Institute of Molecular Animal Breeding and Biotechnology
Faculty of Veterinary Medicine of the
Ludwig-Maximillians-University Munich
Prof. Dr. E. Wolf

Identification of genes induced by the conceptus in the bovine endometrium during the pre-implantation period

Thesis for the attainment of the title Doctor in Veterinary Medicine
from the Faculty of Veterinary Medicine of the
Ludwig-Maximillians University Munich

by
Claudia Klein
from Wiesbaden

Munich 2006

Aus dem Institut für Tierzucht der
Tierärztlichen Fakultät
der Ludwig-Maximilians-Universität München
Lehrstuhl für Molekulare Tierzucht und Biotechnologie
(Vorstand: Prof. Dr. E.Wolf)

Identifizierung von Genen im bovinen Endometrium, welche durch den Konzeptus im prä-implantatorischen Zeitraum induziert werden

Inaugural Dissertation
zur Erlangung der tiermedizinischen Doktorwürde
der Tierärztlichen Fakultät der Ludwig-Maximillians Universität München

von
Claudia Klein
aus Wiesbaden

München 2006

Gedruckt mit Genehmigung der Tierärztlichen Fakultät der
Ludwig-Maximilians-Universtät München

Dekan:	Univ.-Prof.Dr. E.Märtlbauer
Referent:	Univ.-Prof. Dr. E. Wolf
1. Korreferent:	Univ.-Prof. Dr. J. Braun
2. Korreferent:	Univ.-Prof. Dr. K. Pfister
3. Korreferent:	Priv.-Doz. Dr. B. Schalch
4. Korreferent:	Univ. Prof. Dr. W. Schmahl

Tag der Promotion: 10. Februar 2006

The following manuscript, containing the results of the present thesis, has been published in the journal “Biology of Reproduction”:

Claudia Klein, Stefan Bauersachs, Susanne E. Ulbrich, Ralf Einspanier, Heinrich H.D. Meyer, Susanne E.M. Schmidt, Horst-Dieter Reichenbach, Margarete Vermehren, Fred Sinowatz, Helmut Blum, and Eckhard Wolf

Monozygotic twin model reveals novel embryo-induced transcriptome changes of bovine endometrium in the pre-attachment period

Biol Reprod. 2006 Feb;74(2):253-64. Epub 2005 Oct 5.

Table of contents

List of figures

List of abbreviations

bp	base pairs
BSA	bovine serum albumin
°C	degrees Celcius
cDNA	complementary DNA
cm	centimeter
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
E. coli	Escherichia coli
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
et al.	et alii
e.g.	example given
fig.	figure
x g	relative centrifugal force (rcf)
GE	glandular epithelium
h	hour
i.e.	it est
i.p.	intra peritoneal
IFN	interferon
IFNτ	interferon tau
ips	impulses per second
LE	luminal epithelium
LH	luteinizing hormone
M	molar (mol/l)
min	minute
mM	millimolar (mmol/l)
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
NaOAc	sodium acetate
NaOH	sodium hydroxide
NH_4OAc	ammonium acetate

PCR	polymerase chain reaction
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
RNA	ribonucleic acid
RNaseH	ribonuclease H
RT-PCR	reverse transcriptase PCR
rt	room temperature
SDS	sodium dodecylsulfate
sec	second
SEM	standard error of measurement
SSH	suppression subtractive hybridization
UV	ultraviolett
µl	microliter
V	volt

1. Introduction

Early embryonic development, implantation and maintenance of pregnancy are critically dependent on an intact embryo-maternal communication. In cattle, up to 40% of total embryonic losses occur between days 8 and 17 of pregnancy, indicating that early embryonic mortality is the major cause of reproductive failure and has enormous economic implications (Humblot, 2001). Failures in the embryo-maternal cross-talk are in particular assumed to be responsible for the high failure rate to maintain early pregnancy. In ruminants conceptus-derived IFNτ is well characterized as an important pregnancy recognition signal. It is secreted by trophectoderm cells of blastocysts between days 14 to 26 with maximum secretion occurring around day 17 (Bazer et al., 1997), which coincides with the time of "maternal recognition of pregnancy". IFNτ acts in a paracrine manner on the endometrium to prevent pulsatile release of $PGF_{2\alpha}$ and thereby rescues the *corpus luteum* from regression. The abrogation of the luteolytic mechanism by IFNτ ensures ongoing secretion of luteal progesterone. Progesterone is among other hormones mainly responsible for uterine remodeling and uterine gland morphogenesis, which is a prerequisite for production of histotrophs necessary to nourish the conceptus during the pre-implantation period. Further, IFNτ is known to stimulate the transcription of several genes, which are hypothesized to play roles in endometrial differentiation and conceptus implantation.
Although, the general mechanisms of hormonal regulation in the endometrium during establishment of a pregnancy in ruminants are known, the detailed molecular mechanisms are not completely understood. Therefore, a combination of subtracted cDNA libraries and microarray technology was applied to identify genes upregulated in the bovine endometrium due to the presence of a conceptus in the pre-implantation period. The knowledge of the mechanisms underlying successful establishment of pregnancy could help to reduce embryonic losses, e.g. by supplementation of embryotrophic factors or components of embryo-maternal signaling in optimized formulations such as bioartificial matrices. As a long-term goal, signaling mechanisms identified in the bovine species could also be functionally evaluated in other species.

