*

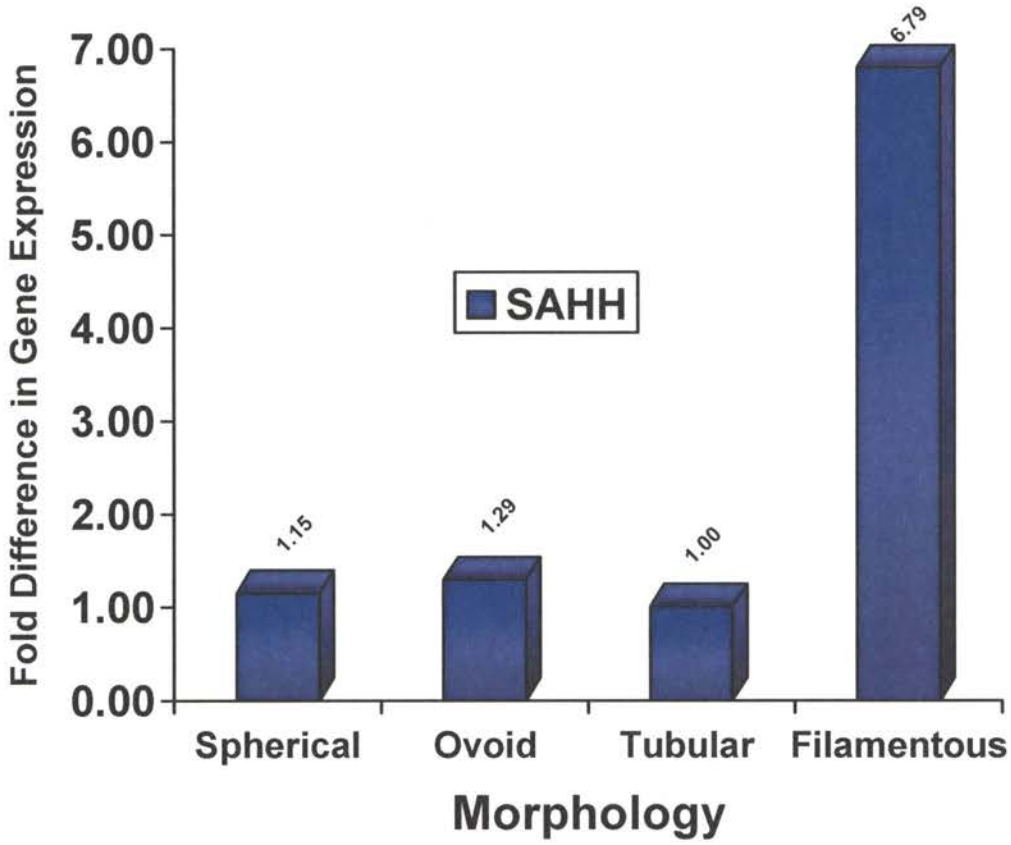

Fold Difference in Gene Expression
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
SAHH
1.15
1.29
1.00
6.79
Spherical
Ovoid
Tubular
Filamentous
Morphology

**Figure 3.4.** Fold difference in conceptus produced gene expression for heat shock cognate 70 kDa detected using one-step real-time RT-PCR (2-8 pools or individual conceptuses/morphology). The fold differences in gene expression were calculated as described in *Materials and Methods.*

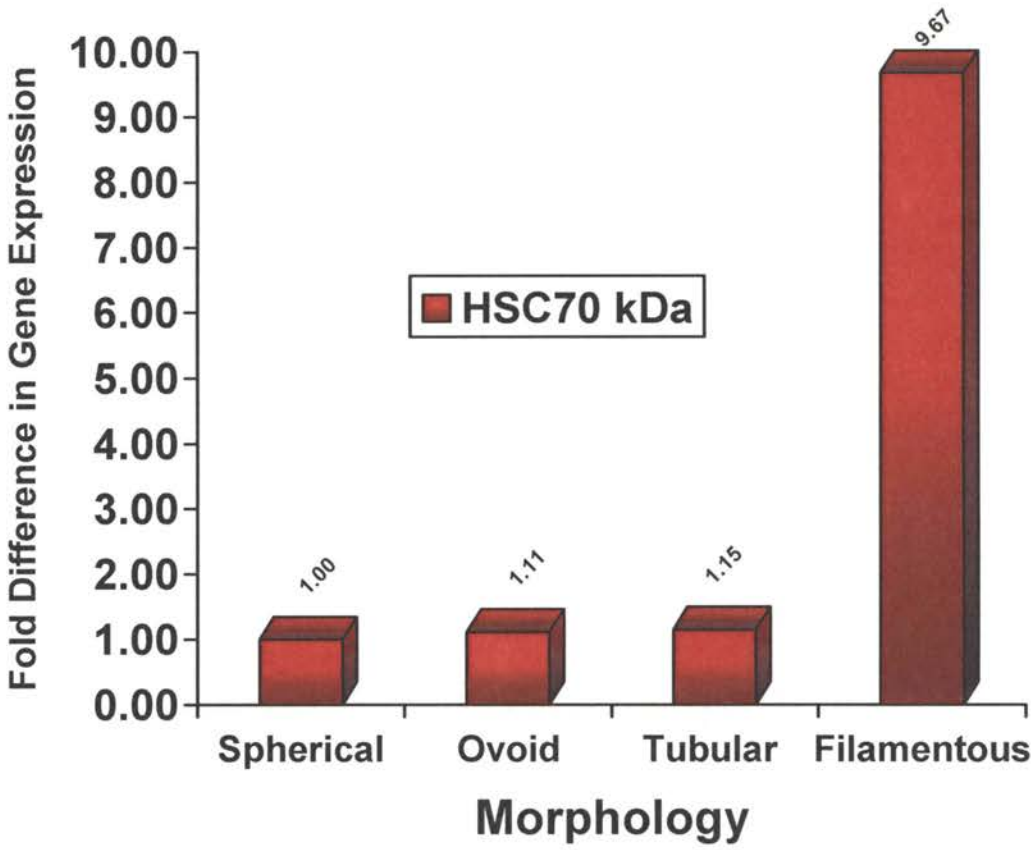
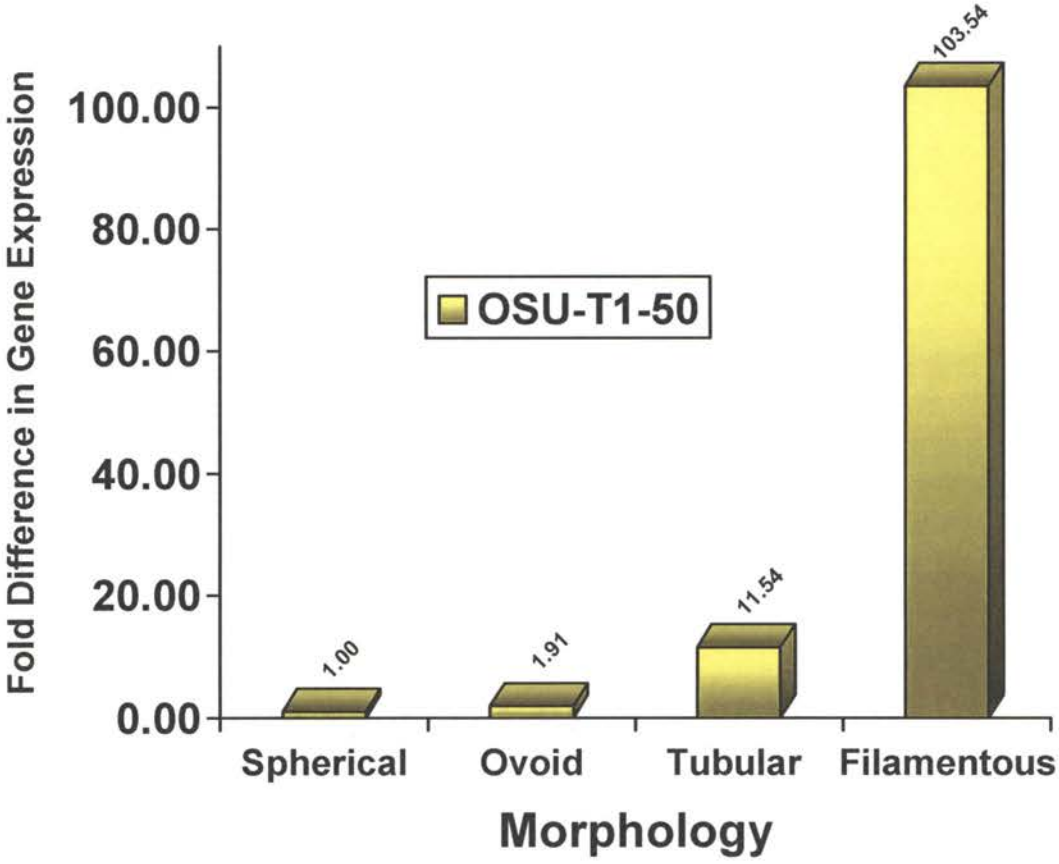

Fold Difference in Gene Expression
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
HSC70 kDa
1.00
1.11
1.15
9.67
Spherical
Ovoid
Tubular
Filamentous
Morphology

**Figure 3.5.** Fold difference in conceptus produced gene expression for the novel gene, OSU-T1-50 detected using one-step real-time RT-PCR (2-8 pools or individual conceptuses/morphology). The fold differences in gene expression were calculated as described in *Materials and Methods.*


Fold Difference in Gene Expression
100.00
80.00
60.00
40.00
20.00
0.00
OSU-T1-50
1.00
1.91
11.54
103.54
Spherical
Ovoid
Tubular
Filamentous
Morphology

## Discussion

During early porcine development a rapid morphological transformation of the conceptus from a spherical (9-10 mm) to filamentous (> 150 mm) morphology is required to establish adequate placenta to uterine contact necessary for survival [Stroband and Van der Lende, 1990]. Characterization of the specific subset of genes regulating peri-implantation conceptus development and trophoblastic elongation in the pig provides valuable information concerning key developmental events essential to embryonic survival after trophoblastic elongation [Pope, 1994].

Previous information regarding genes critical to development in early pig pregnancy is limited. Using semi-quantitative RT-PCR Yelich et al. [1997a] evaluated peri-implantation gene expression profiles for 17α-hydroxylase, aromatase, brachyury and leukemia inhibitory factor receptor, all of which are more greatly expressed in filamentous conceptuses. Messenger RNA for retinoic acid receptor α, retinal binding protein and transforming growth factor β-3 is also increased in filamentous conceptuses [Yelich et al., 1997b]. Using a ribonuclease protection assay, Wilson et al. [2002] indicated enhanced cyclooxygenase-2 expression was specific to filamentous conceptuses. An autocrine effect of conceptus estrogen synthesis has been suggested as filamentous conceptuses have greater gene expression for estrogen receptor β compared to its spherical and tubular counterparts [Kowalski et al., 2002].

Suppression subtractive hybridization allowed isolation of candidate genes expected to be differentially expressed during rapid trophoblastic elongation. Differentially expressed genes identified using SSH striking particular interest were interleukin-1β (IL-1β) and metallopanstimulin-1 (MPS-1), both of which are suspected to

be up-regulated in the elongated conceptus. MPS-1 expression is notable in proliferating cells and has been identified as a nuclear protein that binds to DNA [Fernandez-Pol et al., 1994]. Further analysis indicates cells stimulated with transforming growth factor beta 1 (TGF-β1) exhibit a 8-fold increase in MPS-1 gene expression [Fernandez-Pol et al., 1993]. TGF-β1 and its receptors are localized in the trophectoderm of porcine conceptuses between days 10-14 of gestation [Gupta et al., 1996]. MPS-1 may have roles in transcriptional-mediation of the embryonic response to TGF-β1 having effects on continued intra-uterine elongation. IL-1β was the most predominant clone sequenced during SSH and was also the most abundant clone represented when mapping expressed sequence tags derived from a porcine early embryonic cDNA library in a study by Smith et al. [2001]. We have more thoroughly examined and confirmed IL-1β gene and ligand expression in developing conceptuses as well as the endometrium [Ross et al., 2003a]. Changes in conceptus IL-1β gene expression and ligand release in the uterine horn dramatically increase during the process of elongation [Ross et al., 2003a]. Increase in IL-1β gene expression was specific to the conceptus as endometrial expression was unchanged between cyclic and pregnant gilts. Following completion of conceptus elongation, IL-1β gene expression and secretion rapidly decline and expression levels are more than 2000-fold lower in day 15 compared to day 12 filamentous conceptuses [Ross et al., 2003a]. It is possible IL-1β plays an important role in triggering conceptus elongation and initiating uterine-conceptus "cross-talk" in the pig.

In the present study, three transcripts; OSU-T1-50, SAHH and HSC70 were confirmed to be differentially expressed analogous to the pattern predicted using SSH. Although no confirmed identity was found in GenBank, the novel gene, OSU-T1-50

displayed a very dynamic enhancement of gene expression during trophoblastic elongation. When compared to spherical conceptuses there was almost a 12-fold increase in mRNA expression for OSU-T1-50 in tubular conceptuses while expression in filamentous conceptuses increased over 100-fold. Given the substantial increase in gene expression and temporal relationship to conceptus development, OSU-T1-50 may play an important role in trophoblastic elongation. Future studies will attempt to identify the gene and its translated protein to determine the biological function during conceptus development.

Gene expression of SAHH was similar between spherical, ovoid and tubular conceptuses, but there was a near 7-fold increase in gene expression in filamentous conceptuses. SAHH may have significant impact on the conceptuses ability to use folates during this transitional stage of development. Folates have long been known to be an essential requirement for developing embryos, predominately during neurulation [Van der Put, 2000]. Vallet and coworkers [1998] have previously shown the increase in maternal folate binding protein activity occurs in the uterine lumen of cyclic and pregnant gilts between days 10-12 post-estrus. The increase of folate in the uterine lumen is temporally associated with the increase in conceptus SAHH gene expression.

S-adenosylhomocysteine (SAH) is the resultant product following the release of a methyl group from s-adenosylmethionine (SAM), a universal methyl donor [Cantoni, 1975]. Methyl donation from SAM has crucial developmental impacts governing DNA methylation [Fenech, 2001] as well as methylation of amino acids, proteins, carbohydrates and polysaccharides [Cantoni, 1975]. S-adenosylhomocysteine hydrolase is the only known enzyme capable of SAH hydrolysis. The breakdown of SAH, which is

reversible, results in the release of free homocysteine which is converted to methionine while being used as a substrate for the reduction of 5-methylenetetrahydrofolate (5-MTHF) to tetrahydrofolate (THF). Elevated levels of methionine can then be used in the synthesis of SAM while THF has downstream effects on uracil to thymidine conversions involved with DNA repair and synthesis [Fenech, 2001].

SAHH functions as a protective enzyme for adenosine toxicity by preventing nuclear accumulation of SAH [Hershfield and Kredich, 1978] and may explain the nuclear localization of SAHH in cells that are transcriptionally active in Xenopus embryos [Radomski et al., 1999]. Vanaerts et al. [1994] demonstrated that high concentrations of homocysteine are associated with embryotoxicity during early gestation in rats. These authors suggest the toxicity may be associated with the reverse hydrolysis of homocysteine to SAH resulting in the dramatic reduction of the SAM/SAH ratio to a point where methylation reactions are inhibited. Miller and co-workers [1994] have revealed that the lethal nonagouti ($a^x$) mutation in mice is characterized by deletion of the SAHH gene resulting in embryonic death prior to implantation. Addition of an inhibitor to SAHH, 3-deazaaristeromycin, inhibits inner cell mass proliferation and differentiation during in vitro development of non-mutant embryos [Miller et al., 1994].

As in other species, SAHH is likely a biological regulator of the SAM/SAH ratio controlling the occurrence of transmethylation reactions to the degree of which they are necessary for successful porcine conceptus development. Establishment of the maternal-fetal interface is extremely competitive among littermates in early swine gestation. Advanced conceptuses, those that elongate first, have a much greater advantage with regards to acquiring ample placental:uterine contact and also limit the available uterine

capacity for those conceptuses lagging in development [Stroband and Van der Lende, 1990]. While gene expression for SAHH was present at all stages evaluated, an approximate 7-fold increase in relative expression for this transcript over a 2-3 hour time period is noteworthy. It is possible that increased expression of SAHH by filamentous conceptuses serves as a protective function at the level of the nucleus by reducing SAH to homocysteine thereby maintaining the SAM/SAH ratio at appropriate levels for SAM-mediated transmethylation reactions to occur. Increased SAHH expression by advanced conceptuses suggests there would also be increased homocysteine released into the uterine lumen that may have an embryo-toxic effect on neighboring conceptuses lagging in development.

Gene expression for HSC70 was similar in spherical, ovoid and tubular conceptuses followed by a 10-fold increase in filamentous conceptuses. Gene expression changes for HSC70 during early development have previously been associated with neurulation in Xenopus [Lang et al., 2000] and chick [Rubio et al., 2002] embryos. Negative mutations of HSC70 in the nervous system of Drosophila larvae resulted in both developmental defects and lethality [Elefant and Palter, 1999]. The increased HSC70 gene expression during trophoblastic elongation is temporally associated with neural tube development in pig conceptuses.

Traditionally, heat shock proteins are known for their function during cellular stress as molecular chaperones responsible for the folding, re-folding and transport of newly synthesized proteins. HSC70 is a constitutively expressed member of the 70 kDa heat-shock protein (HSP70) family. Members of the HSP70 family have also been proposed to be involved with HSP90 chaperones regulating signal transduction pathways

[Richtner and Buchner, 2001]. Unlike HSP70, HSC70 does not exhibit increased gene expression when exposed to heat stress or other agents such as sodium arsenate in the gastrula and neurula Xenopus embryo [Ali et al., 1996]. Tsang [1993] suggested because of their ability to bind and transport folded and un-folded cellular proteins, HSP70 family members may function as a cross-linker to couple cellular proteins to the cytoskeletal matrix. Association of HSP70 with the cytoskeleton suggests that conceptus produced HSC70 may be directly involved with the complex process of conceptus remodeling during trophoblastic elongation.

Through the method of SSH, we have detected several genes that may serve a vital role in differentiation, neurulation, as well as attachment and maintenance of pregnancy in the pig. Detection and confirmation of IL-1β, SAHH, HSC70 and OSU-T1-50 as being differentially expressed during the period of rapid trophoblastic elongation contributes important information towards understanding the mechanisms involved with this essential biological event in the pig.

## Chapter IV.

# CHARACTERIZATION OF THE INTERLEUKIN-1β SYSTEM DURING PORCINE TROPHOBLASTIC ELONGATION AND EARLY PLACENTAL ATTACHMENT


### Abstract

The establishment and maintenance of pregnancy in the pig involves intricate communication between the developing conceptuses and maternal endometrium. Conceptus-uterine communication is generally established during trophoblastic elongation when the conceptus synthesizes and releases estrogen, the maternal recognition signal in the pig. We recently identified IL-1β as a gene that is differentially expressed during rapid trophoblastic elongation in the pig. The objective of the current investigation was to determine conceptus and endometrial changes in gene and ligand expression of IL-1β and other genes regulating the IL-1β system during peri-implantation development. Using quantitative real time RT-PCR, gene expression of IL-1β, IL-1 receptor antagonist (IL-1Rant), IL-1 receptor type 1 (IL-1RT1) and IL-1 receptor accessory protein (IL-1RAP) was analyzed in developing peri- and post-implantation conceptuses, as well as uterine endometrium collected from cyclic and pregnant gilts. Conceptus IL-1β gene expression was significantly greater during the period of rapid trophoblastic elongation compared to earlier spherical conceptuses followed by a dramatic decrease in post-elongated day 15 conceptuses. IL-1RT1 and IL-1RAP gene expression in conceptuses was greater in filamentous day 12 and 15 conceptuses compared to earlier morphologies while IL-1Rant gene expression was unchanged by conceptus development. The uterine lumenal content of IL-1β increased during the

process of trophoblastic elongation on day 12. Uterine IL-1β content declined on day 15, reaching a nadir by day 18 of pregnancy. IL-1β gene expression in porcine conceptuses was temporally associated with an increase in endometrial IL-1RT1 and IL-1RAP gene expression in pregnant gilts. Endometrial IL-1β and IL-1Rant gene expression were lowest during days 10 to 15 of the estrous cycle and pregnancy. The temporal expression of IL-1β during conceptus development and the initiation of conceptus-uterine communication suggests conceptus IL-1β synthesis plays an important role in porcine conceptus trophoblastic elongation and the establishment of pregnancy in the pig.

## Introduction

On approximately day 12 of pregnancy, porcine conceptuses initiate attachment to the uterine lumenal surface following a rapid morphological rearrangement of the trophoblast [Geisert et al., 1982a]. The rapid alteration in morphology, termed trophoblastic elongation is initiated when a conceptus becomes an approximate 9-10 mm sphere. Upon reaching this spherical diameter, the conceptus rapidly transforms into a long filamentous thread greater than 150 mm in length within 2-3 h [Geisert et al., 1982a]. Rapid elongation of the trophoblast is not regulated by cellular mitosis [Geisert et al., 1982a; Pusateri et al., 1990] but rather occurs through cellular remodeling of the trophectoderm and endoderm layers [Geisert et al., 1982a; Mattson et al., 1990]. This dramatic transformation in structural morphology coincides with the elevated estrogen synthesis and release by the conceptus [Geisert et al., 1982b] which is required for the establishment of pregnancy in the pig.

Although the biological mechanisms involved with the initiation of trophoblastic remodeling are largely unknown, a few genes proposed to be involved with the rapid

transformation of porcine conceptuses have been investigated. Gene expression for retinoic acid receptors (RAR) α, β and γ, as well as retinal binding proteins (RBP) has been evaluated during porcine conceptus development and trophoblastic elongation [Yelich et al.1997b]. Results from a semi-quantitative PCR evaluation of gene expression indicated RARα and RBP increase during transition to the filamentous morphology. Porcine conceptus expression of growth factors such as transforming growth factor-α, epidermal growth factor and interleukin 6 have been reported [Vaughan et al., 1992, Mathialagan et al., 1992, Anegon et al., 1994, Modric et al., 2000]. Pregnancy specific endometrial expression of LIF is initiated during the period of conceptus elongation and could effect conceptus development since peri-implantation porcine conceptuses express LIF-receptor β [Anegon et al., 1994; Modric et al., 2000]. Conceptus aromatase gene expression also increased during trophoblastic elongation [Yelich et al., 1997a] and may have an autocrine effect on development through the increase in conceptus estrogen receptor β expression [Kowalski et al., 2002]. Conceptus elongation is largely associated with increased prostaglandin production [Geisert et al., 1986]. Interestingly, conceptus cyclooxygenase-2 (COX-2) gene expression increases in filamentous conceptuses following rapid trophoblastic elongation [Wilson et al., 2002].

Recently, interleukin-1β (IL-1β), a pro-inflammatory cytokine, was identified through utilization of suppression subtractive hybridization (SSH) as a gene differentially expressed during the process of rapid trophoblastic elongation [Ross et al., 2003b]. It is possible that IL-1β serves an important role in trophoblastic elongation and initiation of placental-uterine interfacing needed for the establishment of pregnancy. Previously, Tuo et al. [1996] reported that conceptus IL-1β gene expression was high during porcine peri-

implantation development on days 10-12 of pregnancy. Peri-implantation expression of IL-1β has also been documented to increase prior to initiation of blastocyst implantation in the mouse [Takacs and Kauma, 1996; Kruessel et al., 1997], and suggested as the initiator of conceptus-uterine cross-talk during pregnancy in the human [Lindhard et al., 2002]. Many of the endometrial responses evoked by porcine conceptuses during trophoblastic elongation and subsequent adhesion to the uterine apical surface resemble the IL-1 mediated acute-phase responses induced during inflammation of tissue [Geisert and Yelich, 1997].

The present study was undertaken to evaluate changes in gene expression of IL-1 family members to better understand the role of IL-1β in embryonic development and establishment of pregnancy in pigs. Gene expression for IL-1β, IL-1 receptor type 1 (IL-1RT1), IL-1 receptor accessory protein (IL-1RAP) and IL-1 receptor antagonist (IL-1Rant) was analyzed in peri- and post-implantation conceptuses as well as from the endometrium of cyclic and pregnant gilts. Uterine lumenal content of IL-1β was also quantitated during the estrous cycle and early pregnancy. The study was designed to specifically target uterine lumen IL-1β fluctuation occurring during conceptus transition from spherical to filamentous morphology.

## Materials and Methods

### Animals

Research was conducted in accordance with and approved by the Oklahoma State Institutional Animal Care and Use Committee. Cyclic, large white gilts of similar age (8-10 mo) and weight (100-130 kg) were checked for estrous behavior twice daily in the

presence of an intact boar. Onset of estrus was designated day 0 of the estrous cycle. Gilts assigned to be bred were naturally mated with fertile boars at the onset of their second estrus (day 0 of estrous cycle) and again 24 h later.

**Collection of endometrial tissue and conceptuses**

Cyclic and pregnant gilts (4 animals/status per day) were hysterectomized through midventral laporatomy as previously described by Gries et al. [1989]. Cyclic gilts were hysterectomized on days 0, 5, 10, 12, 15 and 18 of the estrous cycle while pregnant gilts were hysterectomized on days 10, 12, 15 and 18 of gestation. Immediately following removal, each uterine horn was flushed with 20 mL of a physiological saline and conceptuses were removed from pregnant gilts. Conceptus morphology was recorded and pools of conceptuses of identical morphologies were transferred to cryogenic vials, snap-frozen in liquid nitrogen and transferred to -80°C for long-term storage. Uterine flushings were transferred to a 50 mL conical tube and centrifuged at 1000 rpm for 1 min to remove cell debris. Uterine flushings were stored at -80°C until utilized in an IL-1β ELISA protein assay. Following conceptus removal, one uterine horn was cut along its anti-mesometrial border, and endometrium (5-10 g) was removed with sterile scissors. Endometrium was snap-frozen in liquid nitrogen and stored at -80°C until analyzed.

**Collection of Elongating Porcine Conceptuses**

Since the 9-10 mm spherical to filamentous transition occurs within a short period of time (2-3 h), collection of tubular conceptuses required utilization of a unilateral hysterectomy procedure previously described by Geisert et al. [1982a]. For that reason, fifteen additional pregnant gilts were utilized to collect conceptuses during the

transitional period between day 11 and 12 of gestation. Conceptuses flushed from the uteri were separated based on morphological development stage (i.e. spherical, ovoid, tubular, filamentous). Conceptuses and uterine flushings were collected and stored at -80°C as described above.

**RNA Isolation**

Total RNA was extracted from conceptus pools following the extraction method previously described by our laboratory [Ross et al., 2003b]. Conceptuses were denatured for 15 min on ice using 500 µl of denaturing solution (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-β-mercaptoethanol), 500 µl phenol, 70 µl 2M sodium acetate (pH 4.0), and 140 µl chloroform/iso-amyl-alcohol (49:1 fresh dilution). The aqueous phase was recovered following centrifugation at 14 000 rpm for 20 min at 4°C and added to a tube containing 500 µl of chloroform, and centrifuged at 10 000 rpm for 10 min at 4°C. The aqueous phase was recovered, placed in a sterile tube, and 7 µl of Rnaid binding matrix (BIO 101, LaJolla, CA) was added, vortexed briefly, and gently agitated for 25 min at 22-25°C. Following rotation, the suspension was centrifuged at 10,000 rpm for 2 min and the aqueous phase was discarded. The remaining pellet containing the glass beads bound to total RNA was washed three times using 250 µl of 50% RNA wash (BIO 101, LaJolla, CA) and 50% ethanol solution followed by centrifugation at 10 000 rpm for 2 min at 22-25°C. The pellet was dried at 22-25°C for 10 min and resuspended in 50 µl of nuclease-free $H_2O$. The resuspended solution was heated at 56°C for 5 min and centrifuged at 10 000 rpm for 2 min. Approximately 40 µl of the aqueous phase containing the purified total RNA was

transferred to a sterile tube and stored at -80°C. Endometrium tissue total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturers recommendations as previously described [Vonnahme et al., 1999]. Approximately 500 mg of endometrium was homogenized in 5 mL TRIzol reagent using a Virtishear homogenizer (Virtis Company Inc., Gardiner, NY). RNA pellets were rehydrated in nuclease-free $H_2O$ and stored at -80°C. RNA content was estimated spectrophotmetrically and purity determined by the 260:280 ratio.

**Quantitative 1-Step RT-PCR**

Quantitative analysis of IL-1β, IL-1RT1, IL-1RAP and IL-1Rant mRNA were assayed using quantitative real-time RT-PCR and a fluorescent reporter as previously described [Hettinger et al., 2001]. Endometrial tissue was assayed in addition to pools (2-7) of conceptuses at the four morphologically distinct stages during rapid trophoblastic elongation; spherical (n=8), ovoid (n=2), tubular (n=5), and filamentous (n=6), and during late peri-implantation development; day 15 (n=2) and 18 (n=5). The PCR amplification was conducted using the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). The transcripts were evaluated using dual-labeled probes designed to have a 5' reporter dye (6-FAM) and a 3' quenching dye (TAMRA). One hundred nanograms of total RNA were assayed for each sample in duplicate. Thermal cycling conditions were 48°C for 30 min and 95°C for 10 min, followed by 40 repetitive cycles of 95 for 15°C sec and 60°C for 1 min. 18S ribosomal RNA was assayed as a normalization control to correct for loading discrepancies for all samples assayed. GenBank accession numbers representing full-length cDNA sequences or expressed sequence tags used to generate primer and probe sequences for the

amplification of IL-1β, IL-1RT1, IL-1RAP and IL-1Rant are presented in Table 4.1. Primer and probe sequences generated for IL-1β using the available porcine GenBank sequence (accession # M86725) efficiently amplified endometrial IL-1β. However, amplification of conceptus IL-1β gene expression with the primers and probe was not exponential. The amplification profile of conceptus IL-1β indicated there were mismatches between the primer/ probe set and conceptus IL-1β mRNA. The IL-1β cDNA sequence previously isolated in our laboratory has 90% homology to the known porcine IL-1β sequence (GenBank accession # M86725). The region in which the primers and probe were constructed (exon number 5) to amplify IL-1β was not totally homologous (81%) between the porcine lung IL-1β in GenBank and conceptus sequence isolated using SSH [Ross et al., 2003]. The partial IL-1β cDNA sequence isolated in our laboratory from conceptus tissue represented exons 4, 5, 6 and 7 of pro-interleukin-1β (GenBank accession # X74568) containing 97, 99, 97 and 96 percent homology, respectively. A second set of primers and probe were designed in exon number 7 using the isolated IL-1β cDNA sequence from conceptuses (Table 4.1).

The difference between the ability to amplify endometrial but not conceptus IL-1β with the original primer/probe design suggests the possibility that an alternate form of IL-1β may exist. However, a comparison between porcine endometrial and conceptus IL-1β sequences needs to be evaluated. Template amplification was quantified by determining the threshold cycle ($C_T$) based on the fluorescence detected within the geometric region of the semilog plot. In the geometric region, one cycle is equivalent to the doubling of the PCR target template. Using the comparative $C_T$ method [Hettinger et

**Table 4.1.** Primer and probe sequences for IL-1β, IL-1RT1, IL-1RAP and IL-1Rant used for real time quantitative RT-PCR

| Target | Primers | Probe | Product Size (bp) | GenBank Accession # |
|---|---|---|---|---|
| Interleukin-1β Endometrial[a] | 5'-TGCCAACGTGCAGTCTATGG-3'<br>5'-TGGGCCAGCCAGCACTAG-3' | 5'-TGCAAACTCCAGGACAAAGACCACAAATC--3' | 70 | M86725 |
| Interleukin-1β Conceptus[a] | 5'-GGCCGCCAAGATATAACTGA-3'<br>5'-CCCTCTGGGTATGGCTTTC-3' | 5'-TTCACCATGGAAGTCCTCTCTCCCTAA--3' | 68 | Unpublished Sequence |
| Interleukin-1 Receptor Type I | 5'-AATGCACTTCCTAGGCTTTCTG-3'<br>5'-GGAACAGGATGTGGTGACAA-3' | 5'-CCTGAATTGCCCTGGCCTGCTA-3' | 65 | BI182393 |
| Interleukin-1Receptor Accessory Protein | 5'-AAATGCCAAAGGGGAGGTT-3'<br>5'-TGCTGTGTGCATCCATTACC-3' | 5'-ACAGACCGGCCAAGGTGAAACAGA-3' | 66 | BE013056 |
| Interleukin-1 Receptor Antagonist | 5'-TTCCTCCTTTTCCTGTTCCA-3'<br>5'-GCATCCTGCAAGGTCTCTTT-3' | 5'-CTCAGAGACTGCCTGCCACCCCT-3' | 67 | L38849 |
| Interleukin-1β Converting Enzyme | 5'-CCCCTCAGACAGTACAAGACAA-3'<br>5'-AGCTTGAGGCTCCCTCTTG-3' | 5'-CCCAGTTAAGCCTGCGTCTTCAGAGC-3' | 67 | AB027296 |

[a]Due to sequence homology differences in conceptus and endometrial IL-1β, a dual-labeled probe and primer set was designed specifically for each tissue type.

al., 2001] relative quantification and fold gene expression differences were determined for different conceptus stages (Table 4.2) and endometrial status during days of the estrous cycle and early pregnancy (Tables 4.3 and 4.4).

**Enzyme Linked Immunosorbent Assay**

IL-1β protein content in uterine lumenal flushings of day 12 and 15 cyclic gilts and pregnant gilts with spherical, ovoid, tubular, day 15 and 18 conceptuses was quantified using a commercially available ELISA (R&D Systems, Minneapolis, MN). The assay employs the quantitative sandwich enzyme immunoassay technique using a monoclonal antibody specific to porcine IL-1β pre-coated onto a microplate. Due to concentrations that exceeded the standard curve, a number of samples were diluted with calibrator diluent RD6-33 (R&D Systems, Minneapolis, MN) to place the samples within the sensitivity of the assay (10-2500 pg/mL). All samples, standards and controls were assayed in duplicate. The assay was conducted according to the manufacturers recommendations (R&D Systems, Minneapolis, MN). Briefly, 50 µl of assay diluent was added to each well followed by the addition of 100 µl of standard, control, or sample (stock or diluted). The plate was gently tapped for 1 min and incubated at 22-25°C for 2 h. Following incubation, each well was washed five times using 400 µl of wash buffer. The plate was tapped dry and 200 µl of porcine IL-1β conjugate was added and allowed to incubate for 2 h at 22-25°C. Washes were repeated as above and 120 µl of substrate solution (1 part stabilized hydrogen peroxide : 1 part stabilized tetramethylbenzidine) was added and allowed to incubate at 22-25°C for an additional 30 min. Following binding of substrate, 120 µl of stop solution was added and optical density of each well was

determined within 30 min using the VMax Kinetic Microplate Reader (Molecular Devices, Sunnyvale, CA) set at 450 nm with a correction wavelength of 562 nm. IL-1β concentrations were calculated based on the generated standard curve. The intraassay coefficient of variation of the IL-1β ELISA was 4.8 %.

### Statistical Analysis

Quantitative RT-PCR $\Delta C_T$ values were analyzed using PROC MIXED of the Statistical Analysis System [SAS User's Guide, 1985]. Analysis of conceptus gene expression tested for the fixed effect of morphology. The analysis of endometrial gene expression tested for the effect of status, day and status x day interaction. The effect of status, day and status x day interaction was evaluated for IL-1β protein in uterine flushings from day 12 and 15 pregnant and cyclic gilts. The fixed effect of conceptus morphology was tested for IL-1β protein in uterine flushings from pregnant uteri during trophoblastic elongation (day 11-12), day 15 and 18. Significance ($P < 0.05$) was determined by probability differences of least squares means. Satterthwaite's approximation was used for means with heterogeneous variance to calculate the effective degrees of freedom for the error term [Steel et al., 1996]. Results are presented as arithmetic means ± SEM.

## Results

### Quantitative RT-PCR

To investigate possible mechanisms of IL-1β action during trophoblastic elongation and attachment to the uterine surface, gene expression profiles were generated

from conceptus and uterine endometrium mRNA for IL-1β, IL-1RT1, IL-1RAP and IL-1Rant using quantitative real time RT-PCR. Conceptus gene expression was evaluated in spherical, ovoid, tubular and filamentous conceptuses collected between days 10-12 of pregnancy as well as during the period of attachment to the surface on day 15 and 18 of gestation. Uterine endometrium from cyclic gilts (days 0, 10, 12, 15 and 18 of estrous cycle) and pregnant gilts (days 10, 12, 15 and 18) were evaluated to determine gene expression during the estrous cycle and critical time-points during early pregnancy.

*Interleukin-1β*

A significant difference ($P < 0.0001$) in IL-1β gene expression between morphological variants during porcine trophoblast development was detected using quantitative RT-PCR (Table 4.2). Selectively comparing fold differences in IL-1β gene expression only during the period of trophoblastic elongation (Figure 4.1, insert), there was an approximate 2-fold increase in IL-1β gene expression during conceptus transition from spherical to tubular conceptuses, which increased to 6-fold in filamentous conceptuses. Comparing all morphologies evaluated, greatest IL-1β gene expression was detected in day 12 filamentous conceptuses, which was over 2000-fold greater compared to conceptuses collected on day 15 and 18 of pregnancy (Figure 4.1). IL-1β gene expression was extremely low in day 15 and 18 conceptuses as spherical conceptuses expressed over 300-fold greater IL-1β compared to post-elongation conceptuses (Figure 4.1). No significant status or day x status interaction was detected in endometrial IL-1β gene expression (Table 4.3). However, there was a tendency for a day effect ($P < 0.06$) as

**Table 4.2**. Quantitative RT-PCR analysis comparing gene expression of IL-1β, IL-1 Receptor Antagonist, IL-1 Receptor Type I and IL-1 Receptor Accessory in conceptuses during rapid trophoblastic elongation and late peri-implantation development.

| Target | Morphology | Average Target $C_T$* | Average 18S rRNA $C_T$* | $\Delta C_T$‡¶ | $\Delta\Delta C_T$§ |
|---|---|---|---|---|---|
| **Interleukin-1β** | Spherical | 19.12± 0.37 | 16.89± 0.21 | 2.23 ± 0.33$^{c}$ | -8.57 |
| | Ovoid | 18.59 ± 1.08 | 16.54 ± 0.43 | 2.05 ± 0.65$^{bc}$ | -8.75 |
| | Tubular | 17.29 ± 0.44 | 16.21 ± 0.14 | 1.08 ± 0.33$^{b}$ | -9.72 |
| | Filamentous | 16.02 ± 0.36 | 16.38 ± 0.05 | -0.37 ± 0.39$^{a}$ | -11.17 |
| | Day 15 | 27.00 ± 0.06 | 16.20 ± 0.00 | 10.80 ± 0.07$^{d}$ | 0.00 |
| | Day 18 | 26.08 ± 0.50 | 16.21 ± 0.13 | 9.87 ± 0.47$^{d}$ | -0.94 |
| **Interleukin-1 Receptor Antagonist** | Spherical | 32.60± 0.52 | 16.89± 0.21 | 15.71 ± 0.49 | -1.79 |
| | Ovoid | 33.08 ± 2.60 | 16.54 ± 0.43 | 16.54 ± 2.18 | -0.97 |
| | Tubular | 31.86 ± 1.71 | 16.21 ± 0.14 | 15.65 ± 1.58 | -1.85 |
| | Filamentous | 32.63 ± 0.78 | 16.38 ± 0.05 | 16.25 ± 0.79 | -1.25 |
| | Day 15 | 33.70 ± 0.11 | 16.20 ± 0.00 | 17.50 ± 0.11 | 0.00 |
| | Day 18 | 31.15 ± 0.40 | 16.21 ± 0.13 | 14.94 ± 0.39 | -2.56 |
| **Interleukin-1 Receptor Type I** | Spherical | 31.61± 0.19 | 16.89± 0.21 | 14.72 ± 0.23$^{b}$ | -1.60 |
| | Ovoid | 30.72 ± 0.98 | 16.54 ± 0.43 | 14.17 ± 1.41$^{abc}$ | -2.15 |
| | Tubular | 32.53 ± 0.26 | 16.21 ± 0.14 | 16.32 ± 0.34$^{c}$ | 0.00 |
| | Filamentous | 28.58 ± 0.66 | 16.38 ± 0.05 | 12.20 ± 0.69$^{a}$ | -4.12 |
| | Day 15 | 29.01 ± 0.02 | 16.20 ± 0.00 | 12.81 ± 0.01$^{a}$ | -3.51 |
| | Day 18 | 29.64 ± 0.36 | 16.21 ± 0.13 | 13.43 ± 0.30$^{a}$ | -2.89 |
| **Interleukin-1 Receptor Accessory Protein** | Spherical | 30.41± 0.37 | 16.89± 0.21 | 13.52 ± 0.31$^{c}$ | -1.39 |
| | Ovoid | 30.30 ± 0.09 | 16.54 ± 0.43 | 13.76 ± 0.52$^{cd}$ | -1.15 |
| | Tubular | 31.12 ± 0.23 | 16.21 ± 0.14 | 14.91 ± 0.32$^{d}$ | 0.00 |
| | Filamentous | 29.05 ± 0.46 | 16.38 ± 0.05 | 12.67 ± 0.48$^{bc}$ | -2.24 |
| | Day 15 | 26.56 ± 0.08 | 16.20 ± 0.00 | 10.36 ± 0.07$^{a}$ | -4.55 |
| | Day 18 | 28.39 ± 0.45 | 16.21 ± 0.13 | 12.18 ± 0.35$^{b}$ | -2.73 |

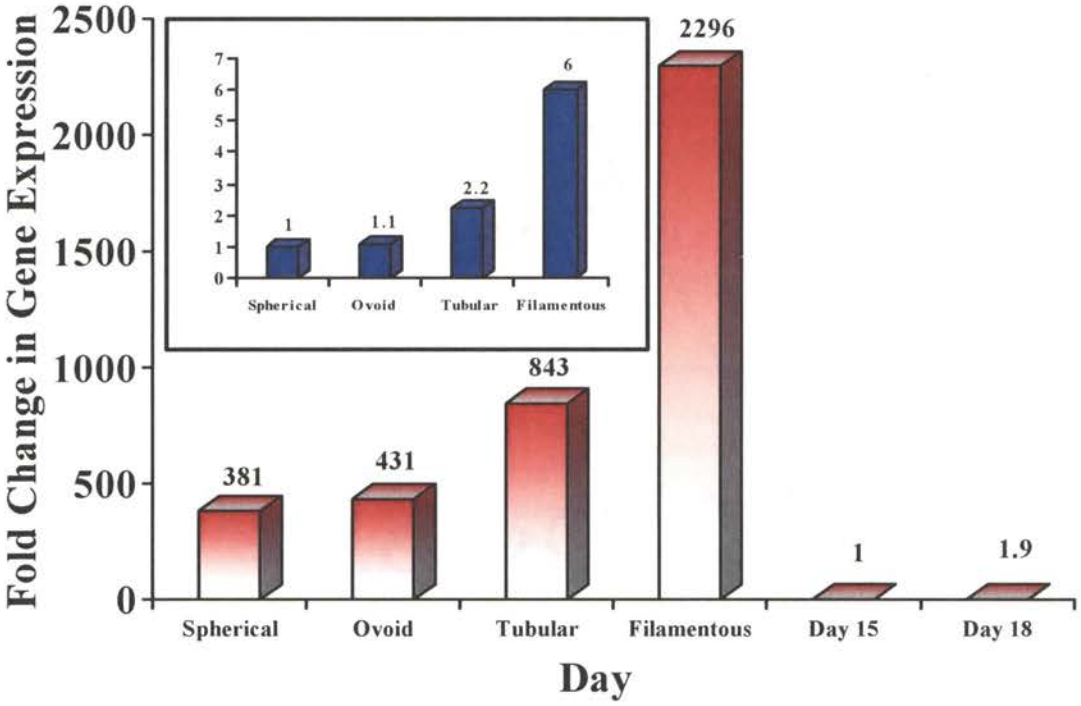
* $C_T$ = Cycle Threshold. Indicates cycle number in which amplification crosses the threshold set in the geometric portion of amplification curve.

‡ $\Delta C_T$ = Target transcript $C_T$ – 18S ribosomal $C_T$: Normalization of $C_T$ for target gene relative to ribosomal 18S RNA $C_T$.

¶ Statistical analysis of normalized expression levels between morphologies. Values with different superscripts for each of the target genes differ significantly ($P < 0.05$).

§ $\Delta\Delta C_T$ = Mean $\Delta C_T$ – highest mean $\Delta C_T$ value: The mean value for the morphology with highest $\Delta C_T$ (lowest expression levels for target) was used as a calibrator to set the baseline for comparing mean differences in the $\Delta C_T$ values across all morphologies.

**Figure 4.1.** Fold gene expression changes of IL-1β in porcine conceptuses during rapid trophoblastic elongation and late peri-implantation development generated using Quantitative 1-Step RT-PCR. Gene expression for day 15 conceptuses was set as the baseline and fold gene expression was calculated as described in *Materials and Methods*. The inset depicts fold differences in IL-1β gene expression during rapid trophoblastic elongation (day11-12) by setting gene expression of spherical conceptuses as the baseline.


Fold Change in Gene Expression
2500
2000
1500
1000
500
0
381
431
843
2296
1
1.9
Spherical
Ovoid
Tubular
Filamentous
Day 15
Day 18
Day
7
6
5
4
3
2
1
0
1
1.1
2.2
6
Spherical
Ovoid
Tubular
Filamentous

gene expression for IL-1β was greater during estrus and early diestrus (day 5) compared to the later days of the estrous cycle and early pregnancy (Figure 4.2).

*Interleukin-1 Receptor Type I*

Conceptus IL-1 RT1 gene expression was affected ($P < 0.003$) by morphological stage of development (Table 4.2). IL-1RT1 expression was lowest in tubular conceptuses compared to all other morphologies (Table 4.2). Similar to IL-1β gene expression, filamentous conceptuses expressed greater IL-1RT1 mRNA when compared to spherical and tubular conceptuses (Table 4.2). IL-1RT1 mRNA expression was approximately 6-fold greater in day 12 filamentous conceptuses compared to spherical conceptuses and approximately 17-fold greater than tubular conceptuses (Figure 4.3).