2. Review of the literature

2.1. Implantation

After fertilization in the oviduct, the fertilized ovum moves towards the uterus while undergoing several cell divisions. At the 8-cell-stage, about 72 hours after ovulation, the bovine conceptus reaches the uterus. The morula stage is reached by day 5 to 6 *post ovulationem* and on day 8 the first differentiation, formation of the blastocyst, occurs. The blastocyst, which contains a blastocoel, consists of two distinct cell types: the trophectoderm, the outer layer of rapidly dividing cells, and the inner cell mass (ICM), which comprises undifferentiated cuboidal cells. The ICM gives rise to the embryo plus extra-embryonic membranes such as allantois and amnion, while the trophectoderm contributes to the trophoblast layers of the placenta. On day 9 *post conceptionem* (p.c.) the blastocyst hatches from the zona pellucida and on day 13 p.c. rapid elongation starts. After elongation the ruminant conceptus occupies almost the entire surface of the uterine luminal epithelium. During this pre-attachment period the blastocyst is free floating in the uterus until about day 18 to 19 of gestation when implantation starts (Rüsse and Sinowatz, 1998). Implantation is the process through which a developing conceptus attaches to the endometrium. It is a temporally and spatially restricted process that involves a precise cross-talk between the embryo and the receptive maternal endometrium. A variety of cytokines and growth factors have become increasingly implicated in embryonic implantation.

2.1.1. Cytokines and implantation

Leukemia inhibitory factor

Many cytokines are expressed in the uterus, but only few have been shown to be required for embryonic implantation. The clearest example is the requirement, at least in the murine and human species, of maternally produced leukemia inhibitory factor (LIF), a pleiotropic cytokine. In mice, the sites of most abundant LIF expression are the uterine endometrial glands, specifically on day 4 of pregnancy, which coincides with the time of implantation. Analysis of LIF expression in pseudopregnant mice and in those undergoing implantation showed that it is under maternal control and coincides with blastocyst formation and always precedes implantation (Bhatt et al., 1991). Blastocysts of female mice lacking a functional LIF gene fail to implant and

do not develop. The blastocysts, however are viable and, when transferred to wild-type pseudopregnant recipients, they can implant and develop to term (Stewart et al., 1992). Hence, blastocyst implantation in mice depends on maternal expression of LIF. Chen et al. (2000) showed that in LIF-deficient mice up to the onset of implantation changes in uterine cell proliferation, hormone levels, blastocyst localization, as well as expression of lactoferrin and Mucin-1, do not differ from wild-types. However, the uterus fails to respond to the presence of embryos or artificial stimuli by the process of decidualization. They further showed that uterine expression of LIF is upregulated by estrogen and that LIF can replace nidatory estrogen at inducing both implantation and decidualization in ovariectomized mice. Implantation of LIF-deficient embryos in LIF-deficient females with normal development to term is rescued by i.p. injection of LIF. Leukemia inhibitory factor is therefore neither required by the embryo for development nor for the maintenance of pregnancy, but it is absolutely necessary for implantation in mice. In human, LIF expression is also coincident with the onset of implantation (Aghajanova, 2004) as well as in the mink (Song et al., 1998) and rabbit uterus (Yang et al., 1994). In the bovine species the gene expression of LIF was investigated using quantitative RT-PCR in bovine endometrial tissues during early and mid-pregnancy (Oshima et al., 2003). The LIF gene was expressed in all samples examined. The highest expression was observed in the intercaruncular endometrium at days 48-49 and 74-140 of pregnancy and the expression was greater than at days 13-14 of the estrous cycle and at other days of pregnancy. In the ovine endometrium a decrease in mRNA encoding LIF is observed during early pregnancy (on days 12-14) followed by an increase to reach high expression levels of LIF mRNA on days 16-20 of pregnancy (Vogiagis et al., 1997a). Immunoreactive LIF was mainly localized in the caruncular and intercaruncular luminal epithelium, and moderate staining was observed in the glandular epithelium and intercaruncular stroma. Passive immunization of ewes against human recombinant LIF resulted in a reduced pregnancy rate in comparison to control animals (Vogiagis et al., 1997b). In ruminants LIF may not be obligatory for implantation but it does appear to have a role during the establishment of pregnancy.