A day x status interaction ($P < 0.0001$) was detected in endometrial IL-1RT1 gene expression (Table 4.4). Endometrium from day 12 of gestation contained greater ($P < 0.04$) mRNA levels of IL-1RT1 compared to all other days of the estrous cycle and pregnancy (Table 4.4). Endometrial IL-1RT1 gene expression was enhanced 2 to 4-fold on day 12 of pregnancy compared to all days of estrous cycle and pregnancy (Figure 4.4).

*Interleukin-1 Receptor Accessory Protein*

Conceptus IL-1RAP gene expression was affected ($P < 0.0001$) by morphological stage of development (Table 4.2). IL-1RAP gene expression increased and was maintained following trophoblastic elongation (Figure 4.3). The greatest IL-1RAP mRNA expression occurred in day 15 conceptuses compared to all other morphologies evaluated while tubular conceptuses contained the lowest IL-1RAP mRNA (Table 4.2). Compared

**Table 4.3.** Quantitative RT-PCR analysis of endometrial gene expression of IL-1β and IL-1 Receptor Antagonist during the estrous cycle and early pregnancy.

| Target | Day | Status | Average Target $C_T$* | Average 18S rRNA $C_T$* | $\Delta C_T$‡¶ | $\Delta\Delta C_T$§ |
|---|---|---|---|---|---|---|
| **Interleukin-1β** | 0 | Cycling | 25.72 ± 1.07 | 17.05 ± 0.20 | 8.67 ± 1.24 | -4.24 |
| | 5 | Cycling | 26.57 ± 1.79 | 16.94 ± 0.09 | 9.63 ± 1.88 | -3.28 |
| | 10 | Cycling | 26.92 ± 0.63 | 16.80 ± 0.13 | 10.13 ± 0.75 | -2.79 |
| | 10 | Pregnant | 28.42 ± 1.29 | 16.49 ± 0.08 | 11.93 ± 1.35 | -0.98 |
| | 12 | Cycling | 28.32 ± 0.69 | 16.65 ± 0.17 | 11.67 ± 0.71 | -1.24 |
| | 12 | Pregnant | 29.29 ± 0.47 | 16.39 ± 0.02 | 12.91 ± 0.47 | 0.00 |
| | 15 | Cycling | 28.85 ± 0.59 | 16.41 ± 0.05 | 12.44 ± 0.64 | -0.47 |
| | 15 | Pregnant | 28.52 ± 0.68 | 16.47 ± 0.08 | 12.06 ± 0.63 | -0.85 |
| | 18 | Cycling | 27.52 ± 0.36 | 16.65 ± 0.26 | 10.87 ± 0.10 | -2.04 |
| | 18 | Pregnant | 28.27 ± 0.51 | 16.60 ± 0.33 | 11.68 ± 0.49 | -1.24 |
| **Interleukin-1 Receptor Antagonist** | 0 | Cycling | 27.13 ± 0.88 | 17.05 ± 0.20 | 10.08 ± 1.06$^{a}$ | -6.45 |
| | 5 | Cycling | 27.84 ± 1.55 | 16.94 ± 0.09 | 10.91 ± 1.64$^{ab}$ | -5.63 |
| | 10 | Cycling | 30.07 ± 0.86 | 16.80 ± 0.13 | 13.27 ± 0.96$^{bc}$ | -3.26 |
| | 10 | Pregnant | 32.40 ± 1.02 | 16.49 ± 0.08 | 15.91 ± 1.06$^{cd}$ | -0.62 |
| | 12 | Cycling | 31.09 ± 0.71 | 16.65 ± 0.17 | 14.43 ± 0.81$^{cd}$ | -2.10 |
| | 12 | Pregnant | 32.41 ± 0.24 | 16.39 ± 0.02 | 16.02 ± 0.26$^{cd}$ | -0.51 |
| | 15 | Cycling | 32.94 ± 0.63 | 16.41 ± 0.05 | 16.53 ± 0.65$^{d}$ | 0.00 |
| | 15 | Pregnant | 30.56 ± 0.67 | 16.47 ± 0.08 | 14.09 ± 0.60$^{c}$ | -2.44 |
| | 18 | Cycling | 29.75 ± 0.24 | 16.65 ± 0.26 | 13.10 ± 0.49$^{bc}$ | -3.43 |
| | 18 | Pregnant | 31.75 ± 0.63 | 16.60 ± 0.33 | 15.15 ± 0.46$^{cd}$ | -1.38 |

* $C_T$ = Cycle Threshold. Indicates cycle number in which amplification crosses the threshold set in the geometric portion of amplification curve.

‡ $\Delta C_T$ = Target transcript $C_T$ – 18S ribosomal $C_T$: Normalization of $C_T$ for target gene relative to ribosomal 18S RNA $C_T$.

¶ Statistical analysis of normalized expression levels between days and statuses. There was a day x status interaction ($P < 0.03$) for endometrial IL-1Rant gene expression. Values with different superscripts within the analysis of each target gene differ significantly ($P < 0.05$)

§ $\Delta\Delta C_T$ = Mean $\Delta C_T$ – highest mean $\Delta C_T$ value: The mean value for the day/status with the highest $\Delta C_T$ (lowest expression levels for target) was used as a calibrator to set the baseline for comparing mean differences in the $\Delta C_T$ values across all days and statuses.

**Figure 4.2.** Fold gene expression changes of IL-1β in endometrium from cyclic and pregnant gilts generated using Quantitative 1-Step RT-PCR. Gene expression levels for day 12 pregnant endometrium was set as the baseline and fold gene expression was calculated as described in *Materials and Methods.*

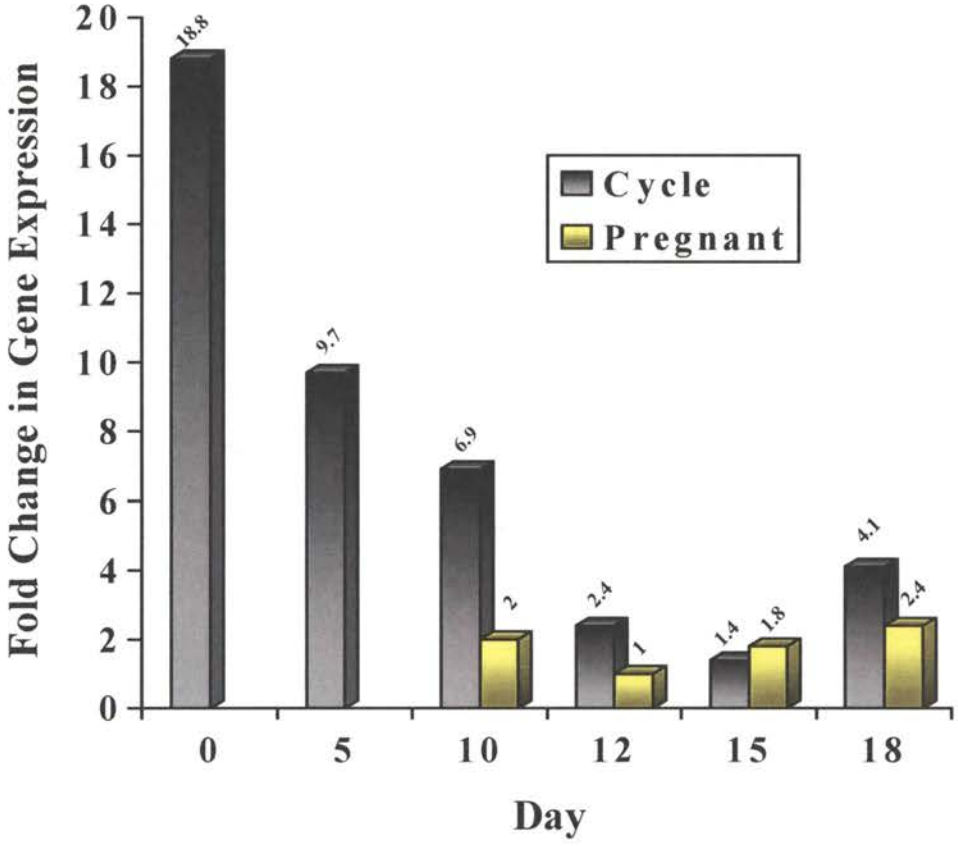

Fold Change in Gene Expression
20
18
16
14
12
10
8
6
4
2
0
18.8
9.7
6.9
2
2.4
1
1.4
1.8
4.1
2.4
Cycle
Pregnant
0
5
10
12
15
18
Day

**Figure 4.3.** Fold gene expression changes of IL-1 Receptor Type 1 and Receptor Accessory Protein in porcine conceptuses during rapid trophoblastic elongation and late peri-implantation development generated using Quantitative 1-Step RT-PCR. Tubular gene expression was set as the baseline for both transcripts and fold gene expression was calculated as described in *Materials and Methods*.

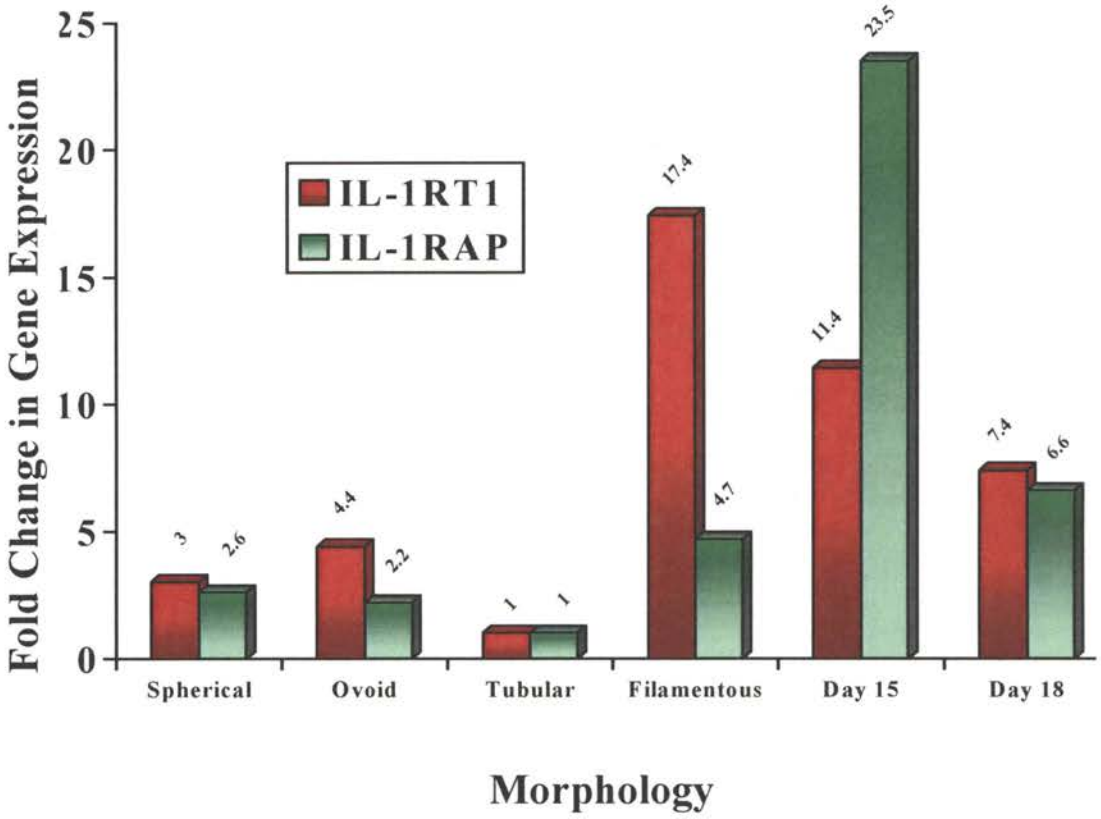

Fold Change in Gene Expression
25
20
15
10
5
0
IL-1RT1
IL-1RAP
3
2.6
4.4
2.2
1
1
17.4
4.7
11.4
23.5
7.4
6.6
Spherical
Ovoid
Tubular
Filamentous
Day 15
Day 18
Morphology

**Table 4.4.** Quantitative RT-PCR analysis of endometrial gene expression for IL-1 Receptor Type 1 and IL-1 Receptor Accessory Protein during the estrous cycle and early pregnancy.

| Target | Day | Status | Average Target $C_T$* | Average 18S rRNA $C_T$* | $\Delta C_T$‡¶ | $\Delta\Delta C_T$§ |
|---|---|---|---|---|---|---|
| **Interleukin-1 Receptor Type I** | 0 | Cycling | 22.39 ± 0.32 | 17.05 ± 0.20 | $5.34 \pm 0.24^{cd}$ | -1.29 |
| | 5 | Cycling | 22.63 ± 0.16 | 16.94 ± 0.09 | $5.69 \pm 0.14^{cde}$ | -0.94 |
| | 10 | Cycling | 21.24 ± 0.34 | 16.80 ± 0.13 | $4.45 \pm 0.36^{b}$ | -2.19 |
| | 10 | Pregnant | 20.88 ± 0.19 | 16.49 ± 0.08 | $4.40 \pm 0.17^{b}$ | -2.24 |
| | 12 | Cycling | 21.92 ± 0.42 | 16.65 ± 0.17 | $5.26 \pm 0.34^{cd}$ | -1.37 |
| | 12 | Pregnant | 19.91 ± 0.22 | 16.39 ± 0.02 | $3.52 \pm 0.20^{a}$ | -3.11 |
| | 15 | Cycling | 21.54 ± 0.20 | 16.41 ± 0.05 | $5.13 \pm 0.24^{bc}$ | -1.50 |
| | 15 | Pregnant | 22.45 ± 0.14 | 16.47 ± 0.08 | $5.98 \pm 0.21^{de}$ | -0.65 |
| | 18 | Cycling | 22.01 ± 0.22 | 16.65 ± 0.26 | $5.37 \pm 0.26^{cd}$ | -1.27 |
| | 18 | Pregnant | 23.23 ± 0.28 | 16.60 ± 0.33 | $6.63 \pm 0.29^{e}$ | 0.00 |
| **Interleukin-1 Receptor Accessory Protein** | 0 | Cycling | 23.60 ± 0.78 | 17.05 ± 0.20 | $6.55 \pm 0.96^{bc}$ | -1.13 |
| | 5 | Cycling | 22.93 ± 0.78 | 16.94 ± 0.09 | $5.99 \pm 0.87^{ab}$ | -1.69 |
| | 10 | Cycling | 23.18 ± 0.27 | 16.80 ± 0.13 | $6.38 \pm 0.37^{bc}$ | -1.30 |
| | 10 | Pregnant | 24.17 ± 0.45 | 16.49 ± 0.08 | $7.68 \pm 0.48^{c}$ | 0.00 |
| | 12 | Cycling | 23.10 ± 0.33 | 16.65 ± 0.17 | $6.45 \pm 0.43^{bc}$ | -1.23 |
| | 12 | Pregnant | 21.02 ± 0.29 | 16.39 ± 0.02 | $4.63 \pm 0.28^{a}$ | -3.05 |
| | 15 | Cycling | 23.52 ± 0.37 | 16.41 ± 0.05 | $7.11 \pm 0.35^{bc}$ | -0.57 |
| | 15 | Pregnant | 22.86 ± 0.23 | 16.47 ± 0.08 | $6.40 \pm 0.28^{bc}$ | -1.28 |
| | 18 | Cycling | 23.88 ± 0.43 | 16.65 ± 0.26 | $7.23 \pm 0.37^{bc}$ | -0.45 |
| | 18 | Pregnant | 22.59 ± 0.29 | 16.60 ± 0.33 | $5.99 \pm 0.13^{ab}$ | -1.69 |

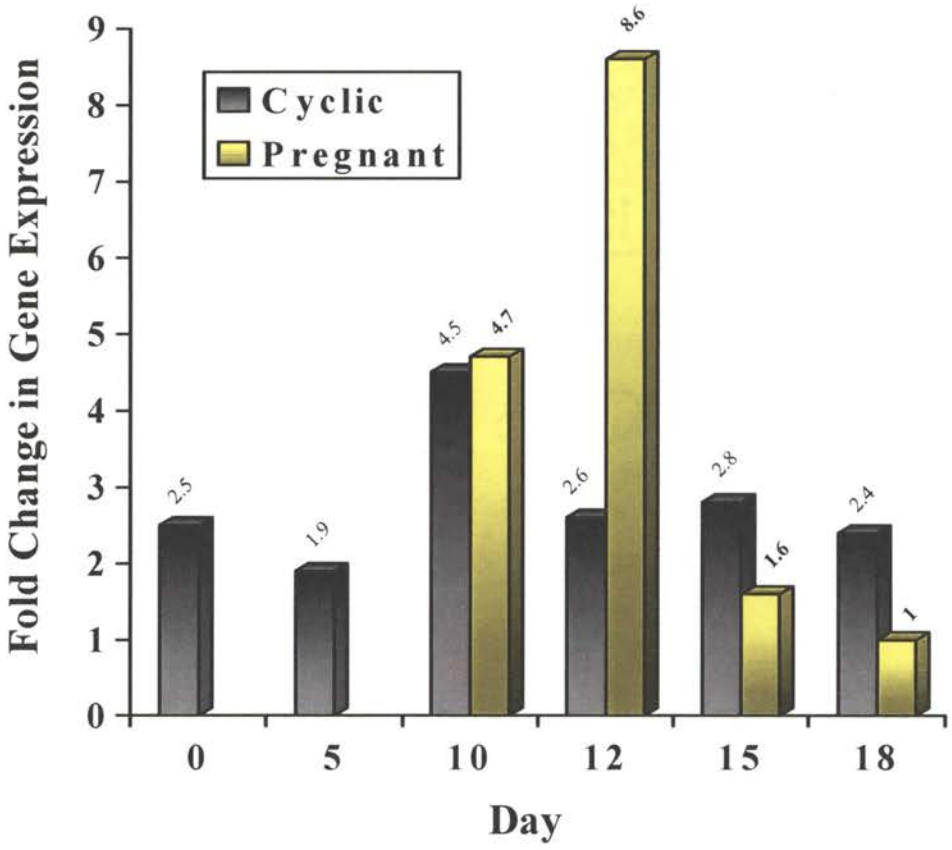
* $C_T$ = Cycle Threshold. Indicates cycle number in which amplification crosses the threshold set in the geometric portion of amplification curve.

‡ $\Delta C_T$ = Target transcript $C_T$ – 18S ribosomal $C_T$: Normalization of $C_T$ for target gene relative to ribosomal 18S RNA $C_T$.

¶ Statistical analysis of normalized expression levels between day and status. There was a day x status interaction in gene expression levels of both IL-1RT1 ($P < 0.0001$) and IL-1RAP ($P < 0.03$). Values with different superscripts within the analysis of each target gene differ significantly ($P < 0.05$)

§ $\Delta\Delta C_T$ = Mean $\Delta C_T$ – highest mean $\Delta C_T$ value: The mean value for the day/status with the highest $\Delta C_T$ (lowest expression levels for target) was used as a calibrator to set the baseline for comparing mean differences in the $\Delta C_T$ values across days of the estrous cycle and pregnancy.

**Figure 4.4.** Fold gene expression changes of IL-1 Receptor Type 1 in endometrium from cyclic and pregnant gilts generated using Quantitative 1-Step RT-PCR. Gene expression levels for day 18 pregnant endometrium was set as the baseline and fold gene expression was calculated as described in *Materials and Methods*.


Fold Change in Gene Expression
9
8
7
6
5
4
3
2
1
0
Cyclic
Pregnant
2.5
1.9
4.5
4.7
2.6
8.6
2.8
1.6
2.4
1
0
5
10
12
15
18
Day

to tubular conceptuses, IL-1RAP expression was approximately 5, 23 and 7-fold greater in filamentous (day 12), day 15 and 18 conceptuses, respectively (Figure 4.3). Evaluation of endometrial IL-1RAP gene expression (Table 4.4) detected a day x status interaction ($P > 0.03$). The highest IL-1RAP mRNA expression was detected in endometrium collected on day 12 of pregnancy (Table 4.4). IL-1RAP gene expression was enhanced 3 to 8-fold on day 12 of gestation (Figure 4.5).

*Interleukin-1 Receptor Antagonist*

Conceptus gene expression for IL-1Rant was not affected ($P < 0.74$) by morphological stage of development (Table 4.2). A day x status interaction ($P < 0.03$) was detected for endometrial IL-1Rant gene expression (Table 4.3). Endometrial IL-1Rant gene expression was greatest on day 0 of the estrous cycle (Table 4.3). IL-1Rant endometrial gene expression was high during estrus and early diestrus (day 5) rapidly declining during diestrus and early pregnancy (Figure 4.6). Differences in gene expression were approximately 50 to 90-fold greater on day 0 and 5 compared to endometrium during the time of peak conceptus IL-1β gene expression occurring on day 12 of gestation (Figure 4.6).

**IL-1β Protein**

Analysis of IL-1β protein in uterine flushings of pregnant and cyclic gilts from days 12 and 15 of gestation or the estrous cycle indicated a status effect ($P < 0.003$). The uterine concentration of IL-1β was below the detectable range of the ELISA (10 pg/mL) in flushings from all cyclic gilts while the concentration of IL-1β in uterine flushings averaged $184 \pm 50.3$

**Figure 4.5.** Fold gene expression changes of IL-1 Receptor Accessory Protein in endometrium from cyclic and pregnant gilts generated using Quantitative 1-Step RT-PCR. Gene expression levels for day 10 pregnant endometrium was set as the baseline and fold gene expression was calculated as described in *Materials and Methods*.

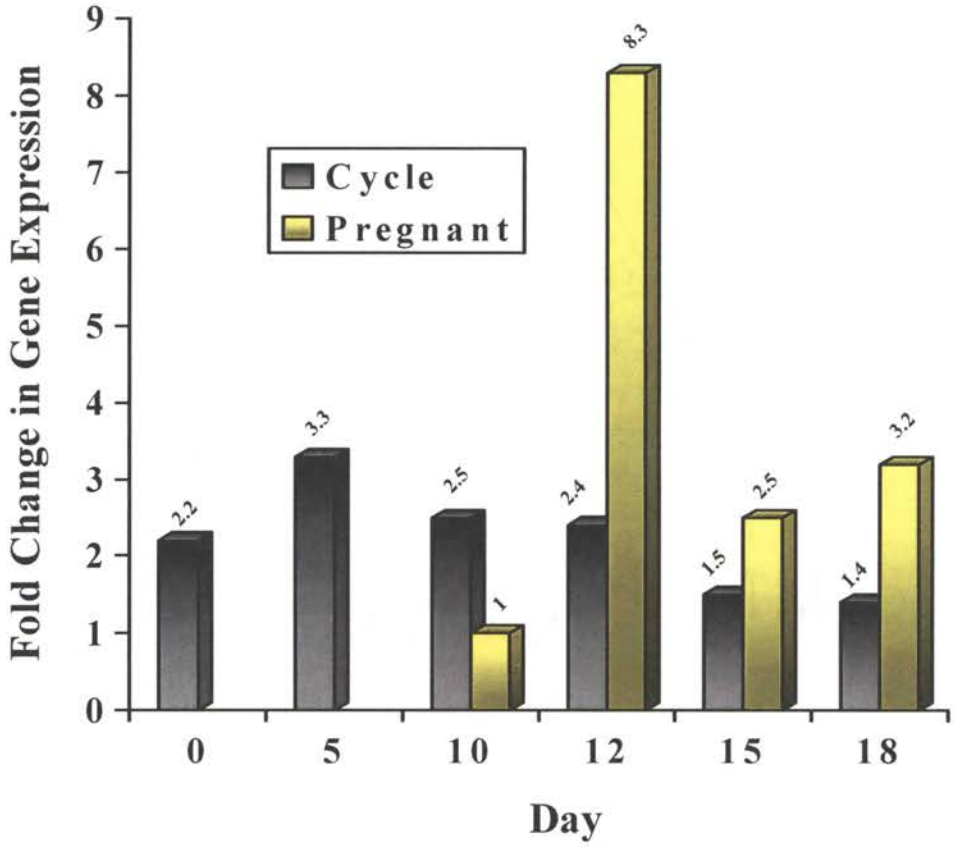
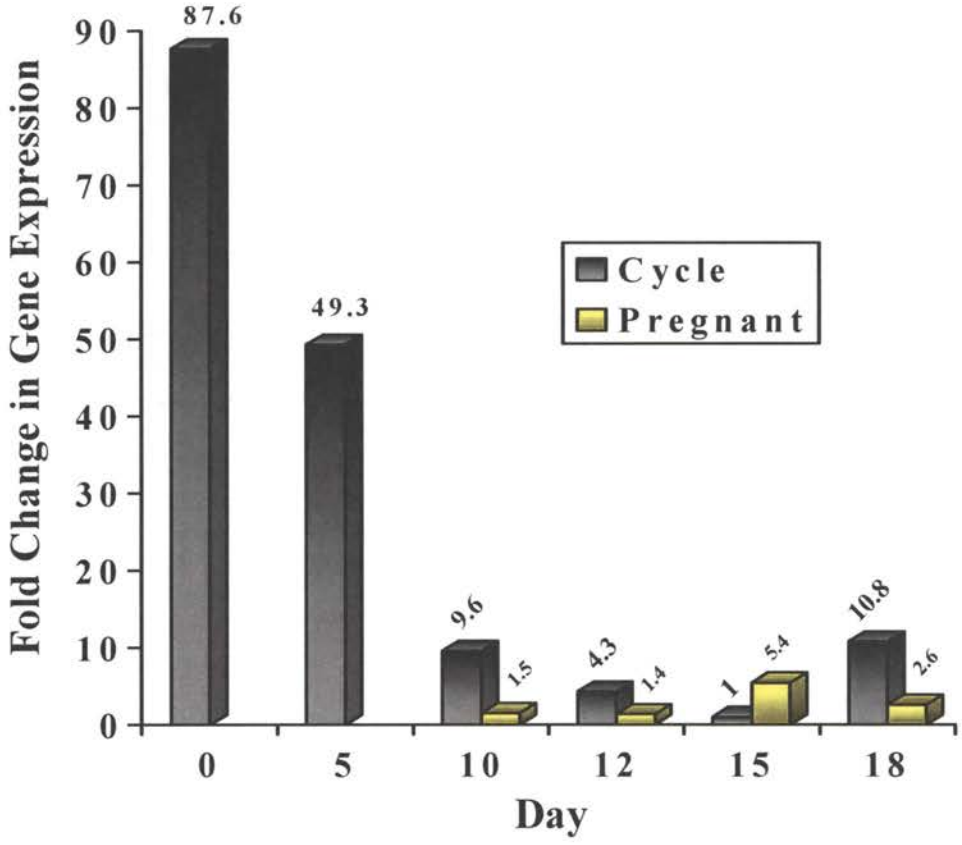

Fold Change in Gene Expression
9
8
7
6
5
4
3
2
1
0
Cycle
Pregnant
2.2
3.3
2.5
1
2.4
8.3
1.5
2.5
1.4
3.2
0
5
10
12
15
18
Day

**Figure 4.6.** Fold gene expression changes of IL-1 Receptor Antagonist in endometrium from cyclic and pregnant gilts generated using Quantitative 1-Step RT-PCR. Gene expression levels for day 15 cyclic endometrium was set as the baseline and fold gene expression was calculated as described in *Materials and Methods*.

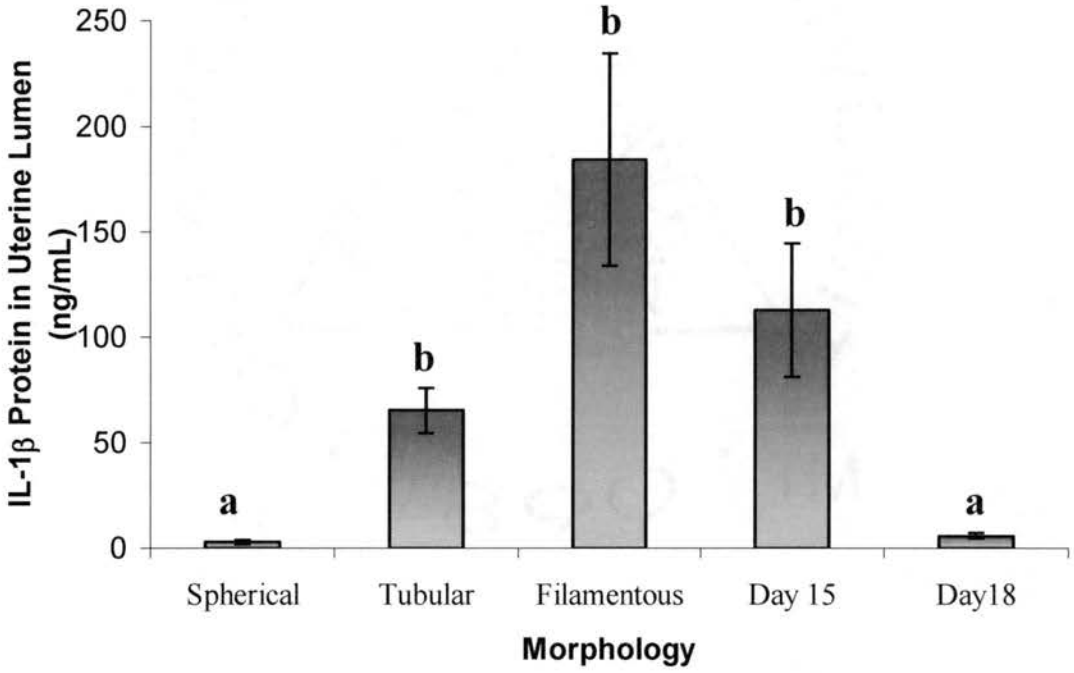

Fold Change in Gene Expression
90
80
70
60
50
40
30
20
10
0
87.6
49.3
9.6
1.5
4.3
1.4
1
5.4
10.8
2.6
Cycle
Pregnant
0
5
10
12
15
18
Day

and 112 ± 31.4 ng/mL on day 12 and 15 of pregnancy, respectively. When data were analyzed for pregnant gilts only, there was an effect ($P < 0.003$) of conceptus morphology in the uterine horn on the concentration of IL-1β protein in the uterine flushings. Concentration of IL-1β in the uterine flushings from pregnant gilts increased during the transition from spherical (2.9 ± 1.0 ng/mL) to tubular (65.4 ± 10.7 ng/mL) morphology and peaked during the presence of day 12 filamentous conceptuses (Figure 4.7). IL-1β protein content in the uterine lumen declined slightly by day 15 and returned to concentrations similar to uteri containing spherical conceptuses on day 18 (5.9 ± 1.5 ng/mL) of gestation (Figure 4.7).

## Discussion

Interleukin-1, previously termed leukocyte endogenous mediator, was first identified as a mediator of the acute-phase inflammatory response [Mantovani et al., 1998]. The ability of IL-1β to invoke inflammation is dependent upon the expression of the vast members of the IL-1 system, which consists of two receptors; IL-1RT1 (functional) and IL-1RT2 (pseudo-receptor), converting enzymes, receptor accessory proteins, and multiple isoforms of receptor antagonists [Mantovani et al., 1998]. The effects of IL-1 as an inducer of the acute-phase response is similar, but to a lesser degree, to the actions of IL-6 [Mantovani et al., 1998] that is also expressed in preimplantation pig conceptuses from days 11 to 21 of pregnancy [Mathialagan et al., 1992, Modric et al., 2000]. IL-1β gene expression in porcine conceptuses was first identified by Tuo et al. [1996]. The importance of IL-1β in porcine conceptus development was suggested when IL-1β was identified as a gene differentially expressed during rapid trophoblastic

**Figure 4.7**. IL-1β protein content ± SEM detected in uterine flushings from pregnant gilts corresponding to morphological stage during the period rapid trophoblastic elongation (day 11-12) and late peri-implantation development (days 15 and 18). Morphologies with different superscripts are significantly different ($P < 0.05$).


IL-1β Protein in Uterine Lumen (ng/mL)
250
200
150
100
50
0
a
b
b
b
a
Spherical
Tubular
Filamentous
Day 15
Day18
Morphology

elongation using SSH [Ross et al., 2003b]. IL-1β was the most abundantly expressed gene isolated using SSH and was one of the most abundant clones represented when mapping expressed sequence tags derived from a porcine early embryonic cDNA library in a study by Smith et al. [2001].

IL-1β is a pro-inflammatory cytokine associated with implantation in the mouse [Takacs and Kauma, 1996; Kruessel et al., 1997] and has been suggested as the initiator of cross-talk between human embryos and the endometrium during initiation of implantation [Lindhard et al., 2002]. Deletion of IL-1RT1 in knockout mice results in only a slightly reduced litter size [Abbondanzo et al., 1996]. However, repeated injections of IL-1Rant into pregnant mice prior to implantation caused implantation failure [Simon et al., 1994a]. Regardless of the receptor pathway, these studies indicate the importance for IL-1β signaling during implantation in the mouse. Detection of porcine conceptus IL-1β gene expression led us to investigate expression patterns of IL-1β, IL-1RT1, IL-1RAP and IL-1Rant in conceptuses and uterine endometrium during peri-implantation development and the establishment of pregnancy in the pig.

The present study clearly demonstrates the presence of IL-1β in the uterine lumen during the time of conceptus elongation and maternal recognition of pregnancy on day 12 of gestation. Uterine lumenal content of IL-1β increased during the initiation of rapid trophoblastic elongation. Absence of IL-1β protein in the uterine lumen on days 12 and 15 of the estrous cycle with the increase in conceptus IL-1β gene expression detected during trophoblastic elongation suggests the IL-1β in the uterine lumen originates from peri-implantation conceptuses. Following the initial sharp increase in IL-1β on day 12, conceptus gene expression and release of IL-1β is greatly reduced by day 15 and 18 of

gestation. IL-1β gene expression was more than 2000-fold less in day 15 conceptuses compared to day 12 filamentous conceptuses. Similarly, IL-1β protein availability in the uterine lumen is declining on day 15 and returned to pre-elongation concentrations by day 18 of gestation. This transient pattern of conceptus IL-1β gene expression and protein secretion is temporally and spatially associated with IL-1RT1 and IL-1RAP gene expression in elongated conceptuses (days 12-15) and in endometrium from pregnant gilts on day 12 of gestation. Conceptus expression for IL-1RT1 and IL-1RAP is greater in filamentous conceptuses compared to earlier morphologies and tends to decline in day 18 conceptuses.

IL-1β ligand binding to both IL-1RT1 and IL-1RAP is needed to elicit a biological response [Cullinan et al., 1998] although as indicated above, IL-1β may function through multiple receptors. The upregulation of IL-1RT1 gene expression in both the endometrium and conceptuses may be in part due to the actions of IL-1β itself, which is known to upregulate the expression of its own receptor, IL-1RT1, in human endometrial stromal and glandular cells [Simon et al., 1994b]. Endometrial IL-1RT1 and IL-1RAP gene expression decreased following conceptus trophoblastic elongation and initial apposition to the uterine apical surface on day 12 of gestation whereas conceptus IL-1RT1 and IL-1RAP gene expression continued to be elevated following conceptus expansion. Endometrial IL-1RT1 gene expression during the time of blastocyst attachment to the uterine surface is similarly expressed with respect to implantation in the mouse [Reese et al., 2001] and human [Simon et al., 1993]. In contrast to the pig, IL-1RAP gene expression in human endometrium is constitutively expressed throughout the

menstrual cycle without significant variation although its presence is more intense in glandular and lumenal epithelium [Bigonnesse et al., 2001].

Based on temporal IL-1RT1, IL-1RAP and IL-1Rant gene expression, conceptus IL-1β gene expression and presence in the uterine lumen likely induces biological actions in both the porcine conceptus and endometrium. IL-1β is a potent stimulator of aromatase activity and subsequent estrogen ($E_2$) synthesis in human cytotrophoblasts [Nestler, 1993] and also stimulates progesterone production in JEG-3 choriocarcinoma cells [Feinberg et al., 1994]. Aromatase gene expression is increased in day 12 filamentous pig conceptuses compared to earlier morphologies [Yelich et al., 1997a]. Estrogen production in porcine conceptuses sharply increases during elongation on day 12, declines rapidly on day 13 and then initiates a second more sustained increase on days 16 to 25 of gestation [Geisert and Yelich, 1997]. Conceptus IL-1β gene expression and protein secretion is temporally associated with initiation of conceptus $E_2$ production during the process of rapid trophoblastic elongation, suggesting IL-1β may be involved with the $E_2$ increase in elongating conceptuses. However, conceptus IL-1RT1 and IL-1 RAP gene expression and continued IL-1β presence in the uterine lumen through day 15 suggests IL-1β could have effects on conceptus and uterine function after trophoblastic elongation. Although the initial conceptus enhancement of $E_2$ synthesis may involve IL-1β, our data suggest the second sustained increase in porcine conceptus $E_2$ release initiated on day 16 is mediated by an alternative mechanism, as IL-1β gene expression is almost absent in day 15 and 18 conceptuses.

IL-1β, an inducer of phospholipase A2 [Kol et al., 1999], may also regulate the release of arachidonic acid from the phosholipid bilayer allowing membrane fluidity

necessary for remodeling of the trophectoderm during elongation and its conversion to prostaglandins needed for placental attachment during the establishment of pregnancy [Davis and Blair, 1993; Geisert and Yelich, 1997]. IL-1β induces cyclo-oxygenase-2 (COX-2) gene expression in human endometrial stromal cells [Huang et al., 1998] and amnion-derived WISH cells [Albert et al., 1994]. Filamentous (day 12) porcine conceptuses express elevated levels of COX-2 mRNA [Wilson et al., 2002] temporally associated to the IL-1β gene expression we report. While conceptus production of prostaglandins may not be necessary for inducing trophoblastic elongation [Geisert et al., 1986], prostaglandins have been demonstrated to be essential for placental attachment and survival following elongation [Kraeling et al., 1985].

During attachment in the pig, the conceptus invokes an acute phase inflammatory response [Geisert and Yelich, 1997]. At this time, the formation of a conceptus-uterine extracellular matrix is essential. Tumor necrosis factor (TNF)-stimulated gene (TSG)-6 gene expression is induced by TNFα and IL-1β [Lee et al., 1992] as well as prostaglandin E2 ($PGE_2$) [Fujimoto et al., 2002]. TSG-6, which is strongly anti-inflammatory [Wisniewski et al., 1996], is thought to be involved in cumulus-oocyte matrix formation in mice because of its ability to bind to both hyaluronic acid (HA) and the heavy chain of inter-α-trypsin inhibitor (IαI) stabilizing the covalent bond between the two [Richards et al., 2002]. IαI has been reported in the porcine endometrium and hypothesized to assist in attachment of the conceptus to the uterine surface by stabilizing the uterine epithelial surface glycocalyx [Geisert and Yelich, 1997]. It is possible that conceptus IL-1β production may initially stimulate TSG-6 production during conceptus elongation and

induces COX-2 expression for continued stimulation of TSG-6 through placental $PGE_2$ release.

The significance of the inflammatory response induced via IL-1β lies in the effects IL-1β may have on the regulation of the maternal T helper cell (TH) population at the fetal-maternal interface allowing a permissive response to conceptus antigens. In mice, shifting from predominately a TH1 population, which is not compatible with pregnancy [Raghupathy, 1997], to a TH2 population, is generally associated with successful pregnancies [Wegmann et al., 1993]. TH2 proliferation in mice requires IL-1 expression by antigen-presenting cells [Weaver et al., 1988] and recurrent pregnancy loss in women has been linked to TH1-type immunity to trophoblastic antigens due to polymorphisms in the IL-1β promoter region of the mother [Wang et al., 2002] suggesting that IL-1β may be involved in regulating a maternal immune response which is permissive to conceptus antigens in the pig.

Utilizing quantitative real-time RT-PCR and a commercial ELISA assay we have clearly demonstrated changes in porcine conceptus IL-1β gene expression, synthesis and release during rapid trophoblastic elongation and its potential signaling pathways in both the uterine endometrium and conceptus. Based on current literature and the dynamic conceptus IL-1β gene expression and synthesis temporally and spatially associated with IL-1RT1 and IL-1RAP gene expression in the uterine endometrium, we suggest IL-1β is an imperative conceptus signaling component required for the establishment of a successful pregnancy in the pig.

# Chapter V.

## Summary and Conclusion

In the pig, the most notable pre-attachment conceptus developmental phenomena occurs on day 11-12 of gestation when a 9-10 mm spherical conceptus rapidly transforms into a long filamentous thread (> 150 mm) within 2-3 h [Geisert et al., 1982a]. Due to development of a non-invasive, diffuse, epitheliochorial type of placenta, rapid trophoblastic elongation is essential for individual littermates to acquire ample placental-uterine surface contact to attain adequate nutrition necessary to remain viable throughout gestation [Stroband and Van der Lende, 1990]. The limited time frame between rapid trophoblastic elongation and implantation represents the period when the majority of embryonic loss occurs in the pig [Pope, 1994].

Characterization of the specific subset of genes regulating peri-implantation conceptus development and trophoblastic elongation in the pig provides valuable information concerning key developmental events essential to embryonic survival during and after trophoblastic elongation. Determining and understanding the functional roles of genes involved with regulating rapid trophoblastic elongation and the ensuing effects their expression has on maternal recognition of pregnancy, attachment, and the maintenance of a successful pregnancy in the pig could result in future applications to manipulate reproduction in the pig.

As presented previously, information regarding genes critical to development in early pig pregnancy is limited. Utilization of suppression subtractive hybridization in the present investigation allowed isolation of candidate genes expected to be differentially expressed during rapid trophoblastic elongation. Several mRNAs identified during this

endeavor had not been previously characterized during early porcine conceptus development. SAHH, HSC70, IL-1β and the novel OSU-T1-50 were confirmed to be differentially expressed using one-step real-time RT-PCR. Real-time RT-PCR proved to be an extremely sensitive and effective method to quantify differences in mRNA expression between varying developmental morphologies.

Of specific interest in the present study was that significant sequence homology for OSU-T1-50 was not contained in either the non-redundant or EST GenBank database of NCBI. Of the 702 bp sequence BLAST searched, a 209 bp region contained 87% homology to uncharacterized sequence information gained during the sequencing of the human genome. Gene expression changes for OSU-T1-50 are interesting due to the dramatic (100-fold) increase in gene expression occurring during the onset and continuation of trophoblastic elongation. These data indicate that OSU-T1-50 is likely highly involved with the mechanisms controlling rapid trophoblastic elongation in the pig. It would be prudent to make efforts to further determine gene and protein expression for this novel molecule throughout development as well as the possible mechanisms through which it acts.

HSC70 may be of crucial developmental importance as noted by its near 10-fold increase in gene expression in filamentous conceptuses compared to earlier morphological stages. Neurulation, gastrulation and early somite development in the inner cell mass begin coincidentally during elongation of the trophoblast on day 12 of gestation in the pig. Increased mRNA for HSC70 has been reported during neurulation in Xenopus [Lang et al., 2000] and chick [Rubio et al., 2002] embryos. Using Drosophila as a model, Elefant and Palter [1999] have shown interesting effects of negative mutations

of HSC70 causing both developmental defects and lethality. Expression of two mutant HSC70 transcripts, endoplasmic reticulum hsc3 and cytoplasmic hsc4, in the nervous system of developing larvae resulted in severe behavioral abnormalities and subsequent death. Drosophila expressing mutant hsc4 in mesoderm/muscle cells had severe muscle pattern defects which was not noted in individuals expressing mutant hsc3 indicating that the cytoplasmic HSC70 has developmental impacts on muscle development. The transcript identified in the pig conceptus through SSH has not been classified as a cytoplasmic or endoplasmic reticulum HSC70. Regardless, it is interesting that the pig conceptus has increased HSC70 gene expression corresponding to the time of mesodermal outgrowth and somite development. The onset of neurulation in the pig conceptus at this time may also be dependant upon HSC70 gene expression. Using TUNEL and anti-HSC70 immunofluorescence, Rubio et al. [2002] have shown that HSC70 shows up predominantly in areas that have a high incidence of apoptosis but is not present in dying cells in the neurulating chick embryo. They confirmed this finding by showing that HSC70 antisense oligonucleotide treatment of neurulating embryos both *in vitro* and *in ovo* resulted in a higher incidence in apoptosis detected using TUNEL.

Gene expression of SAHH was similar between spherical, ovoid and tubular conceptuses, however, there was nearly a 7-fold increase in filamentous conceptuses. SAHH is intricately involved in biochemical pathways regulating the use of folates for DNA methylation as well as RNA and DNA repair [Cantoni, 1975; Fenech, 2001]. Folates have long been known as essential requirements for developing embryos, predominately during neurulation [Van der Put, 2000]. Increased maternal folate binding

protein activity in the pig during the period of trophoblastic elongation indicates a folate requirement at this stage of gestation [Vallet et al., 1998].

Deletion of the SAHH gene through the nonagouti ($a^x$) mutation in mice is characterized by embryonic death prior to implantation [Miller et al., 1994]. Addition of an inhibitor to SAHH, 3-deazaaristeromycin, inhibits inner cell mass proliferation and differentiation during in vitro development of non-mutant embryos [Miller et al., 1994]. The nonagouti ($a^x$) mutation may limit the ability of SAHH to function as a protective enzyme known for preventing nuclear accumulation of SAH [Hershfield and Kredich, 1978]. Accumulation of SAH, which has been shown to be embryotoxic in rats, is likely due to the reverse hydrolysis of homocysteine to SAH reducing the SAM/SAH ratio to a point where methylation reactions are inhibited [Vanaerts et al. 1994].

As in other species, SAHH is likely a biological regulator of the SAM/SAH ratio controlling the occurrence of transmethylation reactions to the degree of which they are necessary for successful porcine conceptus development. Establishment of the maternal-fetal interface is extremely competitive among littermates in early swine gestation. It is possible that increased expression of SAHH by conceptuses who elongate first may serve a protective function at the level of the nucleus maintaining the appropriate SAM/SAH ratio necessary for development to occur. However, increased SAHH expression by advanced conceptuses suggests increased homocysteine release into the uterine lumen would occur that may have an embryo-toxic effect on neighboring conceptuses lagging in development and not producing adequate amounts of SAHH.