Interleukin-1

Interleukin-1 (IL-1) is another cytokine that is assumed to play an important role in implantation of the early embryo since blockade of the IL-1 receptor prevents implantation in the mouse by interfering with embryonic attachment (Simon et al.,

1994). In mice, IL-1 bioactivity increases from a low concentration on day 3 of pregnancy to a peak between days 4 and 5, the time of blastocyst implantation and decreases thereafter to low concentrations on days 7 and 8 (De et al., 1993). Besides IL-1, the interleukin-1 type I receptor is found in the mouse uterus around the time of implantation (Simon et al., 1994). In human endometrial tissue, IL-1 type I receptor mRNA is expressed throughout the entire menstrual cycle. However, IL-1 type I receptor mRNA levels were found to be significantly higher during both early and late luteal phase than in follicular and midluteal phase (Simon et al., 1993). Interleukin-1 was also detected in human endometrium during the implantation process (Kauma et al., 1990). However, the precise role of IL-1 in the context of implantation is not clearly defined, as mice lacking a functional type 1 IL-1 receptor do not exhibit any profound alterations in their reproduction, apart from a slight reduction in mean litter size (Abbondanzo et al., 1996).

Macrophage-colony stimulating factor

Macrophage-colony stimulating factor (M-CSF or CSF-1) is a glycoprotein growth factor required for the proliferation and differentiation of mononuclear phagocytotic cells (Flanagan and Lader, 1998). Detection of high concentrations of M-CSF in the mouse uterus during pregnancy led to the assumption that this cytokine also plays a role outside the haematopoietic system (Bartocci et al., 1986). Results from M-CSF null mutant mice suggest that M-CSF is required for pregnancy as homozygous mutant crosses are consistently infertile. However, maternally derived M-CSF is not an absolute requirement for embryonic survival and development, since null mutant females are fertile when mated to heterozygous males (Pollard et al., 1991). Pollard and coworkers (1987) showed that uterine M-CSF concentration is regulated by the synergistic action of the female sex steroids estradiol-17 beta and progesterone. These findings indicate that M-CSF, under hormonal influence, plays a role in implantation. In the bovine endometrium no significant changes occur in M-CSF gene expression in the caruncular area during early to mid-pregnancy. In the intercaruncular endometrium M-CSF gene expression is highest at days 74-140 compared to earlier pregnancy states (Oshima et al., 2003).

HOXA10

Hox genes are well known transcriptional regulators that play an essential role in embryonic development. The expression of Hoxa10 increases dramatically during the

midsecretory phase of the human menstrual cycle, overlapping with the time of implantation (Taylor et al., 1998). In mice Hoxa10 gene expression in the uterus is strongly activated by progesterone and this activation is blocked by the progesterone receptor antagonist RU486 (Ma et al., 1998). Female mice that are homozygous for a targeted disruption of the Hoxa10 gene exhibit infertility. Female null mutant mice ovulate normally, but about 80% are sterile because of embryonic death between days 2.5 and 3.5 *post coitum* (Satokata et al., 1995). While expression of LIF and heparin-binding epidermal growth factor (HB-EGF) appears unaffected in the mutant uteri, a decrease is observed in the intensity and number of blue dye reactions, an indicator of increased vascular permeability in response to implantation. In addition mutant uteri exhibit decreased decidualization in response to artificial stimuli (Benson et al., 1996). One mechanism through which HOXA10 might influence the process of implantation is by direct regulation of the beta3-integrin subunit expression in the endometrium. Seven consensus HOXA10 binding sites were identified 5´ of the beta 3-integrin gene and direct binding of HOXA10 protein to four sites has been demonstrated (Daftary et al., 2002).

2.1.2. Growth factors and implantation

Growth hormone

Pituitary growth hormone (GH) is known to control postnatal growth and differentiation. For a long time embryonic growth and development has been assumed to be independent of GH because hypophysectomized mouse, rat and rabbit fetuses display almost normal intrauterine growth (Gluckman et al., 1981). However, litter sizes in growth hormone receptor (GHR) knockout mice are reduced (Danilovich et al., 1999) and several experiments have shown that GH is able to influence embryonic development, differentiation, and metabolism. For example, culture of murine pre-implantation embryos in the presence of GH supports their development in vitro (Drakakis et al., 1995). Growth hormone is able to modulate apoptosis during early bovine in vitro embryogenesis and consequently increases thereby the cell number in the inner cell mass and the trophectoderm (Kolle et al., 2002). Further, Kolle et al. (2004) showed that treatment with GH induced the elimination of glycogen storage in the inner cell mass and stimulated the exocytosis of lipid vesicles in the inner cell mass and the trophectoderm. Overall, treatment with

GH results in an improvement of the ultrastructural features of embryos produced *in vitro*. In human endometrium intense staining for GH was observed in the mid and late luteal phase of the menstrual cycle, while no staining was observed during the proliferative or early luteal phase which implies a role of GH in blastocyst implantation (Sbracia et al., 2004). In the sheep, both endometrium and conceptus express GH and GHR during early pregnancy (Lacroix et al., 1999). The bovine conceptus has been shown to express GH and GHR (Kolle et al., 1998; Kolle et al., 2001) and the bovine endometrium produces low levels of GHR mRNA during the first trimester of gestation which gradually increase to peak at the end of gestation (Kolle et al., 1997).

Epidermal growth factor

Accumulated evidence suggests that growth factors of the epidermal growth factor (EGF) family, including epidermal growth factor (EGF), amphiregulin (AR), heparin-binding epidermal growth factor (HB-EGF), and transforming growth factor alpha (TGF-alpha) play an important role in the implantation process in a variety of species. The EGF members signal through a family of receptor tyrosine kinases (Ullrich and Schlessinger, 1990), which lead to a signal transduction cascade that regulates diverse cellular functions including cell proliferation, survival, adhesion, migration and differentiation (Yarden, 2001). In the human endometrium HB-EGF mRNA and protein expression is low during the proliferative stage of the menstrual cycle and increases in the secretory phase, with highest expression immediately prior to the window of implantation (Yoo et al., 1997; Stavreus-Evers et al., 2002; Lessey et al., 2002). In the mouse, HB-EGF is expressed in the luminal epithelium solely at the presumptive sites of blastocyst attachment (Das et al., 1994). Highest levels of EGF, TGF-alpha, HB-EGF, AR, and EGF receptor (EGF-R) were detected on days 9 and 11 of pregnancy in the rhesus monkey uterus, with primarily localization in glandular and luminal epithelium, which suggests that they play a role in monkey implantation (Yue et al., 2000). Transforming growth factor alpha and EGF were found to be present in the caprine endometrium during the peri-implantation period (Flores et al., 1998). In the ovine and rat species, TGF-alpha and EGF-R have been detected around the time of implantation in the endometrium and conceptuses (Gharib-Hamrouche et al., 1995; Tamada et al., 1997; Tamada et al., 2002). The elongating bovine blastocyst expresses EGF-R, EGF, TGF-alpha, and HB-EGF. Further, at day 13 of gestation, the expression of EGF-R, EGF, and TGF-alpha could be detected in

the bovine endometrium (Kliem et al., 1998). It has been shown that EGF and TGF-alpha stimulate the proliferation of mouse uterine epithelial cells *in vitro* (Komatsu et al., 2003) and, additionally, directly promote trophoblast development (Machida et al., 1995). Intraluminal injection of EGF is sufficient to initiate implantation and decidualization in ovariectomized progesterone-treated delayed implanting rats (Tamada et al., 1994), whereas intraluminal injection of anti-TGF-alpha antibodies into uterine horns prior to implantation decreases the number of rats showing implantation (Tamada et al., 1997). However, regardless of the conserved expression pattern of members of the EGF family, their precise role is not clear yet. Gene targeting experiments have demonstrated that mice null for EGF, AR (Luetteke et al., 1999), or TGF-alpha (Mann et al., 1993) are fertile. In addition, compound knockouts for all three alleles are still fertile (Troyer et al., 2001).