IL-1β was the most predominant clone sequenced during SSH. Changes in conceptus IL-1β gene expression and ligand release in the uterine lumen dramatically

increase during the process of elongation. However the dramatic decline in IL-1β mRNA synthesis and protein secretion following elongation indicates that the necessity for IL-1β signaling is very short lived. Interestingly, the increase and decrease of IL-1β synthesis and secretion by the porcine conceptus occurs simultaneously with onset and completion of trophoblastic elongation. Furthermore, IL-1β signaling is temporally and spatially associated with the transient increase in epithelium IL-1RT1 and IL-1RAP gene expression. IL-1β has been suggested to be involved in implantation and conceptus uterine cross-talk in multiple species [Takacs and Kauma, 1996; Kruessel et al., 1997; Lindhard et al., 2002].

Repeated injections of IL-1Rant in pregnant mice prior to implantation causes implantation failure [Simon et al., 1994a] indicating the importance of IL-1β signaling. However, gene knockout of IL-1RT1 results only in a slightly reduced litter size in mice [Abbondanzo et al., 1996]. It is assumed that receptors other than IL-1RT1 are capable of mediating an IL-1β biological action.

In addition to the uterine epithelium, this transient pattern of conceptus IL-1β gene expression and protein secretion is also temporally and spatially associated with IL-1RT1 and IL-1RAP gene expression in elongated conceptuses (greatest on days 12-15). Conceptus expression for IL-1RT1 and IL-1RAP is greater in filamentous conceptuses compared to earlier morphologies and tends to decline by day 18 conceptuses.

We have clearly documented the establishment of an IL-1β communication pathway between the conceptus and uterus during the time of trophoblastic elongation in the pig. The increased expression of IL-1RT1 and IL-1RAP by both elongated conceptuses and endometrium form pregnant gilts on day 12 of gestation suggests that

conceptus IL-1β may simultaneously serve more than one dynamic function. The potential functions of IL-1β are vast. IL-1β is known to induce aromatase gene expression and estrogen production [Nestler, 1993], phospholipase A2 gene expression [Kol et al., 1999], and cycoloxygenase-2 gene expression [Huang et al., 1998] in reproductive tissues of various species. All of these factors are known to be essential regulators of conceptus development in the pig.

The inflammatory response invoked by the conceptus, most likely through the actions of the pro-inflammatory cytokine IL-1β, is possibly the most fundamental and essential mechanism caused by conceptus IL-1β signaling to the uterine endometrium. Both estrogen and prostaglandin $E_2$ are anti-inflammatory factors that could regulate a short-lived immune response to the conceptus. While a uterine immunological response to the conceptus is imperative, the transient presence of IL-1β is accompanied by conceptus secretion of estrogen and prostaglandin $E_2$. The significance of the inflammatory response induced via IL-1β, lies in the effects IL-1β may have on the regulation of the maternal T helper cell (TH) population at the fetal-maternal interface allowing a permissive response to conceptus antigens. Shifting the maternal T-cell repertoire from predominately a TH1 population to a TH2 population is generally associated with successful pregnancies [Wegmann et al., 1993; Raghupathy, 1997]. TH2 proliferation in mice requires IL-1 expression by antigen-presenting cells [Weaver et al., 1988] and recurrent pregnancy loss in women has been linked to TH1-type immunity to trophoblastic antigens due to a polymorphism in the IL-1β promoter region of the mother [Wang et al., 2002]. These data suggest that IL-1β may be involved in regulating a maternal immune response which is permissive to conceptus antigens in the pig. The

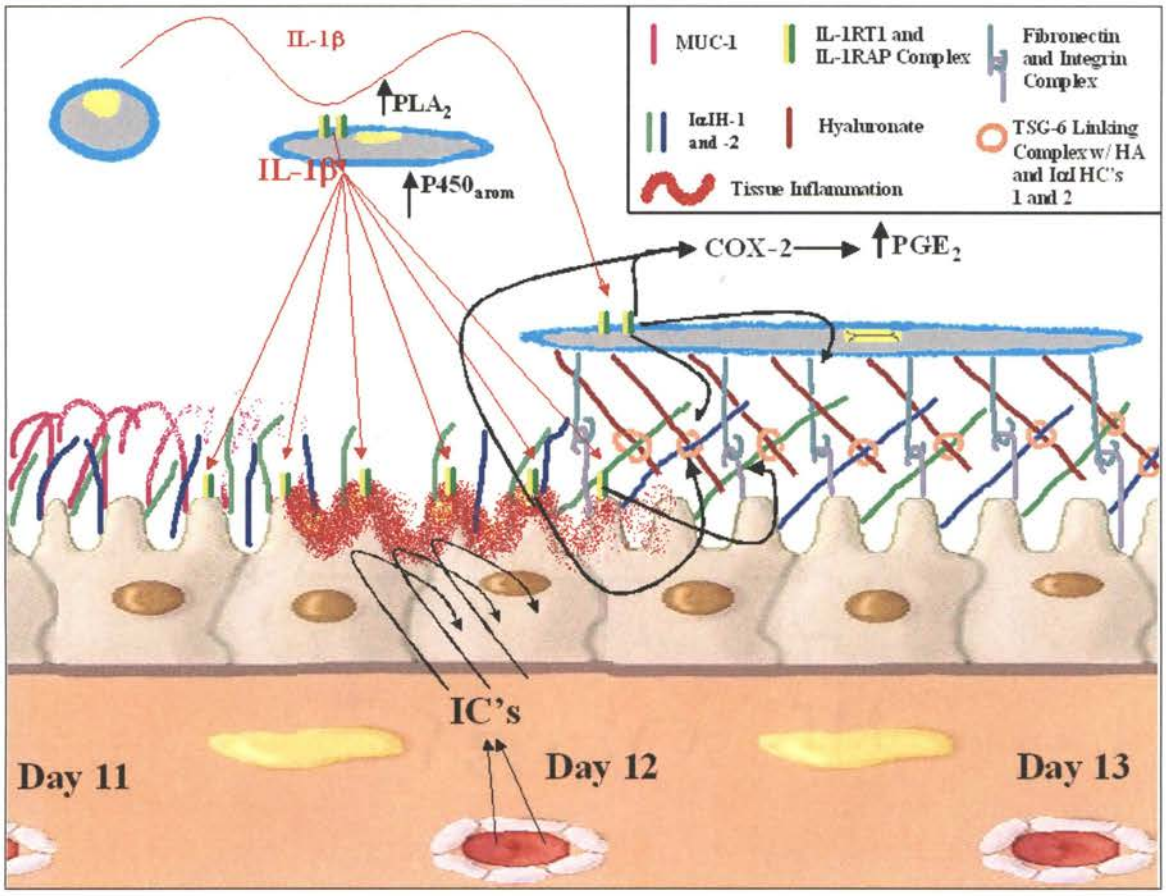
necessity for the appropriate maternal TH cell repertoire at the site of attachment is to insure the maternal immune response is permissive to the presence of a conceptus expressing paternal alloantigens [Mellor and Munn, 2000]. During the initial contact between the conceptus and endometrium, inflammation is unavoidable, largely in part to IL-1β. However, the induced inflammation is not necessarily devastating to the conceptus as it stimulates counter regulatory responses limiting immunological damage to the conceptus while encouraging shifts in the maternal T cell population lasting throughout pregnancy [Mellor and Munn, 2000].

Formation of a conceptus-endometrium extracellular matrix follows uterine immunological stimulation. IL-1β may also be intricately involved regulating the courtship between the conceptus and uterine endometrium. Yang et al. [1999] demonstrated the ability of IL-1β to promote the production of extracellular matrix in cultured human peritoneal mesothelial cells by inducing the expression of fibronectin, a cell surface integrin-binding factor. The expression of fibronectin by porcine conceptuses is thought to be partially involved in conceptus attachment in the pig [Jaeger et al., 2001]. Tumor necrosis factor (TNF)-stimulated gene (TSG)-6 gene expression is inducible by TNFα and IL-1β [Lee et al., 1992] as well as $PGE_2$ [Fujimoto et al., 2002]. TSG-6, which is strongly anti-inflammatory [Wisniewski et al., 1996], is thought to be involved in cumulus-oocyte matrix formation in mice because of its ability to bind to both hyaluronic acid (HA) and the heavy chain of inter-α-trypsin inhibitor (IαI) stabilizing the covalent bond between the two [Richards et al., 2002]. IαI has been reported in the porcine endometrium and hypothesized to assist in attachment of the conceptus to the uterine surface by stabilizing the uterine epithelial surface glycocalyx

[Geisert and Yelich, 1997]. IαI heavy chains -1, -2 and -4 are present in the uterine endometrium of pigs although gene expression is not different between days and pregnancy status (Geisert et al., unpublished data). However, both bikunin [Hettinger et al., 2001] and tissue kallikrein [Vonhamme et al., 1999] gene expression and enzymatic activity increase in the porcine endometrium during days 12 to 18 of pregnancy, and may be the responsible for the biological activity of the IαI heavy chains. It is possible conceptus IL-1β production may initially stimulate TSG-6 production during conceptus elongation and induce COX-2 expression for continued stimulation of TSG-6 through placental $PGE_2$ release. It is evident that IL-1β gene expression is elevated in 6 mm spherical conceptuses as even they express several hundred fold greater IL-1β compared to day 15 or 18 conceptuses. The stimuli inducing IL-1β gene expression in porcine conceptuses is open to speculation, however, based on current literature, the effect of IL-1β signaling by porcine conceptuses is more discernable and should be discussed. A proposed hypothetical model predicting the actions of IL-1β signaling during day 12 of gestation is presented in Figure 5.1.

Through the utilization of SSH, we have detected several genes that may serve vital roles in early conceptus development, attachment and the establishment of a successful pregnancy in the pig. The use of quantitative real-time RT-PCR confirmed the gene expression of IL-1β, SAHH, HSC70 and OSU-T1-50 as being differentially expressed during the period of rapid trophoblastic elongation contributing important information towards understanding the mechanisms involved with this essential biological event in the pig.

**Figure 5.1.** Proposed model to depict the interactions between the conceptus and the uterine endometrium occurring as a result of IL-1β on days 11 to 13 of gestation. Typically the degradation of mucin-1 (MUC-1) on the surface epithelium on day 10 to 11 of gestation indicates the opening of the implantation window and the capability of the uterine epithelium to receive conceptus signals, particularly IL-1β. Interleukin-1 (IL-1) receptor type 1 (IL-1RT1) and IL-1 receptor accessory protein (IL-1RAP) complexes appear in the elongating conceptus and may result in increased expression of phospholipase A2 ($PLA_2$), P450 aromatase ($P450_{arom}$), cyclooxygenase-2 (COX-2), tumor necrosis factor stimulated gene-6 (TSG-6), as well as the extracellular matrix forming components, fibronectin and integrins. The increase of both $PLA_2$ and $P450_{arom}$ may partially regulate conceptus elongation and growth. $PLA_2$ functions to cleave arachidonic acid from the phospholipid bilayer, possibly increasing membrane fluidity essential for trophoblastic elongation to occur. $P450_{arom}$ is an essential enzyme converting androgen into estrogen, the maternal recognition signal in the pig which may also augment conceptus growth through estrogen receptor β mediated pathways. The transient increase in both IL-1RT1 and IL-1RAP in the uterine epithelium on day 12, simultaneous with peak IL-1β synthesis and release from the conceptus suggest a specific and necessary communication pathway. IL-1β stimulation of endometrial tissue likely results in the recruitment of immune cells (IC's), as well as the stimulation of factors similar to what would be stimulated in the conceptus such as COX-2, TSG-6, fibronectin and integrins. The primary function of IL-1β signaling from the conceptus is the recruitment of maternal IC's to the site of conceptus attachment. The immunological attack by epithelial IC's provoked by the conceptus is quickly called off with the dramatic reduction of endomtrial IL-1RT1 and IL-1RAP expression by day 13 coupled with the increase in anti-inflammatory TSG-6, as well as COX-2 mediated increase in prostaglandin $E_2$ ($PGE_2$), which is also highly anti-inflammatory. This model proposes that the recruitment of IC's to the site of implantation in the pig is two-fold. First, to regulate the differentiation of T helper cells shifting the T cell repertoire from predominantly TH1 to TH2. Secondly, this short exposure of conceptuses paternal antigens will prevent a sustained immunological attack from the maternal immune system throughout the remainder of gestation. This sort of "crying wolf" by the conceptus ensures that similar, more sustained immunological responses at the site of implantation are prevented. Furthermore the expression of TSG-6 by both the conceptus and uterine epithelium functions to promote the preservation and buildup of the extracellular matrix by linking inter-α–trypsin inhibitor (IαI) heavy chains (H) 1 and 2, which are constitutively expressed by the epithelium, with hyaluronate on the conceptus trophectoderm. Also, IL-1β induction of integrin and fibronectin expression in both the conceptus and epithelium further stabilizes the extracellular matrix between the conceptus trophectoderm and uterine epithelium. Collectively, these events result in the establishment of immunologically safe contact between the conceptus and dam required for successful pregnancy.


IL-1β
PLA2
IL-1β
P450arom
MUC-1
IL-1RT1 and IL-1RAP Complex
Fibronectin and Integrin Complex
IαIH-1 and -2
Hyaluronate
TSG-6 Linking Complex w/ HA and IαI HC's 1 and 2
Tissue Inflammation
COX-2
PGE2
IC's
Day 11
Day 12
Day 13

Utilizing quantitative real-time RT-PCR and a commercial ELISA assay we have clearly demonstrated changes in porcine conceptus IL-1β gene expression, synthesis and release during rapid trophoblastic elongation as well as the potential signaling pathways in both the uterine endometrium and conceptus. Based on current literature and due to the dynamic conceptus IL-1β gene expression and synthesis temporally and spatially to IL-1RT1 and IL-1RAP gene expression in the uterine endometrium, we suggest IL-1β is an imperative maternal signaling component required for the establishment of a successful pregnancy in the pig.

This investigation has resulted in the isolation and characterization of expression patterns for numerous genes in the pig conceptus which appear to mediate trophoblastic elongation and establishment of pregnancy. While the proteins translated by the genes indicated in this study are ideal targets for understanding the underlying mechanisms controlling trophoblastic elongation and the establishment of pregnancy it would be precocious to conclude the exact functions of the indicated genes without further investigation. Techniques such as immunocytochemistry, fluorescent *in situ* hybridization, and protein antibody arrays would lend great support identifying the location and expression patterns of proteins encoded by the genes characterized in this study. However, understanding the expression patterns and localization of translated proteins will not significantly add to our understanding of the necessity of the appropriate gene expression and its impacts on trophoblastic elongation and establishment of pregnancy in the pig.

Recently, Fire et al. [1998] injected double-stranded RNA into *C. elegans* resulting in the inhibition of endogenous gene expression of the specific gene

corresponding to the sequence of the injected dsRNA. This intriguing discovery is now termed RNA interference (RNAi). RNAi is mainly the result of two key enzymes endogenously present in all cells. While it is a naturally occurring, the exact explanation for this phenomena is not fully understood, it is thought to occur as a mechanism responsible for maintaining the integrity of an individual organisms genome. The two major enzymes responsible for creating RNAi, DICER and RISC, function to degrade dsRNA in the cell and destroy normally expressed mRNA in the cell containing the identical sequence, respectively [see review Kennedy, 2002]. The utilization of RNAi to study early development in the pig has recently been demonstrated by Cabot and Prather [2003]. These researchers injected dsRNA into single cell and two-cell porcine embryos and evaluated the early effects of silencing the effects of a specific gene. However the impact of RNAi via dsRNA injections typically only last for 4 to 5 cell divisions and the effects of injecting dsRNA this early may not be detectable by day 11 to 12 of gestation. Recently, Tuschl [2002] described using plasmid DNA transfection to cause the permanent endogenous expression of dsRNA in mammalian cells. Using this approach, it may be possible to expand RNAi to silence genes that are suspected to be involved with trophoblastic elongation and maternal recognition of pregnancy on day 11 to 12.

Because of the extensive cost associated with genetically deleting genes in large mammalian species, RNAi provides a much more cost effective means to systematically assess the phenotypic consequence when expression for a specific gene is inhibited. By doing so, one could irrefutably determine the mechanism and effects that expression of a specific gene has in porcine conceptus development. This knowledge would provide research directions for investigators interested in controlling this aspect of pig

reproduction with hopes of reducing the high embryonic mortality rate in the pig and subsequently increasing litter size.

# Appendix

## RNA Extraction using RNaid Kit

Solutions:

- Denaturing Solution(DS):
  4 M Guanidine Thiocyanate
  25 mM Sodium Citrate pH 7
  .5 % Sarcosyl

- Active Denaturing Solution:
  5 ml Denaturing Solution
  36 µl .1 M, 2-beta mercaptoethanol

Procedure:

1. Add 500 µl Active DS, to embryo prep, vortex, then add in the following order:
   a. 500 µl phenol (pH 5.1), vortex,
   b. 70 µl 2 M sodium acetate, pH 4.0, vortex,
   c. 140 µl chloroform:iso-amyl-alcohol (49:1, fresh dilution), vortex
2. Final vortex (15 sec.), cool on ice for 15 minutes
3. Spin @ 15,000 rpm for 20 min. at 4° C
4. Recover lysate ( ~ 400 µl, upper clear solution)
   Note: Recover all lysates with a long tipped pipette tip to prevent contamination
5. Add 500 µl chloroform:iso-amyl-alcohol, vortex.
6. Centrifuge at 15,000 rpm for 10 minutes at 4° C
7. Recover lysate ( ~ 250 µl upper clear solution)

*Prepare .5 ml RNaid washing conc. + .5 ml 100 % EtOH per tube being extracted*

8. Add 7 µl of glass beads (RNaid Kit, BIO101, Catalog # 1007-200), vortex lightly
9. Gently agitate at RT for 10- 25 minutes
10. Centrifuge at 15,000 rpm for 5 minutes at RT
11. Discard supernatant.
12. Add 300 µl washing solution mix (50:50 EtOH : Rnaid Wash conc.)
13. Mix gently with pipette until pellet is fully broken
14. Centrifuge at 15,000 rpm for 3 min at RT
15. Discard supernatant

**Repeat steps 12-15 at least once, up to three times (using 200 µl wash) to ensure all salt is discarded**

16. Carefully remove all liquid from the tube
17. Dry pellet for 5 minutes
18. Add 50 µl DEPC H2O
19. Break up pellet via pipetting action
20. Incubate at 56° C for 5 min in an air incubator
21. Centrifuge at Max speed for 5 min. at RT
22. Remove upper 50 µl and discard remaining glass bead pellet
23. Centrifuge 50 µl at max speed for 5 min at RT
24. Remove upper 40 µl and use for RNA stock. STORE at –80° C.
25. Save bottom 10 µl to run electrophoresis gel.
26. Add 1 µl of RNA stock to 49 µl of TE Buffer to spectrophotometrically estimate quantity.

## Suppression Subtractive Hybridization

### RNA analysis

After total and poly A+ RNA isolation, examine the RNA's integrity by electrophoresing samples on a agarose/EtBr gel. Total mammalian RNA typically exhibits two bright bands, which correspond to ribosomal 28S and 18S RNA at ~4.5 and 1.9 kb, respectively, with a ratio of intensities of about 1.5–2.5:1. If the ratio is <1:1, test all RNA isolation reagents for RNases or find another source of tissue or cells for RNA isolation. Mammalian poly A+ RNA appears as a smear from 0.5–12 kb with weak ribosomal RNA bands at approximately 1.9 and 4.5 kb. Poor quality RNA will cause high background in the subtraction procedure and should not be used.

**Reagents:**

**First Strand Synthesis**

AMV Reverse Transcriptase (20 units/μl)

cDNA Synthesis Primer (10μM)—(5'-TTTTGTACAAGCTT$_{30}$N$_1$N-3')

5X First-Strand Buffer

| | | |
|---|---|---|
| 250 | mM | Tris-HCl (pH 8.5) |
| 40 | mM | $MgCl_2$ |
| 150 | mM | KCl |
| 5 | mM | Dithiothreitol |

**Second Strand Synthesis**

20X Second-Strand Enzyme Cocktail

(DNA polymerase I, 6 units/μl; RNase H, 0.25 units/μl; E. coli DNA ligase, 1.2 units/μl)

5X Second-Strand Buffer

| | | |
|---|---|---|
| 500 | mM | KCl |
| 50 | mM | Ammonium sulfate |
| 25 | mM | $MgCl_2$ |
| 0.75 | mM | β-NAD |
| 100 | mM | Tris-HCl (pH 7.5) |
| 0.25 | mg/mL | BSA |

T4 DNA Polymerase (3 units/μl)

**Rsa I Digestion**

10X Rsa I Restriction Buffer

Rsa I (10 units/μl)

### Adaptor Ligation

T4 DNA Ligase (400 units/μl; contains 3mM ATP)

5X DNA Ligation buffer

| | | |
|---|---|---|
| 250 | mM | Tris-HCl (pH 7.8) |
| 50 | mM | $MgCl_2$ |
| 10 | mM | DTT |
| 0.25 | mg/mL | BSA |

Adaptor 1 (10 μM)
(5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3')
Adaptor 2R (10μM)
(5'-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3')

**Hybridization**

Hybridization Buffer
Dilution Buffer

| | | |
|---|---|---|
| 20 | mM | HEPES-HCl (pH 8.3) |
| 50 | mM | NaCl |
| 0.2 | mM | EDTA (pH 8.0) |

**PCR Amplification**

| | |
|---|---|
| PCR Primer 1 (10 μM) | (5'-CTAATACGACTCACTATAGGGC-3') |
| Nested PCR Primer 1 | (5'-TCGAGCGGCCGCCCGGGCAGGT-3') |
| Nested PCR Primer 2R | (5'-AGCGTGGTCGCGGCCGAGGT-3') |

**Control Reagents**

| | |
|---|---|
| G3PDH 5' Primer | (5'-ACCACAGTCCATGCCATCAC-3') |
| G3PDH 3' Primer | (5'-TCCACCACCCTGTTGCTGTA-3') |

**General Reagents**

dNTP mix (10 mM each of dATP, dCTP, dGTP, dTTP)
20X EDTA/Glycogen Mix (0.2 M EDTA; 1 mg/mL glycogen)
$NH_4Oac$ (4 M)
Sterile $H_2O$
70% EtOH
95% EtOH
Phenol:Chloroform:Isoamyl Alcohol (25:24:1)

1. Melt Phenol
2. Equilibrate with an equal volume of sterile TNE buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA)
3. Incubate the mixture at room temperature for 2-3 hours.
4. Remove and discard the top layer.
5. Add an equal volume of chloroform:isoamyl alcohol (24:1) to the remaining layer. Mix thoroughly. Remove and discard the top layer.
6. Store the bottom layer of phenol:chloroform:isoamyl alcohol at 4°C away from light for a maximum of two weeks.

Chloroform:isoamyl alcohol (24:1)

**Protocol:**

**A. First-Strand cDNA Synthesis**

1. For each tester and driver, combine the following components in a sterile 0.5-ml microcentrifuge tube. (Do not use a polystyrene tube):

| | |
|---|---|
| Poly A+ RNA (2 μg) | 2–4 μl |
| cDNA Synthesis Primer (10 μM) | 1 μl |

2. Incubate the tubes at 70°C in a thermal cycler for 2 min.
3. Cool the tubes on ice for 2 min.
4. Briefly centrifuge the tubes.
5. Add the following to each reaction tube:

| | |
|---|---|
| 5X First-Strand Buffer | 2 μl |
| dNTP Mix (10 mM each) | 1 μl |
| Sterile H2O | 1 μl |
| AMV Reverse Transcriptase (20 units/μl) | 1 μl |

6. Gently vortex and briefly centrifuge the tubes.
7. Incubate the tubes at 42°C for 1.5 hr in an air incubator.
   **Note**: Do not use a water bath or thermal cycler (unless it has a heated lid). Evaporation could reduce the reaction mixture volume, reducing the reaction efficiency.
8. Place the tubes on ice to terminate first-strand cDNA synthesis and immediately proceed to Section B.

**B. Second-Strand cDNA Synthesis**

Perform the following procedure with each first-strand tester and driver cDNA.

1. Add the following components to the first-strand synthesis reaction tubes (containing 10 μl):

| | |
|---|---|
| Sterile $H_2O$ | 48.4 μl |
| 5X Second-Strand Buffer | 16.0 μl |
| dNTP Mix (10 mM) | 1.6 μl |
| 20X Second-Strand Enzyme Cocktail | 4.0 μl |

2. Mix contents and briefly spin tubes. The final volume should be 80 μl.
3. Incubate tubes at 16°C (water bath or thermal cycler) for 2 hr.
4. Add 2 μl (6 units) of T4 DNA Polymerase. Mix contents well.
5. Incubate the tube at 16°C for 30 min in a water bath or thermal cycler.
6. Add 4 μl of 20X EDTA/Glycogen Mix to terminate second-strand synthesis.
7. Add 100 μl of phenol:chloroform:isoamyl alcohol (25:24:1).
8. Vortex thoroughly, and centrifuge the tubes at 14,000 rpm for 10 min at room temperature.
9. Carefully remove the top aqueous layer and place in a clean (sterile) 0.5-ml microcentrifuge tube. Discard the interphase and lower phase.
10. Add 100 μl of chloroform:isoamyl alcohol (24:1) to the aqueous layer.
11. Repeat steps 8 and 9.
12. Add 40 μl of 4 M NH4OAc and 300 μl of 95% ethanol.
    **Note:** Proceed immediately with precipitation. **Do not** store tubes at -20°C.

Prolonged exposure to this temperature can precipitate unwanted salts.

13. Vortex thoroughly and centrifuge the tube at 14,000 rpm for 20 min at room temperature.
14. Remove the supernatant carefully.
15. Overlay the pellet with 500 µl of 70% ethanol.
16. Centrifuge the tube at 14,000 rpm for 10 min.
17. Remove supernatant carefully and repeat steps 15-16 twice.
18. Carefully remove supernatant and air-dry the pellet for about 10 min to evaporate residual ethanol.
19. Dissolve precipitate in 50 µl of $H_2O$.
20. Transfer 6 µl to a fresh microcentrifuge tube. Store this sample at –20°C until after *Rsa I* digestion for agarose gel electrophoresis to estimate yield and size range of ds cDNA products synthesized (refer to Clonetech User Manual, Sections V.A and V.B for expected results).
21. Proceed to Section C.

**C. Rsa I Digestion**

Perform the following procedure with each experimental ds tester and driver cDNA. This step generates shorter, blunt-ended ds cDNA fragments, which are optimal for subtraction and necessary for adaptor ligation in Section F.

1. Add the following reagents into the tube:

| | |
|---|---|
| ds cDNA | 43.5 µl |
| 10X Rsa I Restriction Buffer | 5.0 µl |
| Rsa I (10 units/ µl) | 1.5 µl |

2. Mix by vortexing and centrifuging briefly.
3. Incubate at 37°C for 3-4 hr.
4. Set aside 5 µl of the digest mixture to analyze the efficiency of Rsa I digestion as described in Section V.B.
5. Add 2.5 µl of 20X EDTA/Glycogen Mix to terminate the reaction.
6. Add 50 µl of phenol:chloroform:isoamyl alcohol (25:24:1).
7. Vortex thoroughly.
8. Centrifuge the tubes at 14,000 rpm for 10 min to separate phases.
9. Remove the top aqueous layer and place in a clean 0.5-ml tube.
10. Add 50 µl of chloroform:isoamyl alcohol (24:1) and vortex thoroughly.
11. Centrifuge the tubes at 14,000 rpm for 10 min to separate phases.
12. Remove the top aqueous layer and place in a clean 0.5-ml tube.
13. Add 25 µl of 4 M NH4OAc and 187.5 µl of 95% ethanol.
    **Note:** Proceed immediately with precipitation. **Do not** store tubes at -20°C. Prolonged exposure to this temperature can precipitate unwanted salts.
14. Vortex the mixture thoroughly.
15. Centrifuge the tubes for 20 min at 14,000 rpm at room temperature.
16. Remove the supernatant carefully.
17. Gently overlay the pellets with 200 µl of 70% ethanol.
18. Centrifuge at 14,000 rpm for 5 min.
19. Remove supernatant carefully and repeat steps 17-18 twice.

20. Remove the supernatant carefully and air-dry the pellets for 5–10 min.
21. Dissolve the pellet in 6.5 µl of H2O and store at –20°C.

    These 6.5- µl samples of *Rsa I* digested cDNA will serve as your **experimental driver cDNA**. In the next section, these samples will be ligated with adaptors to create your tester cDNAs for forward, control, and reverse (if applicable) subtractions.
22. Spectrophotometrically estimate the yield of each digested cDNA. Yield of each sample should be equal and having a minimum of 300 ng/µl (dilute most concentrated samples to have equal concentration as the least concentrated sample as long as the minimum concentration remains above 300 ng/µl.

23. Check your *Rsa* I digested cDNA from Step 4 using agarose/EtBr gel electrophoresis, as described in Section B (refer to Clonetech User Manual, Section V.B for expected results). Proceed to Section D to finish preparing your experimental **tester cDNAs**.

## D. Adaptor Ligation

Each cDNA is aliquotted into two separate tubes: one aliquot is ligated with Adaptor 1 (Tester 1-1 and 2-1), and the second is ligated with Adaptor 2R (Tester 1-2 and 2-2). After the ligation reactions are set up, portions of each tester tube are combined so that the cDNA is ligated with both adaptors (Unsubtracted tester control 1-c and 2-c). Each Unsubtracted tester control cDNA serves as a positive control for ligation, and later serves as a negative control for subtraction. **Note:** Through the rest of the procedure, be sure to label tubes using the nomenclature described in this User Manual. Labeling the tubes of intermediate products with the step number in which they were created may prove helpful as well. **Adaptors will not be ligated to the driver cDNA.**

1. Dilute 1 µl of each Rsa I-digested **experimental** cDNA (Step E.21) with 5 µl of sterile H2O.

**Prepare your adaptor-ligated tester cDNA:**

2. Prepare a ligation Master Mix by combining the following reagents in a 0.5-ml microcentrifuge tube. To ensure that you have sufficient Master Mix, prepare enough for all ligations plus one additional reaction.

   per rxn:

| | |
|---|---|
| Sterile H2O | 3 µl |
| 5X Ligation Buffer | 2 µl |
| T4 DNA Ligase (400 units/µl) | 1 µl |

   **Note:** The ATP required for ligation is in the T4 DNA Ligase (3 mM initial, 300 µM final).

3. For each experimental tester cDNA and for the control skeletal muscle tester cDNA, combine the reagents in Table I in the order shown in 0.5-ml microcentrifuge tubes. Pipet mixture up and down to mix thoroughly.

**TABLE I: SETTING UP THE LIGATION REACTIONS**

| Tube # | 1 | 2 |
|---|---|---|
| | Tester 1-1 | Tester 1-2* |
| **Component:** | (µl) | (µl) |
| Diluted Tester cDNA | 2 | 2 |
| Adaptor 1 (10 mM) | 2 | - |
| Adaptor 2R (10 mM) | - | 2 |
| Master Mix | 6 | 6 |
| **Final Volume** | 10 | 10 |

***Use the same setup for Tester 2-1 and 2-2.**

4. In a fresh microcentrifuge tube, mix 2 µl of Tester 1-1 and 2 µl of Tester 1-2. After ligation is complete, this will be your Unsubtracted tester control 1-c. Do the same for each additional experimental tester cDNA. After ligation, approximately 1/3 of the cDNA molecules in each Unsubtracted tester control tube will bear two different adaptors.
5. Centrifuge tubes briefly, and incubate at 16°C overnight.
6. Stop ligation reaction by adding 1 µl of EDTA/Glycogen Mix.
7. Heat samples at 72°C for 5 min to inactivate the ligase.
8. Briefly centrifuge the tubes. Preparation of your experimental and control **Adaptor-Ligated Tester cDNAs** and your **Unsubtracted tester controls** is now complete.
9. Remove 1 µl from each Unsubtracted tester control (1-c, 2-c, 3-c) and dilute into 1 ml of H2O. These samples will be used for PCR (Section G).
10. Store samples at –20°C.

Perform the ligation efficiency analysis, which is described in Section V.C of the Clontech User Manual. Following the ligation of adaptors conduct this test to determine if appropriate ligation of adaptors occurred. The G3PDH primers available in the kit do not work well with pig cDNA. PCR parameters may need to be optimized. If this analysis fails, you may wish to proceed throughout the remainder of the protocol as the limiting reagents have already been used. It is possible that hybridization will work effectively even if the analysis of ligation fails.

## E. First Hybridization

**Important note:** Perform the ligation efficiency analysis (Section V.C of Clontech User Manual) **before proceeding with the hybridizations described below**. If your ligation did not work well, you should repeat the ligation before performing the hybridizations. In the following procedure, an excess of driver cDNA is added to each tester cDNA, and the samples are heat denatured and allowed to anneal. The remaining ss cDNAs (available for the second hybridization) are dramatically enriched for differentially expressed sequences, as non-target cDNAs present in the tester and driver cDNA form hybrids. **Important:** Before you begin the hybridization, **make sure the 4X hybridization buffer has been allowed to warm to room temperature for at least 15–20 min**. Be sure there is no visible pellet or precipitate before using the buffer. If necessary, heat the buffer at 37°C for ~10 min to dissolve any precipitate.

1. For each of the experimental subtraction, combine the reagents in Table II (below) in 0.5-ml tubes in the order shown.
2. Overlay samples with one drop of mineral oil and centrifuge briefly.
3. Incubate samples in a thermal cycler at 98°C for 1.5 min.
4. Incubate samples at 68°C for 8 hr,* then proceed **immediately** to Section F.
   * Samples may hybridize for as little as 6 hr, or as much as 12 hr. **Do not let the incubation exceed 12 hours.**

**TABLE II: SETTING UP THE FIRST HYBRIDIZATION**

| Component | Hybridization Sample 1 (µl) | Hybridization Sample 2 (µl) |
|---|---|---|
| Rsa I-digested driver cDNA | 1.5 | 1.5 |
| Adaptor 1-ligated Tester 1-1 | 1.5 | - |
| Adaptor 2R-ligated Tester 1-2 | - | 1.5 |
| 4X Hybridization Buffer | 1.0 | 1.0 |
| **Final Volume** | **4.0** | **4.0** |

**F. Second Hybridization**

The two samples from the first hybridization are mixed together, and fresh denatured driver DNA is added to further enrich for differentially expressed sequences. New hybrid molecules are formed which consists of differentially expressed cDNAs with different adaptors on each end. **Important:** Do not denature the primary hybridization samples at this stage. Also, do not remove the hybridization samples from the thermal cycler for longer than is necessary to add fresh driver. Repeat the following steps for each experimental tester cDNA.

1. Add the following reagents into a sterile tube:
   Driver cDNA (Step IV.E.21) 1 µl
   4X Hybridization Buffer 1 µl
   Sterile H2O 2 µl
2. Place 1 µl of this mixture in a 0.5-ml microcentrifuge tube and overlay it with 1 drop of mineral oil.
3. Incubate in a thermal cycler at 98°C for 1.5 min.
4. Remove the tube of freshly denatured driver from the thermal cycler.

Use the following procedure to simultaneously mix the driver with hybridization samples 1 and 2 (prepared in Section IV.G; see Table II). This ensures that the two hybridization samples mix together only in the presence of freshly denatured driver.

a. Set a micropipettor at 15 µl.
b. Gently touch the pipette tip to the mineral oil/sample interface of the tube containing hybridization sample 2.
c. Carefully draw the entire sample partway into the pipette tip. Don't worry if a small amount of mineral oil is transferred with the sample.
d. Remove the pipette tip from the tube, and draw a small amount of air into the tip, creating a slight air space below the droplet of sample.

e. Repeat steps b–d with the tube containing the freshly denatured driver. The pipette tip should now contain both samples separated by a small pocket of air.
f. Transfer the entire mixture to the tube containing hybridization sample 1.
g. Mix by pipetting up and down.

5. Briefly centrifuge the tube if necessary.
6. Incubate reaction at 68°C overnight.
7. Add 200 µl of dilution buffer to the tube and mix by pipetting.
8. Heat in a thermal cycler at 68°C for 7 min.
9. Store at –20°C.

**G. PCR Amplification**

Differentially expressed cDNAs are selectively amplified during the reactions described in this section. Prior to thermal cycling, you will fill in the missing strands of the adaptors by a brief incubation at 75°C; this creates the binding site for PCR Primer 1. In the first amplification, only ds cDNAs with different adaptor sequences on each end are exponentially amplified. In the second, nested PCR is used to further reduce background and to enrich for differentially expressed sequences. A minimum of seven PCR reactions are recommended for: (1) your forward-subtracted experimental cDNA, (2) the unsubtracted tester control (1-c), (3) your reverse-subtracted experimental cDNA, (4) the unsubtracted tester control for the reverse subtraction (2-c).

**Notes:**

• All cycling parameters were optimized using a Perkin-Elmer GeneAmp PCR Systems 2400/9600. If a different type of thermal cycler is used, the cycling parameters must be optimized for that machine.

• If you do not use Advantage cDNA Polymerase Mix, you can use Taq DNA polymerase alone; however, 3–5 more cycles will be needed in primary and secondary PCR. You must also use a hot start. You can perform hot start as follows: (1) Prepare the primary PCR Master Mix without Taq Polymerase. (2) Mix PCR samples and heat the reaction mix to 75°C for 1 min. (3) Quickly add the necessary amount of Taq polymerase. (4) **Incubate the reaction at 75°C for 5 min.** (5) Perform PCR as described in step 8 below.

1. Prepare the PCR templates:
   a. Aliquot 1 µl of each diluted cDNA (i.e., each subtracted sample from Step I.F and the corresponding diluted Unsubtracted tester control from Step D) into an appropriately labeled tube.
   b. Aliquot 1 µl of the PCR control subtracted cDNA (provided in the kit) into an appropriately labeled tube.

2. Prepare a Master Mix for all of the primary PCR tubes plus one additional tube. For each reaction planned, combine the reagents in Table III in the order shown:

**TABLE III: PREPARATION OF THE PRIMARY PCR MASTER MIX**

| Reagent | Amount per* Reaction (µl) |
|---|---|
| Sterile H2O | 19.5 |
| 10X PCR reaction buffer | 2.5 |
| dNTP Mix (10 mM) | 0.5 |
| PCR Primer 1 (10 µM) | 1.0 |
| 50X Advantage cDNA Polymerase Mix | 0.5 |
| **Total Volume** | **24.0** |

* Prepare Master Mix for one additional reaction.

5. Mix well by vortexing, and briefly centrifuge the tube.
6. Aliquot 24 µl of Master Mix into each of the reaction tubes prepared in step 1.
7. Incubate the reaction mix in a thermal cycler at 75°C for 5 min to extend the adaptors. (Do not remove the samples from the thermal cycler.)
   **Note:** This step "fills in" the missing strand of the adaptors and thus creates binding sites for the PCR primers.

8. Immediately commence thermal cycling:

   - 94°C 25 sec

   27 cycles:

   - 94°C 10 sec
   - 66°C 30 sec
   - 72°C 1.5 min

9. Analyze 8 µl from each tube on a 2.0% agarose/EtBr gel run in 1X TAE buffer. (See Section II.D for expected results.) Alternatively, you can set these 8- µl aliquots aside and run them on the same gel used to analyze the secondary PCR products (step 16).
10. Dilute 3 µl of each primary PCR mixture in 27 µl of H2O. This diluted primary PCR product will be used in the PCR Select Differential Screening procedure (if applicable).
11. Aliquot 1 µl of each diluted primary PCR product mixture from Step 9 into an appropriately labeled tube.
12. **Prepare Master Mix for the secondary PCR plus one additional reaction by combining the reagents in Table IV in the order shown:**

**TABLE IV: PREPARATION OF THE SECONDARY PCR MASTER MIX**

| Reagent | Amount per reaction (µl) |
|---|---|
| Sterile H2O | 18.5 |
| 10X PCR reaction buffer | 2.5 |
| Nested PCR Primer 1 (10 mM) | 1.0 |
| Nested PCR Primer 2R (10mM) | 1.0 |
| DNTP Mix (10 mM) | 0.5 |
| 50X Advantage cDNA Polymerase Mix | 0.5 |
| **Total Volume** | **24.0** |

* Prepare Master Mix for one additional reaction.

13. Mix well by vortexing, and briefly centrifuge the tube.
14. Aliquot 24 µl of Master Mix into each reaction tube from step 10.
15. Immediately commence thermal cycling:

    10–12 cycles:
    - 94°C 10 sec
    - 68°C 30 sec
    - 72°C 1.5 min
16. Analyze 8 µl from each reaction on a 2.0% agarose/EtBr gel run in 1X TAE buffer. (See Section V.D of Clontech User Manual for expected results. The number of cycles for the secondary PCR may vary. Depending on the number of differentially expressed genes the optimal number of cycles for the 2$^{nd}$ary PCR will result distinctive bands with limited background when visualized using a 2% agarose gel.
17. Store reaction products at –20°C.

The PCR mixture is now enriched for differentially expressed cDNAs. In addition, differentially expressed transcripts that varied in abundance in the original mRNA sample should now be present in roughly equal proportions. Refer to Sections V.D and V.E (Clontech User Manual) for Analysis of Results: It is strongly recommend that you perform this subtraction efficiency test.

The uncloned subtracted mixture is an ideal hybridization probe for screening libraries of genomic DNA, full-length cDNA, YAC, BAC, or cosmid clones (Diatchenko et al., 1996). For all other applications, you should clone the products to make a subtracted cDNA library. The cDNAs can be directly inserted into a T/A cloning vector. Alternatively, use the Not I (Sma I, Xma I) site on Adaptor I and the Eag I site on Adaptor 2R for site-specific cloning, or use the Rsa I site at the adaptor/cDNA junction for blunt-end cloning.

## Topo Cloning Subtracted Products

LB Agar Plates

1. Q.S. 8 grams of LB Agar (Fisher Scientific, Catalog # BP1425-500) to 200 mL using distilled $H_2O$
2. Autoclave
3. Let cool at RT
4. When temperature is approximately 45-55°C add the following and make sure is evenly distributed:
    a. Kanamycin (final concentration of 50 μg/mL) **or** ampicillin (final concentration of 100 μg/mL) **or** carbenicillin (final concentration of 100 μg/mL)
    b. IPTG (final concentration of 0.1 mM)
    c. Xgal (final concentration of 20 μg/mL)
5. Evenly pour agar into 100 x 150 mm plates.

Conduct cDNA cloning of PCR amplified subtracted products using the TOPO TA Cloning Kit for Sequencing, PCR-IV Vector (Invitrogen, Catalog #45-0030) manual with the following considerations:

1. Use TOP10 chemically competent *E. coli.*
2. Use .5 to 4 μl of 2$^{nd}$ary PCR product.
    (Note: This PCR reaction must be fresh without any freeze thaw cycles which reduces the 3' A overhangs necessary for successful cloning).
3. Make the TOPO cloning reaction 30 minutes at RT instead of 5 minutes.
4. During the One Shot chemical transformation, step #6, the outgrowth period may need to last 1.5 to 2.0 hours.
5. Spread varying volumes (25 to 100 μl) of transfected *E. coli* cells onto agar plates to determine best colony density formation.
6. Incubate overnight at 37°C.
7. Store plate at +4°C (not long term) wrapped in parafilm to prevent dehydration.

## Plasmid Culture

Terrific Broth

- Add 47.6 grams Terrific Broth (Difco, Catalog # 243820) into 1 liter of $ddH_2O$, dissolve using stir plate and autoclave.
- Cool at RT and store at +4°C up to 1 month
- Just before use add Kanamycin (final concentration of 50 μg/mL) **or** ampicillin (final concentration of 100 μg/mL) **or** carbenicillin (final concentration of 100 μg/mL)

Randomly select 96 clones containing template inserts and inoculate both a 96 deep-well culture plate (2 mL cultures) and a second plate containing 1 mL cultures to be used for freezing back stock cultures. (A single toothpick used to pick a single colony can inoculate both plates).

Culture at 37°C for 16-18 h. Cloudy appearance should be visible indicating successful growth.

**Stock cultures:** combine 800 ml and glycerol (30 % final concentration) into an appropriately labeled cryovial, vortex and store at –80°C

**Plasmid Extraction:** Spin the 96 well plate at 2000 RPM for 30 min bringing cells into a pellet, pour off broth leaving the pellet in the plate, and proceed following the manufacturer's protocol accompanying the Wizard® SV96 Plasmid DNA Purification System (Promega, Catalog #A2255). Use sterile, nuclease-free $H_2O$ to elute the DNA, which can be stored at +4°C (not long term) until used for differential screening.

## Differential Screening

Solutions:

Denaturation Solution
15 mL 5M NaCl
35 mL 1.5M NaOH or (5 mL 5M NaOH +30 mL $ddH_2O$)

Neutralization Solution
15 mL 5M NaCl
35 mL 1M Tris-HCl pH 7.5

20X SSC
175.3 g NaCl
88.2 g NaOAc
800 mL $ddH_2O$
--pH to 7.5, QS to 1 liter, and autoclave

**Membrane Spotting Procedure:**

1. Pre-wet a 8.5 x 11.5 cm nylon, positively charged membrane (Roche, Catalog # 1417240) in $ddH_2O$.
2. Denature DNA in the 96 well elution plate (from plasmid DNA extraction protocol) by adding 250 µl of the Denaturation solution and incubate for 5-10 min at RT.
3. Place membrane in the BioRad BioDot apparatus per assembly protocol.
4. Rehydrate the membrane using 100 µl. Apply vacuum.
5. Apply 77 µl of denatured DNA per well, per membrane (For SSH: 4 membranes per tester population, ~60 µl of sample per well in SV96 well prep, and 250 ml of denaturation solution).
6. Apply a gentle vacuum until solution is pulled through membrane.
7. Rinse the wells with 200 µl neutralization solution, incubate 5-10 min. Apply gentle to full vacuum.
8. Remove the membrane and rinse by setting on blotting paper soaked in 1X SSC for ~1 min.
9. UV cross-link membrane and store at +4°C.

**Probe Labeling**

1. Conduct 6-8 $2^{nd}$ary PCR reactions per the SSH protocol.
2. Run a small aliquot on a 2% agarose gel to ensure appropriate amplification.
3. Combine all tubes and purify using PCR purification kit (Qiagen, Catalog #28104) per the manufacturers protocol, resuspending the purified product in 50 µl of sterile, nuclease free $H_2O$.
4. Add 2.5 µl of RsaI and 5.8 µl of 10X RsaI buffer, vortex, briefly centrifuge and incubate in a 37°C water bath for 3 h (this is to remove adaptors which if labeled would cause a lot of background as the templates spotted on the nylon membrane also contain the adaptors).