Vascular endothelial growth factor

Angiogenesis is a crucial event during the process of implantation and the uterine expression of vascular endothelial growth factor (VEGF) has been characterized in a number of species. The vascular endothelial growth factor is related with the platelet-derived growth factor, PDGF, and exhibits potent angiogenetic activity (Alberts et al., 2004). The temporal and spatial expression pattern of VEGF in the uterus of rhesus monkeys and marmosets implies that this factor may play a critical role in evolving pregnancy in these species (Ghosh et al., 2000; Rowe et al., 2003; Wang et al., 2003). A significant upregulation of the mRNAs of VEGF and its receptors was observed in the endometrium during the peri-implantation period when compared with the pre-implantation period in pigs (Welter et al., 2003). In human endometrium intense immunostaining of VEGF was observed in both glandular epithelial and stromal cells during the mid-secretory phase of the menstrual cycle. Further, strong staining for VEGF was observed in decidual cells of early pregnant uteri (Sugino et al., 2002).

Insulin-like growth factor system

Insulin-like growth factors (IGFs) are growth promoting peptides that share significant structural homology with insulin. The IGF-system comprises an increasingly complex network of ligands (IGF-I and IGF-II), receptors (type I and type II IGF receptor, IGF-IR and IGF-IIR), and high-affinity binding proteins (IGFBP-1 to -6), which modulate bioavailability and effects of the IGFs (Rajaram et al., 1997). The IGF-system has

been implicated to play a role in early embryonic development and decreased expression of certain members of the IGF-system may be one of the major causes of unexplained infertility in women (Wu and Zhou, 2004). Numerous studies have been conducted to describe temporal and spatial expression patterns of components of the IGF-system during early pregnancy in the uterus of humans (Zhou and Bondy, 1992; Strowitzki et al., 1996), baboons (Fazleabas et al., 1989; Tarantino et al., 1992; Hild-Petito et al., 1994), rats (Sadek et al., 1994; Cerro and Pintar, 1997; Korgun et al., 2003; Tamura et al., 2004), cats (Boomsma et al., 1994), mice (Hofig et al., 1991; Markoff et al., 1995; Liu et al., 1995), pigs (Hofig et al., 1991), and rhesus monkeys (Dhara et al., 2001). In the ewe, temporal variations in endometrial expression of mRNAs encoding IGF-I and IGF-II, and IGFBP-1 and -2 were investigated between estrus and day 20 of pregnancy. The IGF-II transcript level was unaffected by day of pregnancy. Insulin-like growth factor I expression was increased at estrus and at day 8 of pregnancy, relative to expression on day 15, whereas IGFBP-2 expression decreased. The insulin-like growth factor binding protein 1 was expressed only on day 15. The temporal variation of the expression of mRNAs encoding IGF-I and IGFBP-2 suggests a role for these factors in the uterine environment during early pregnancy in ewes (Cann et al., 1997). In the bovine species it has been demonstrated that endometrial expression of IGF-II mRNA is greater in pregnant than cycling endometrium on days 15 and 18. Levels of endometrial IGFBP-2 mRNA increased between days 10 and 18 of the estrous cycle and early pregnancy. These results suggest that the presence of the bovine conceptus has a stimulatory effect on endometrial expression levels of IGF-II, whereas progesterone appears to be involved with enhancement of IGFBP-2 (Geisert et al., 1991). In order to determine the role that IGFBPs may have in regulating initial stages of conceptus elongation in cattle, Keller et al. (1998) evaluated the type and relative abundance of IGFBPs in serum, uterine tissues, and uterine fluid from pregnant and non-inseminated cows on days 13 and 15 postestrus and in day 15 conceptuses. Insulin-like growth factor binding proteins in conceptuses and conceptus-conditioned culture media were only faintly detectable, whereas tissue specificity and changes in abundance of IGFBPs indicate the potential importance of endometrial IGFBPs in regulating uterine IGFs during the time period of conceptus elongation in the bovine species.

2.1.3. Blastocyst implantation and the adhesion cascade

In domestic ruminants endometrial invasion, as it takes place in humans or rodents, does not occur; thus, definitive implantation is achieved by tight adhesion of mononuclear trophoblast cells to the endometrial luminal epithelium. Conceptus attachment in the ewe has been studied using scanning and transmission electron microscopy. Three stages could be determined: i) from day 14 on precontact was established and the conceptus appeared to be immobilized in the uterine lumen, ii) on day 15 apposition occurred and iii) between days 16 and 18 adhesion began (Guillomot et al., 1981). Several adhesion molecules have been implicated in the process of embryonic implantation, such as Muc-1, integrins, and selectins.