5. Purify the digested PCR product as in step #3 except eluting DNA in only 30 ml of nuclease free water to increase concentration.
6. Spectrophotometrically estimate the quantity of purified tester DNA (this needs to be at least 62.5 ng/μl with at least 16 μl total volume).
7. Label DNA using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Catalog # 1585614) and the accompanying protocol.
    a. The labeling reaction should last for 20 h to maximize labeled DNA yield
8. Determine labeling efficiency according to protocol to estimate concentration of labeled DNA.

**Hybridization**

1. Conduct the hybridization, stringency washes, and immunological detection following the product protocol.
    a. The purpose of four identically spotted membranes representing each subtracted tester product is to simultaneously "probe" those 96 templates with the following four DIG labeled probes, which were also isolated during the SSH procedure: forward subtracted, forward unsubtracted, reverse subtracted and reverse unsubtracted.
2. Following immunological detection expose to X-ray film (Kodak, X-Omat) for 10 s to 1 min (depending on signal intensity), develop film, and determine genes confirmed differentially expressed.
    a. The exposure time for each of four identical membranes must be the same as signal intensities between them will be compared.
    b. Genes that are confirmed differentially expressed should have 5 times the signal intensity when probed with forward subtracted labeled DNA compared to when probed with reverse subtracted DNA. Also signal intensity should be greater when probed with forward unsubtracted labeled DNA compared to reverse unsubtracted labeled DNA.

## Sequencing

Plasmids containing genes confirmed differentially expressed through differential screening should be re-cultured using frozen plasmid stock described above.

1. Two to four mL cultures of terrific broth (described in plasmid culture section) should be inoculated using frozen plasmid stock corresponding to the templates differentially expressed.
2. Culture at 37°C 16-18 h.
3. Extract plasmid DNA using Wizard Plus Minipreps (Promega, Catalog # A7500) and the accompanying protocol.
4. Subject plasmid DNA to dideoxy chain termination sequencing using the M13 reverse primer.
    a. Sequences longer than 700 bp may need to be sequenced again using M13 forward primer to obtain additional sequence information.
5. Sequencing can be done by the Oklahoma State University Recombinant DNA/Protein Resource Facility.

## Quantitative RT-PCR

### Taqman Probe Assay

Designing dual-labeled probe and primer set:

1. Guidelines for designing both primers and probes:
    a. Select the probe first and design the primers as close as possible to the probe without overlapping it (amplicons of 50-150 base pairs are strongly recommended).
    b. Keep the G/C content in the 30-80% range for both the primers and the probe.
    c. Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more Gs should be avoided.
2. Guidelines specific for designing Probe:
    a. When using Primer Express® software the $T_m$ should be 68-70°C (~8-10°C higher than the $T_m$ of the primers) .
    b. No G on the 5' end.
    c. Select the strand that gives the probe more C than G bases.
    d. Design the Probe to have 6-FAM as the 5' reporting dye and to have TAMRA as the 3' quenching dye.
        -Multiple reporting dyes must be used if multiplexing, however FAM is the most stable dye and should be used whenever assaying for a single target gene within a single reaction.
3. Guidelines specific for designing Primers:
    a. When using Primer Express® software the $T_m$ should be 58-60°C (~8-10°C below the $T_m$ of the probe).
    b. The five nucleotides at the 3' end should have no more than two G and/or C bases.

Master Mix (Using Qiagen's QuantiTect™ Probe RT-PCR Kit, Catalog # 204443)

| **Component** | **Volume/Rxn** | **Final Concentration** |
|---|---|---|
| 2X QuantiTect Probe RT-PCR Master Mix | 12.5 µl | 1X |
| Forward Primer (10 µM) | 1.0 µl | 400 nM |
| Reverse Primer (10 µM) | 1.0 µl | 400 nM |
| Dual-Labeled Probed (10 µM) | 0.5 µl | 200 nM |
| QuantiTect Probe Reverse Transcriptase Mix | 0.25 µl | 0.25 µl/rxn |
| Nuclease-free $H_2O$ | 7.75 µl | -- |
| Total RNA (20 to 50 ng/µl) | 2 µl | 40 to 100 ng/rxn |
| **Total Volume** | **25.0 µl** | **25.0 µl** |

Note: Primer and probe concentrations may vary depending on the abundance of the target sequence. Also primer and probe concentrations can be greatly reduced if using 18s ribosomal RNA to normalize gene expression.

Cycling Parameters (Using ABI's 7700 Sequence Detection System)

| **Step** | **Reverse Transcription** | **Hot Start Reverse Transcriptase Activation** | **PCR Amplification** 45 Repetitive Cycles | |
|---|---|---|---|---|
| | | | Denature | Anneal/Extension |
| Time | 30 min | 15 min | 15 sec | 1 min |
| Temperature | 50°C | 95°C | 95°C | 60°C |

*Flourescent data must be acquired during the combined annealing/extension steps.

**SYBR Green Assay**

Designing primer set for SYBR Green Assay:

1. Keep the G/C content in the 30-80% range for both the primers and the probe.
2. Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more Gs should be avoided.
3. $T_m$ should be 58-60°C.
4. The five nucleotides at the 3' end should have no more than two G and/or C bases.

Master Mix (Using Qiagen's QuantiTect™ SYBR® Green RT-PCR Kit, Catalog # 204243)

| **Component** | **Volume/Rxn** | **Final Concentration** |
|---|---|---|
| 2X QuantiTect SYBR Green RT-PCR Master Mix | 12.5 µl | 1X |
| Forward Primer (10 µM) | 2.5 µl | 1.0 µM |
| Reverse Primer (10 µM) | 2.5 µl | 1.0 µM |
| QuantiTexct SYBR Green Reverse Transcriptase Mix | 0.25 µl | 0.25 µl/rxn |
| Nuclease-free $H_2O$ | 5.25 µl | -- |
| Total RNA (20 to 50 ng/µl) | 2 µl | 40 to 100 ng/rxn |
| **Total Volume** | **25.0 µl** | **25.0 µl** |
| | | |

Note: Primer concentrations may vary depending on the abundance of the target sequence.

Cycling Parameters (Using ABI's 7700 Sequence Detection System)

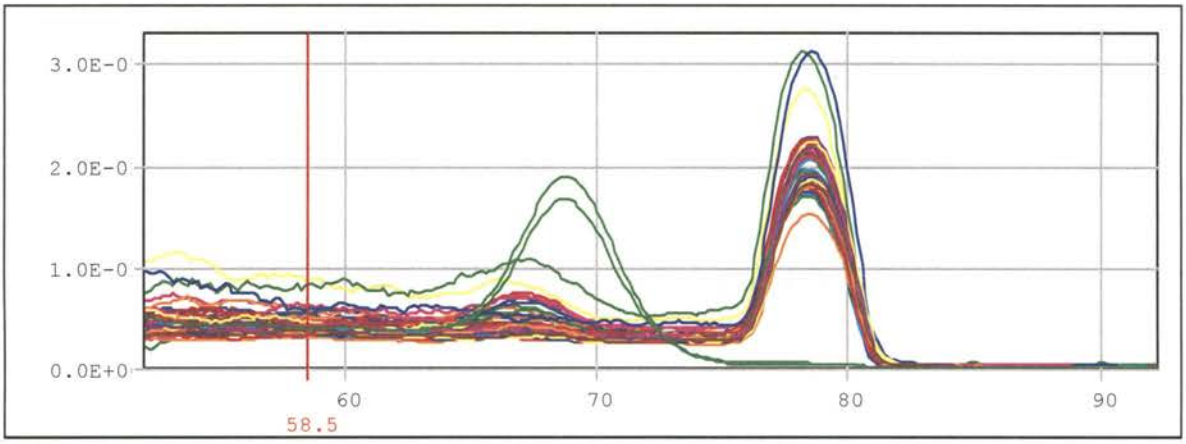
| **Step** | **Reverse Transcription** | **Hot Start Reverse Transcriptase Activation** | **PCR Amplification** 45 Repetitive Cycles Denature/ Anneal/ Extension/ Data Acquisition | | | |
|---|---|---|---|---|---|---|
| Time | 30 min | 15 min | 15 sec | 1 min | 30 sec | 15 sec |
| Temperature | 50°C | 95°C | 95°C | 50-60°C | 72°C | x °C* |

*Flourescent data must be acquired during the data acquisition step. The temperature for this step is variable and depends on the Tm of product being amplified. A melting curve analysis must be ran immediately following the PCR run to determine the Tm of primer dimmers and the amplified gene product to determine what the data acquisition temperature should be set at and to ensure that fluorescence being detected is from the desired PCR product and not primer-dimers.

Melting Curve Analysis Parameters:

| Step | Denature | Anneal | Denature |
|---|---|---|---|
| Time | 15 sec | 30 sec | 15 sec |
| Temperature | 95°C | 50-60°C* | 95°C |

*Following the annealing step, the ramp time for the machine should be set at maximum (19 min:59 sec) with data acquisition occurring throughout the ramp from annealing to denature. This multicomponent data can then be exported and used to create a melting curve plotting the change in fluorescence over time as shown below:




This melting curve analysis indicates that a primer-dimer has a Tm at ~69°C indicated by the two green peaks representing two No Template Controls. The remaining reactions, containing template, produce peaks at ~79°C indicating the Tm of the product. Therefore the data acquisition temperature should be set at ~78°C so that the fluorescence detected during that stage is strictly from the formation of the double-stranded target molecule and not from any primer-dimers, which are completely denatured by ~72°C.

## Literature Cited


Abbondanzo SJ, Cullinan EB, McIntyre K, Labow MA, Stewart CL. Reproduction in mice lacking a functional type 1 IL-1 receptor. Endocrinology (1996) 137:3598-3601.

Albert TJ, Su HC, Zimmermann PD, Iams JD, Kniss DA. Interleukin-1 beta regulates the inducible cyclooxygenase in amnion-derived WISH cells. Prostaglandins (1994) 48:401-416.

Ali A, Salter-Cid L, Flajnik MF, Heikkila JL. Isolation and characterization of a cDNA encoding Xenopus 70-kDA heat shock cognate protein, hsc70.I. Comp Biochem Physiol (1996) 113B:681-687.

Altschul S, Gish W, Miller W, Myers E, Lipman D. Basic local alignment search tool. J Mol Biol (1990) 215:403-410

Anderson LL. Growth, protein content and distribution of early pig embryos. Anat Rec (1978) 190:143-154.

Anegon I, Cuturi MC, Godard A, Moreau M, Terqui M, Martinat-Botte F, Soulillou JP. Presence of leukaemia inhibitory factor and interleukin 6 in porcine uterine secretions prior to conceptus attachment. Cytokine (1994) 6:493-499.

Atalay A, Crook T, Ozturk M, Yulug IG. Identification of genes induced by BRCA1 in breast cancer cells. Biochem Biophys Res Commun (2002) 299:839-846.

Bazer FW, Thatcher WW. Theory of maternal recognition of pregnancy in swine based on estrogen controlled endocrine versus exocrine secretion of prostaglandin $F_{2\alpha}$ by the uterine endometrium. Prostaglandins (1977) 14:397-401.

Bazer FW, Geisert RD, Thatcher WW, Roberts RM. The establishment and maintenance of pregnancy. In: Control of Pig Reproduction (Edited by Cole DJA, Foxcroft GR) Butterworth Scientific, London, UK (1982) pp.227-252.

Bazer FW, Vallet JL, Roberts RM, Sharp DC, Thatcher WW. Role of conceptus secretory products in establishment of pregnancy. J Reprod Fertil (1986) 76:841-850.

Bigonnesse F, Marois M, Maheux R, Akoum A. Interleukin-1 receptor accessory protein is constitutively expressed in human endometrium throughout the menstrual cycle. Mol Hum Reprod (2001) 7:333-339.

Blair RM, Geisert RD, Zavy MT, Yellin T, Fulton RW, Short EC. Endometrial surface and secretory alterations associated with embryonic mortality in gilts administered estradiol valerate on days 9 and 10 of gestation. Biol Reprod (1991) 44:1063-1079.

Bowen JA, Bazer FW, Burghardt RC. Spatial and temporal analyses of integrin and Muc-1 expression in porcine uterine epithelium and trophectoderm in vivo. Biol Reprod (1996) 55:1098-1106.

Bowen JA, Bazer FW, Burghardt RC. Spatial and temporal analyses of integrin and Muc-1 expression in porcine uterine epithelium and trophectoderm in vitro. Biol Reprod (1997) 56:409-415.

Braga VMM, Gendler SJ. Modulation of Muc-1 mucin expression in the mouse uterus during the estrous cycle, early pregnancy and placentation. J Cell Sci (1993) 105:397-405.

Burgess AW. Epidermal growth factor and transforming growth factor alpha. BR Med Bull (1989) 45:401-424.

Burghardt RC, Bowen JA, Newton GR, Bazer FW. Extracellular matrix and the implantation cascade in pigs. In: Control of Pig Reproduction V (Edited by Foxcroft GR, Geisert RD, Doberska C). Cambridge, UK, J Reprod Fertil Suppl (1997) 52:151-164.

Cabot RA, Prather RS. Cleavage stage porcine embryos may have differing developmental requirements for karyopherins alpha2 and alpha3. Mol Reprod Dev (2003) 64:292-301.

Cantoni, J.L. Biological methylation: selected aspects. Annu Rev Biochem (1975) 44:435-451.

Carraway KL, Idris M. Regulation of sialomucin complex/Muc4 in the female rat reproductive tract. Biochem Soc Trans (2001) 29:162-166.

Chastant S, Monget P, Terqui M. Localization and quantification of insulin-like growth factor-I (IGF-I) and IGF-II/mannose-6-phosphate (IGF-II/M6P) receptors in pig embryos during early pregnancy. Biol Reprod (1994) 51:588-596.

Choi I, Simmen RC Simmen FA. Molecular cloning of cytochrome P450 aromatase complementary deoxyribonucleic acid from periimplantation porcine and equine blastocysts identifies multiple novel 5'-untranslated exons expressed in embryos, endometrium, and placenta. Endocrinology (1996) 137:1457-1467.

Christenson RK, Leymaster KA, Young LD. Justification of unilateral hysterectomy-ovariectomy as a model to evaluate uterine capacity in swine. J Anim Sci (1987) 65:738-744.

Corbin CJ, Khalil MW, Conley AJ. Functional ovarian and placental isoforms of porcine aromatase. Mol Cell Endocrinol (1995) 113:29-37.

Corner GW. The problem of embryonic pathology in mammals, with observations upon intrauterine mortality in the pig. Am J Anat (1923) 31:523-545.

Cullinan EB, Kwee L, Nunes P, Shuster DJ, Ju G, McIntyre KW, Chizzonite RA, Labow MA. IL-1 receptor accessory protein is an essential component of the IL-1 receptor. J Immunol (1998) 161:5614-5620.

Czech MP. Structures and functions of the receptors for insulin and the insulin-like growth factors. J Anim Sci (1986) 63(Suppl. 2):27-38.

Dantzer V. Electron microscopy of the initial stages of placentation in the pig. Anat Embryol (1985) 172:281-293.

Dardik A, Schultz RM. Blastocoel expansion in the preimplantation mouse embryo: stimulatory effect of TGF-alpha and EGF. Development (1991) 113:919-930.

Davis AJ, Fleet IR, Harrison FA, Maule Walker FM. Pulmonary metabolism of prostaglandin $F_{2\alpha}$ in the conscious nonpregnant ewe and sow. J Physiol, London (1979) 301:86.

Davis DL, Blair RM. Studies of uterine secretions and products of primary cultures of endometrial cells in pigs. In: Control of Pig Reproduction IV (Edited by Foxcroft GR, Hunter MG and Doberska C). Cambridge, UK, J Reprod Fertil Suppl (1993) 48:143-155.

Davis DL, Pakrasi PL, Dey SK. Prostaglandins in swine blastocysts. Biol Reprod (1983) 28:1114-1118.

Dekaney CM, Ing NH, Bustamante L, Madrigal MM, Jaeger LA. Estrogen and progesterone peri-implantation porcine conceptuses. Biol Reprod (1998) 58 (Suppl. 1):92 (Abstract).

Dhindsa DS, Dzuik PF. Effects of pregnancy in the pig after killing embryos or fetuses in one uterine horn in early pregnancy. J Anim Sci (1968) 27:122-126.

Diatchenko L, Lau YF, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED, Siebert PD. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc Natl Acad Sci USA (1996) 93:6025-6030.

Dziuk PJ. Effect of number of embryos and uterine space on embryo survival in the pig. J Anim Sci (1968) 27:673-676.

Elefant F, Palter KB. Tissue-specific expression of dominant negative mutant Drosophila HSC70 causes developmental defects and lethality. Mol Biol Cell (1999) 10:2101-2117.

Engelhart H, Croy BA, King GJ. Conceptus influences the distribution of uterine leukocytes during early porcine pregnancy. Biol Reprod (2002) 66:1875-1880.

Feinberg BB, Anderson DJ, Steller MA, Fulop V, Derkowitz RS, Hill JA. Cytokine regulation of trophoblast steroidogenesis. J Clin Endo Metab (1994) 78:586-591.

Fenech, M. The role of folic acid and vitamin B12 in genomic stability of human cells. Mutat Res (2001) 475:57-67.

Fernandez-Pol JA, Klos DJ, Hamilton PD. A growth factor-inducible gene encodes a novel nuclear protein with zinc finger structure. J Biol Chem (1993) 268:21198-21204.

Fernandez-Pol JA, Klos DJ, Hamilton PD. Metallopanstimulin gene product produced in a baculovirus expression system is a nuclear phosphoprotein that binds to DNA. Cell Growth Differ (1994) 5:811-825.

Ferrell AD, Malayer JR, Carraway KL, Geisert RD. Sialomucin complex (Muc4) expression in porcine endometrium during the oestrous cycle and early pregnancy. Reprod Dom Anim (2003) 38:1-3.

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature (1998) 391:806-811.

Fischer HE, Bazer FW, Fields MJ. Steroid metabolism by endometrial and conceptus tissues during early pregnancy and psuedopregnancy in gilts. (1985) 75:69-78.

Ford SP Christenson RK. Blood flow to uteri of sows during the estrous cycle and early pregnancy: local effect of the conceptus on the uterine blood supply. Biol Reprod (1979) 21:617-624.

Ford SP, Magness RR, Farley DB, Van Orden DE. Local and systemic effects of intrauterine estradiol-17β on luteal function of nonpregnant sows. J Anim Sci (1982a) 55:657-664.

Ford SP, Christenson RK, Ford JJ. Uterine blood flow and uterine arterial, venous and lumenal concentrations of oestrogens on days 11, 13 and 15 after oestrus in pregnant and nonpregnant sows. J Reprod Fertil (1982b) 64:185-190.

Ford SP, Christenson RK. Direct effects of oestradiol-17β and prostaglandin E-2 in protecting pig corpora lutea from a luteolytic dose of prostaglandin F-2α. J Reprod Fert (1991) 93:203-209.

Ford SP. Embryonic and fetal development in different genotypes in pigs. In: Control of Pig Reproduction V (Edited by Foxcroft GR, Geisert RD, Doberska C.) Cambridge, UK, J Reprod Fertil Suppl (1997) 52:165-176.

Frank M, Bazer FW, Thatcher WW, Wilcox CJ. A study of prostaglandin F2alpha as the luteolysin in swine: III effects of estradiol valerate on prostaglandin F, progestins, estrone and estradiol concentrations in the utero-ovarian vein of nonpregnant gilts. Prostaglandins (1977) 14:1183-96.

Friess AE, Sinowatz F, Skolek-Winnisch R, Trautner W. The placenta of the pig I. Finestructural changes of the placenta barrier during pregnancy. Anat Embryol (1980) 158:179-191.

Fujimoto T, Savani RC, Watari M, Day AJ, Strauss JF. Induction of the hyaluronic acid-binding protein, tumor necrosis factor-stimulated gene-6, in cervical smooth muscle cells by tumor necrosis factor-α and prostaglandin $E_2$. Am J Pathol (2002) 160:1495-1502.

Gardner ML, First NL, Casida LE. Effect of exogenous estrogens on corpus luteum maintenance in gilts. J Anim Sci (1963) 22:132-134.

Garlow JE, Hakhyun Ka, Johnson GA, Burghardt RC, Jaeger LA, Bazer FW. Analysis of osteopontin at the maternal-placental interface in pigs. Biol Reprod (2002) 66:718-725.

Geisert RD, Brenner RM, Moffatt RJ, Harney JP, Yellin T, Bazer FW. Changes in oestrogen receptor protein, mRNA expression and localization in endometrium of cyclic and pregnant gilts. Reprod Fertil Dev (1993) 5:247-260.

Geisert RD, Brookbank JW, Roberts RM, Bazer FW. Establishment of pregnancy in the pig: II. Cellular remodeling of the porcine blastocyst during elongation on day 12 of pregnancy. Biol Reprod (1982a) 27:941-955.

Geisert RD, Brookbank JW, Roberts RM, Bazer FW. Establishment of pregnancy in the pig: I. Interrelationships between preimplantation development of the pig blastocyst and uterine endometrial secretions. Biol Reprod (1982b) 27:925-939.

Geisert RD, Chamberlain CS, Vonnahme KA, Malayer JR, Spicer LJ. Possible role of kallikrein in proteolysis of insulin-like growth factor binding proteins during the oestrous cycle and early pregnancy in pigs. Reproduction (2001) 121:719-28.

Geisert RD, Morgan GL, Zavy MT, Blair RM, Gries LK, Cox A, Yellin T. Effect of asynchronous transfer and oestrogen administration on survival and development of porcine embryos. J Reprod Fertil (1991) 93:475-81.

Geisert RD, Pratt TN, Bazer FW, Mayes JS, Watson GH. Immunocytochemical localization and changes in endometrial progestin receptor protein during the porcine oestrous cycle and early pregnancy. Reprod Fertil Dev (1994) 6:749-760.

Geisert RD, Rasby RJ, Minton JE, Wettemann RP. Role of prostaglandins in development of porcine blastocysts. Prostaglandins (1986) 31:191-204.

Geisert RD, Schmitt RAM. Early embryonic survival in the pig: Can it be improved? J Anim Sci (2000) 80(E. Suppl. 1):1-12.

Geisert RD, Yelich JV. Regulation of conceptus development and attachment in pigs. In: Control of Pig Reproduction V (Edited by Foxcroft GR, Geisert RD, Doberska C.) Cambridge, UK, J Reprod Fertil Suppl (1997) 52:133-149.

Geisert RD, Zavy MT Wettemann RP, Biggers BG. Length of psuedopregnancy and pattern of uterine protein release as influenced by time and duration of oestrogen administration in the pig. J Reprod Fertil (1987) 79:163-172.

Geisert RD, Zavy MT, Moffatt RJ, Blair RM, Yellin T. Embryonic steroids and establishment of pregnancy in pigs. In: Control of Pig Reproduction III (Edited by Cole DJA, Foxcroft GR, Weir BJ) Cambridge, UK, J Reprod Fertil Suppl (1990) 40:293-305.

Graddy LG, Kowalski AA, Simmen FA, Davis SL, Baumgartner WW, Simmen RC. Multiple isoforms of porcine aromatase are encoded by three distinct genes. J Steroid Biochem Mol Biol (2000) 73:49-57.

Green ML, Simmen RC, Simmen FA. Developmental regulation of steroidogenic enzyme gene expression in the periimplantation porcine conceptus: a paracrine role for insulin-like growth factor-I. Endocrinology (1995) 136:3961-70.

Gries LK, Geisert RD, Zavy MT, Garret JE, Morgan GL. Uterine secretory alterations coincident to embryonic mortality in the gilt after exogenous estrogen administration. J Anim Sci (1989) 67:276-284.

Gupta A, Bazer FW, Jaeger LA. Differential expression of beta transforming growth factors (TGF beta 1, TGF beta 2, and TGF beta 3) and their receptors (type I and type II) in peri-implantation porcine conceptuses. Biol Reprod (1996) 55:796-802.

Gupta A, Ing NH, Bazer FW, Bustamante LS, Jaeger LA. Beta transforming growth factors (TGFβ) at the porcine conceptus-maternal interface. Part I: Expression of TGFβ1, TGFβ2, and TGFβ3 messenger ribonucleic acids. Biol Reprod (1998) 59:905-910.

Guthrie HD and Lewis GS. Production of prostaglandin $F_{2\alpha}$ and estrogen by embryonal membranes and endometrium and metabolism of prostaglandin $F_{2\alpha}$ by embryonal membranes, endometrium and lung from gilts. Dom Anim Endocrinol (1986) 3:185-198.

Harney JP, Ali M, Vedeckis WV, Bazer FW. Porcine conceptus and endometrial retinoid-binding proteins. Reprod Fertil Dev (1994) 6:211-219.

Harney JP, Mirando MA, Smith LC, Bazer FW. Retinol-binding protein: A major secretory product of the pig conceptus. Biol Reprod (1990) 42:523-532.

Herrmann BG, Labiet S, Poustka A, King TR, Lehrach H. Cloning of the T gene required in mesoderm formation in the mouse. Nature (1990) 343:617-622.

Hershfield MS and Kredich NM. S-adenosylhomocysteine hydrolase is an adenosine-binding protein: A target for adenosine toxicity. Science (1978) 202:757-760.

Hettinger AM, Allen MR, Zhang BR, Goad DW, Malayer JR, Geisert RD. Presence of the acute phase protein, bikunin, in the endometrium of gilts during estrous cycle and early pregnancy. Biol Reprod (2001) 65:507-513.

Heuser CH, Streeter GL. Early stages in the development of pig embryos, from the period of initial cleavage to the time of appearance of limb-buds. Contrib Embryol Carnegie Inst (1929) 20:3-29.

Hofig A, Simmen FA, Bazer FW, Simmen RC. Effects of insulin-like growth factor-I on aromatase cytochrome P450 activity and oestradiol biosynthesis in preimplantation porcine conceptuses in vitro. J Endocrinol (1991) 130:245-250.

Huang JC, Liu DY, Yadollah S, Wu KV and Dawood MY. Interleukin-1β induces cyclooxygenase-2 gene expression in cultured endometrial stromal cells. J Clin Endo Metab (1998) 83:538-541.

Hynes RO. Integrins: versatility, modulation, and signaling in cell adhesion. Cell (1992) 69:11-25.

Jaeger LA, Johnson GA, Ka H, Garlow JG, Burghardt RC, Spencer TE, Bazer FW. Functional analysis of autocrine and paracrine signaling at the uterine-conceptus interface in pigs. In: Control of Pig Reproduction VI (Edited by Geisert RD, Niemann H, Doberska C) Caimbridge, UK, J Reprod Fertil Suppl (2001) 58:191-207.

Kennedy D. Breakthrough of the year. Science (2002) 298:2283-2284.

Keys JL, King GL. Microscopic examination of porcine conceptus-maternal interface between days 10 and 19 of pregnancy. Am J Anat (1990) 188:221-238.

Kidder HE, Casida LE, Grummer RH. Some effects of estrogen injections on estrual cycle of gilts. J Anim Sci (1955) 14:470-474.

King GJ, Rjamahendran R. Comparison of plasma progesterone profiles in cyclic, pregnant, psuedopregnant and hysterectomized pigs between 8 and 27 days after oestrus. J Endocrinol (1988) 119:111-116.

King GJ, Atkinson BA, Robertson HA. Implantation and early placentation in domestic ungulates. J Reprod Fertil (1982) 31:17-30.

Ko Y, Choi I, Green ML, Simmen FA, Simmen RCM. Transient expression of the cytochrome P450 aromatase gene in elongation porcine blastocysts is correlated with uterine insulin-like growth factor levels during peri-implantation development. Mol Reprod Dev (1994) 37:1-11.

Kol S, Kehat I, Adashi EY. Ovarian interleukin-1-induced gene expression: privileged genes threshold theory. Med Hypotheses (2002) 58: 6-8.

Kowalski AA, Graddy LG, Vale-Cruz DS, Choi I, Katzenellenbogen BS, Simmen FA, Simmen RC. Molecular cloning of porcine estrogen receptor-β complementary DNAs and developmental expression in periimplantation embryos. Biol Reprod (2002) 66:760-769.

Kowalski AA, Graddy LG, Choi I, Katzenellenbogen BS, Simmen FA, Simmen RCM. Expression of estrogen receptor (ER)-α and -β and progesterone receptor (PR) by porcine embryos suggests potential autocrine functions in development. Biol Reprod (2000) 62 (Suppl. 1):106 (Abstract).

Kraeling RR, Rampacek GB, Fiorello NA. Inhibition of pregnancy with indomethacin in mature gilts and prepubertal gilts induced to ovulate. Biol Reprod (1985) 32:105-110.

Kruessel JS, Huang HY, Wen Y, Kloodt AR, Bielfeld P, Polan ML. Different pattern of interleukin-1β-(IL-1β), interleukin-1 receptor antagonist-(IL-1ra) and interleukin-1 receptor type I-(IL-1R tI) mRNA-expression in single preimplantation mouse embryos at various developmental stages. J Reprod Immunol (1997) 33:103-120.

Lang L, Miskovic D, Lo M, Heikkila JJ. Stress-induced, tissue-specific enrichment of hsp70 mRNA accumulation in Xenopus laevis embryos. Cell Stress and Chaperones (2000) 5:36-44.

Lee CY, Green ML, Simmen RC, Simmen FA. Proteolysis of insulin-like growth factor-binding proteins (IGFBPs) within the pig uterine lumen associated with peri-implantation conceptus development. J Reprod Fertil (1998) 112:369-377.

Lee KF, Yao YQ, Kwok KL, Xu JS, Yeung WS. Early developing embryos affect the gene expression pattern in the mouse oviduct. Biochem Biophys Res Commun (2002) 292:564-570.

Lee TH, Wisniewski HG, Vilcek J. A novel secretory tumor necrosis factor-inducible protein (TSG-6) is a member of the family of hyaluronate binding proteins, closely related to the adhesion receptor CD44. J Cell Biol (1992) 116:545-557.

Letcher R, Simmen RCM, Bazer FW, Simmen FA. Insulin-like growth factor-I expression during early conceptus development in the pig. Biol Reprod (1989) 41:1143-1151.

Lindhard A, Bentin-Ley U, Ravn V, Islin H, Hviid T, Rex S, Bangsboll S, Sorensen S. Biochemical evaluation of endometrial function at the time of implantation. Fertil and Steril (2002) 78:221-233.

Long GG, Diekman MA. Effect of purified zearalenone on early gestation in gilts. J Anim Sci (1984) 59:1662-1670.

Maddox-Hyttel P, Dinnyes A, Laurincik J, Rath D, Niemann H, Rosenkranz C, Wilmut I. Gene expression during pre- and peri-implantation embryonic development in pigs. In: Control of Pig Reproduction VI (Edited by Geisert RD, Niemann H, Doberska C) Caimbridge, UK, J Reprod Fertil Suppl (2001) 58:175-189.

Mantovani A, Muzio M, Ghessi P, Colotta C, Introna M. Regulation of inhibitory pathways of the interleukin-1 system. Ann N Y Acad Sci (1998) 840:338-351.

Mathialagan N, Bixby JA, Roberts RM. Expression of interleukin-6 in porcine, ovine, and bovine preimplantation conceptuses. Mol Reprod Dev (1992) 32:324-330.

Mattson BA, Overstrom EW, Albertini DF. Endodermal cytoskeletal rearrangements during preimplantation pig morphogenesis. Biol Reprod (1990) 42:195-205.

McNeer RR, Carraway CAC, Fregien NL, Carraway KL. Characterization of the expression and steroid hormone control of sialomucin complex in the rat uterus: Implications for uterine receptivity. J Cell Physiol (1998) 176:110-119.

Mellor AL, Munn DH. Immunology at the maternal-fetal interface: lessons for T cell tolerance and suppression. Annu Rev Immunol (2000) 18:367-391.

Miller MW, Duhl DM, Winkes BM, Arredondo-Vega F, Saxon PJ, Wolff GL, Epstein CJ, Hershfield MS, Barsh GS. The mouse lethal nonagouti ($a^x$)mutation deletes the s-adenosylhomocysteine hydrolase (AHCY) gene. EMBO J (1994) 13:1806-1816.

Modric T, Kowalski AA, Green ML, Simmen RCM and Simmen FA. Pregnancy-dependent expression of leukaemia inhibitory factor (LIF), LIF receptor-β and interleukin 6 (IL-6) messenger ribonucleic acids in the porcine female reproductive tract. Placenta (2000) 21:345-353.

Moeljono MPE, Bazer FW, Thatcher WW. A study of prostaglandin $F_{2\alpha}$ as the luteolysin in swine: I. Effect of in prostaglandin $F_{2\alpha}$ hysterectomized gilts. Prostaglandins (1976) 11:737-743.

Mohan M, Ryder S, Claypool P, Geisert RD, Malayer JR. Analysis of gene expression in the bovine blastocyst produced in vitro using suppression-subtractive hybridization. Biol Reprod (2002) 67:447-453.

Morgan GL, Geisert RD, Zavy MT, Fazleabas AT. Development and survival of pig blastocysts after oestrogen administration on day 9 or days 9 and 10 of pregnancy. J Reprod Fertil (1987a) 80:133-141.

Morgan GL, Geisert RD, Zavy MT, Shawley RV, Fazleabas AT. Development of pig blastocysts in a uterine environment advanced by exogenous oestrogen. J Reprod Fertil (1987b) 80:125-131.

Nara BS, Darmadja D, First NL. Effect of removal of follicles, corpora lutea or ovaries on maintenance of pregnancy in swine. J Anim Sci (1981) 52:794-801.

Nestler JE. Interleukin-1 stimulates the aromatase activity of human placental cytotrophoblasts. Endocrinology (1993) 132:566-570.

Ni H, Ding N, Harper MJK, Yang Z. Expression of leukemia inhibitory factor receptor and gp130 in mouse uterus during early pregnancy. Mol Reprod Devel (2002) 63:143-150.

Niemann H and Wrenzycki C. Alterations of expression of developmentally important genes in preimplantation bovine embryos by in vitro culture conditions: implications for subsequent development. Theriogenology (2000) 53:21-34.

Perry JS, Heap RB, Amoroso EC. Steroid hormone production by pig blastocysts. Nature, Lond. (1973) 245:45-47.

Perry JS, Heap RB, Burton RD, Gadsby JE. Endocrinology of the blastocyst and its role in the establishment of pregnancy. J Reprod Fertil Suppl (1976) 25:85-104.

Perry JS. The mammalian fetal membranes. Reprod Fertil (1981) 62:321-335.

Polge C, Rowson LEA, Chang MC. The effect of reducing the number of embryos during early stages of gestation on the maintenance of pregnancy in the pig. J Reprod Fertil (1966) 12:395-397.

Pope WF. Embryonic Mortality in Swine. In: Embryonic Mortality in Domestic Species (Edited by Zavy MT, Geisert RD), CRC Press, Boca Raton (1994) pp. 53-78.

Pope WF, Xie S, Broermann DM, Nephew KP. Causes and consequences of early embryonic diversity in pigs. In: Control of Pig Reproduction III (Edited by Cole DJA, Foxcroft GR, Weir BJ) Cambridge, UK, J Reprod Fertil Suppl (1990) 40:251-260.

Pollard JW, Hunt JS, Wiktor-Jedrzejczak W, Stanley ER. A pregnancy defect in the osteopetrotic (op/op) mouse demonstrates the requirement for CSF-1 in female fertility. Dev Biol (1991) 148:273-83.

Pusateri AE, Rothschild MF, Warner CM, Ford SP. Changes in morphology, cell number, cell size and cellular estrogen content or individual littermate pig conceptuses on days 9 to 13 of gestation. J Anim Sci (1990) 68:3727-3735.

Radomski N, Kaufmann C, Dreyer C. Nuclear accumulation of s-adenosylhomocysteine hydrolase in transcriptionally active cells during development of Xenopus laevis. Mol Biol Cell (1999) 10:4283-4298.

Raghupathy R. TH1-type immunity is incompatible with successful pregnancy. Immunol Today (1997) 18:478-482.

Rechler MM. Insulin-like growth factor binding proteins. Vitam Horm (1993) 47:1-114.

Reese J, Das SK, Paria BC, Lim H, Song H, Matsumoto H, Knudtson KL, DuBois RN, Dey SK. Global gene expression analysis to identify molecular markers of uterine receptivity and embryo implantation. J Biol Chem (2001) 276:44137-44145.

Richards JS, Russell DL, Ochsner S, Espey LL. Ovulation: New dimensions and new regulators of the inflammatory-like response. Annu Rev Physiol (2002) 64:69-92.

Richter K and Buchner J. Hsp90: Chaperoning signal transduction. J Cell Physiol (2001) 188:281-290.

Roberts RM. Xie S, Trout WE. Embryo-uterine interactions in pigs during week 2 of pregnancy. In: Control of Pig Reproduction IV (Edited by Foxcroft GR, Hunter MG, Doberska C) Caimbridge, UK, J Reprod Fertil Suppl (1993) 48:171-186.

Ross JW, Malayer JR, Ritchey JW, Geisert RD. Characterization of the Interleukin-1β System During Porcine Trophoblastic Elongation and Early Placental Attachment. Biol Reprod (2003a) Submitted.

Ross JW, Ashworth MD, Hurst AG, Malayer JR, Geisert RD. Analysis and Characterization of Differential Gene Expression During Rapid Trophoblastic Elongation in the Pig Using Suppression Subtractive Hybridization. Reprod Biol Endocrin (2003b) 1:23 http://www.RBEj.com/content/1/1/23.

Rubio E, Valenciano AI, Segundo C, Sanchez N, de Pablo F, de la Rosa EJ. Programmed cell death in the neurulating embryo is prevented by the chaperone heat shock cognate 70. Eur J Neuroscience (2002) 15:1646-1654.

SAS. SAS User's Guide: Statistics (version 5.0). Cary, NC: Statistical Analysis System Institute Inc.; (1985).

Simon C, Frances A, Piquette GN, el Di, Zurawski G, Dang W, Polan ML. Embryonic implantation in mice is blocked by interleukin-1 receptor antagonist. Endocrinology (1994a) 134: 521-528.

Simon C, Piquette GN, Frances A, El-Danasouri I, Irwin JC, Polan ML. The effect of interleukin-1β (IL-1β) on the regulation of IL-1 receptor type I messenger ribonucleic acid and protein levels in cultured human endometrial stromal and glandular cells. J Clin Endocrin Metab (1994b) 78:675-682.

Simon C, Piquette GN, Frances A, Polan ML. Localization of interleukin-1 type 1 receptor and interleukin-1β in human endometrium throughout the menstrual cycle. J Clin Endocrin Metab (1993) 77:549-555.

Simmen FA, Simmen RC, Geisert RD, Martinat-Botte F, Bazer FW, Terqui M. Differential expression, during the estrous cycle and pre- and postimplantation conceptus development, of messenger ribonucleic acids encoding components of the pig uterine insulin-like growth factor system. Endocrinology (1992) 130:1547-56.

Smith TPL, Fahrenkrug SC, Rohrer GA, Simmen FA, Rexroad CE, Keele JW. Mapping of expressed sequence tags from a porcine early embryonic cDNA library. Anim Genet (2001) 32:66-72.

Steel RGD, Torrie JH, Dickey DA. Principles and Procedures of Statistics: A Biometrical Approach, 3rd Edition. McGraw-Hill (1996), Columbus, OH.

Stroband HW, Van der Lende T. Embryonic and uterine development during pregnancy. In: Control of Pig Reproduction III (Edited by Cole DJA, Foxcroft GR, Weir BJ) Cambridge, UK, J Reprod Fertil Suppl (1990) 40:261-277.

Surveyor GA, Gendler SJ, Pemberton L, Das SK, Chakraborty I, Julian J, Pimental RA, Wegner CC, Dey SK, Carson DD. Expression and steroid hormonal control of Muc-1 in the mouse uterus. Endocrinology (1995) 136:3639-3647.

Takacs R and Kauma S. The expression of interleukin-1α, interleukin-1β, and interleukin-1 receptor type I mRNA during preimplantation mouse development. J Reprod Immunol (1996) 32:27-35.

Thompson JN, Howell J, Pitt GAJ. Vitamin A and reproduction in rats. Proceedings of the Royal Society of London Series (1964) B159:510-535.

Tomanek M, Kopecny V, Kanka J. Genome reactivation in developing early pig embryos: an ultrastructural and autoradiographic analysis. Anat Embryol (1989) 180:309-316.

Tuo W, Bazer FW. Expression of oncofetal fibronectin in porcine conceptuses and uterus throughout gestation. Reprod Fertil Dev (1996) 8:1207-13.

Tuo W, Harney JP, Bazer FW. Developmentally regulated expression of interleukin-1β by peri-implantation conceptuses in swine. J Reprod Immunol (1996) 31:185-198.

Tuo W, Harney JP, Bazer FW. Colony-stimulating factor-1 in conceptus and uterine tissues in pigs. Biol Reprod (1995) 53:133-142.

Trout WE, Hall JA, Stallings-Mann ML, Galvin JA, Anthony RV, Roberts RM. Steroid regulation of the synthesis and secretion of retinol-binding protein by the uterus of the pig. Endocrinology (1992) 130:2557-2564.

Tsang TS. New model for 70 kDa heat shock proteins' potential mechanisms of function. FEBS (1993) 323(1,2):1-3.

Tuschl T. Expanding small RNA interference. Nature Biotech (2002) 20:446-448.

Vallet JL, Christenson RK, Klemcke HG. Purification and characterization of intrauterine folate-binding proteins from swine. Biol Reprod (1998) 59:176-181.

Vallet JL, Christenson RK, McGuire WJ. Association between uteroferrin, retinol-binding protein, and transferrin within the uterine and conceptus compartments during pregnancy in swine. Biol Reprod (1996) 55:1172-1178.

Van der Put NMJ, Blom HJ. Neural tube defects and a disturbed folate dependant homocysteine metabolism. Eur J Obstet & Gynecol and Reprod Biol (2000) 92:57-61.

Vanaerts L, Blom H, Deabreu R, Trijbels F, Eskes T, Copius Peereboom-Stegeman J, Noordhoek J. Prevention of neural tube defects by and toxicity of L-homocysteine in cultured postimplantation rat embryos. Teratology (1994) 50:348-360.

Vaughan TJ, James PS, Pascall JC, Brown KD. Expression of the genes for TGFα, EGF and the EGF receptor during early pig development. Development (1992) 116:663-669.

Vonnahme KA, Malayer JR, Spivey HO, Ford SP, Clutter A, Geisert RD. Detection of kallikrein gene expression and enzymatic activity in porcine endometrium during the estrous cycle and early pregnancy. Biol Reprod (1999) 61:1235-1241.

Vonnahme KA, Wilson ME, Foxcroft GR, Ford SP. Impacts on conceptus survival in a commercial swine herd. J Anim Sci (2002) 80:553-559.

Wang ZC, Yunis EJ, De los Santos MJ, Xiao L, Anderson DJ, Hill JA. T helper 1-type immunity to trophoblast antigens in women with a history of recurrent pregnancy loss is associated with polymorphism of the IL1B promoter region. Genes and Immun (2002) 3:38-42.

Weaver CT, Hawrylowicz CM, Unanue ER. T helper cell subsets require the expression of distinct costimulatory signals by antigen-presenting cells. Proc Natl Acad Sci USA (1988) 85:8181-8185.

Wegmann TG, Lin H, Guilbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a $T_H2$ phenomenon? Immunol Today (1993) 14:353-356.

Wiktor-Jedrzejczak W, Bartocci A, Ferrante AW Jr, Ahmed-Ansari A, Sell KW, Pollard JW, Stanley ER. Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (*op/op*) mouse. Proc Natl Acad Sci USA (1990) 87:4828-4832.

Wilson ME, Biensen NJ, Ford SP. Novel insight into the control of litter size in pigs, using placental efficiency as a selection tool. J Anim Sci (1999) 77:1654-1658.

Wilson ME, Fahrenkrug SC, Smith T, Rohrer GA, Ford SP. Differential expression of cyclooxygenase-2 around the time of elongation in the pig conceptus. Animal Reprod Sci (2002) 71:229-237.

Wilson ME, Ford SP. Comparative aspects of placental efficiency. In: Control of Pig Reproduction VI (Edited by Geisert RD, Niemann H, Doberska C) Caimbridge, UK, J Reprod Fertil Suppl (2001) 58:223-232.

Wislocky GB, Dempsey EW. Histochemical reactions of the placenta of the pig. Am J Anat (1946) 78:181-225.

Wisniewski H, Hua J, Poppers DM, Naime D, Vilcek J, Cronstein BN. TNF/IL-1-inducible protein TSG-6 potentiates plasmin inhibition by inter-α-inhibitor and exerts a strong anti-inflammatory effect in vivo. J Immunol (1996) 156:1609-1615.

Wood GW, Hausmann E, Choudhuri R. Relative role of CSF-1, MCP-1/JE, and RANTES in macrophage recruitment during successful pregnancy. Mol Reprod Devel (1997) 46:62-70.

Yelich JV, Pomp D, Geisert RD. Detection of transcripts for retinoic acid receptors, retinol binding protein, and transforming growth factors during rapid trophoblastic elongation in the porcine blastocyst. Biol Reprod (1997b) 57:286-294.

Yelich JV, Pomp D, Geisert RD. Ontogeny of elongation and gene expression in the early developing porcine conceptus. Biol Reprod (1997a) 57:1256-1265.

Ying CW, Hsu WL, Hong WF, Cheng WTK, Yang YC. Progesterone receptor gene expression in preimplantation pig embryos. Eur J Endocrinol (2000) 143:697-703.

# VITA

Jason W. Ross

Candidate for the Degree of

Master of Science

Thesis: IDENTIFICATION AND ANALYSIS OF GENES REGULATING EARLY CONCEPTUS DEVELOPMENT, MATERNAL RECOGNITION OF PREGNANCY AND IMPLANTATION IN THE PIG

Major Field: Animal Science

Personal Data: Born in Grinnell, IA on October 10, 1977, the son of Wayne and Marla Ross

Education: Graduated from Iowa City West High School, Iowa City, Iowa in May of 1996. Earned Bachelor of Science degree from Iowa State University, Ames, Iowa in December 2000. Completed the requirements for the Master of Science Degree with a major in Molecular Reproductive Physiology at Oklahoma State University in May 2003.