Mucin-1

Mucin-1 (Muc-1) is a major epithelial apical surface glycoprotein molecule in the endometrium of various species. It has a large, extended and highly glycosylated ectodomain that contains keratin sulphate chains and has been shown experimentally to inhibit cell-cell interactions by steric hindrance of binding interactions mediated by receptors, including integrins and cadherins (Aplin, 1999). Thus, Muc-1 contributes to a functionally non-receptive uterine environment and high expression levels during the time of blastocyst implantation are unexpected. Indeed, this glycoprotein coat is drastically reduced in the uterus of many species during the time of embryo implantation (Carson et al., 1998). In the murine uterus Muc-1 expression is high in the proestrus and estrus stages and decreases during diestrus. Both Muc-1 mRNA and protein decline to barely detectable levels by day 4 of pregnancy, i.e. before the time of blastocyst attachment (Surveyor et al., 1995). Similar to the observations made in the murine uterus, a decreasing expression of Muc-1 towards the time of implantation was observed in the rat (Isaacs and Murphy, 2003) and porcine uterus (Bowen et al., 1996). In the ovine uterus immunoreactive Muc-1 is highly expressed at the apical surfaces of uterine luminal and glandular epithelium in both cycling and pregnant ewes but decreases dramatically by day 9 and is nearly undetectable by day 17 of pregnancy when intimate contact between the luminal epithelium and trophectoderm begins (Johnson et al., 2001). It has been shown that the expression of Muc-1 is modulated by steroid hormones and that down-regulation of Muc-1 towards the time of blastocyst implantation is a progesterone dominated event (Braga and Gendler, 1993; Surveyor et al., 1995). In

the human and rabbit uterus the situation differs substantially from that described above. In these species Muc-1 expression is stimulated by progesterone so that high expression levels of Muc-1 are present during the time of implantation (Hey et al., 1994; Hoffman et al., 1998; Acosta et al., 2000). However, Muc-1 is locally reduced at implantation sites which is presumably accomplished via signals apparently produced by the blastocyst (Hoffman et al., 1998). Furthermore, Muc-1 has been considered to play a role in unexplained infertility because these women exhibit a smaller Muc-1 allele size than fertile women (Horne et al., 2001).

Integrins

Integrins are transmembrane glycoproteins composed of α and β subunits that exist in close association with the cytoskeleton and signaling proteins (Hillis and MacLeod, 1996; Lukashev and Werb, 1998; Porter and Hogg, 1998). Until now 22 integrin receptors have been identified, and these comprise at least 16 types of α subunits and 8 types of β subunits (Juliano and Haskill, 1993). Integrin receptors interact with a variety of ligands including ECM proteins and cell surface molecules and many of them recognize the tripeptide sequence arginine-glycine-aspartic acid (RGD), which commonly appears in ECM components (Ruoslahti and Pierschbacher, 1987). Altered expression of integrins is associated with several causes of infertility in women (Chen et al., 1998; Skrzypczak et al., 2001) and the temporal and spatial expression pattern of several integrins has been investigated in different species to elucidate their role in reproductive processes. In the human endometrium certain integrins are expressed constitutively throughout the menstrual cycle whereas others, like alpha(4)beta(1) and alpha(v)beta(3) are only expressed during the luteal phase. The cycle dependent expression, which frames the putative window of implantation, suggests a role for these integrins in the establishment of uterine receptivity (Lessey et al., 1994; Nardo et al., 2002). In the rhesus monkey there is evidence that correlated expression of integrins and their ECM ligands might be involved in the regulation of cell proliferation and differentiation and the counterbalanced invasion-accelerating and invasion-restraining processes in trophoblast cells during early stages of pregnancy (Qin et al., 2003). In the rabbit uterus higher levels of mRNA for beta(3) subunit were observed at the implantation site, with reduced expression in nonimplantation sites and in non-pregnant adult and immature endometrium (Illera et al., 2003). During the peri-implantation period, alpha(v)beta(3) integrin and alpha(4) and alpha(5) subunits, as well as the ECM components vitronectin and fibronectin

are expressed in the caprine endometrium and may play a role in the cascade of embryonic implantation (Garcia et al., 2004). In swine, the alpha(4), alpha(5), alpha(v), beta(1), and beta(3) integrin subunits, along with the ECM components fibronectin and vitronectin were detected at sites of attachment between uterine epithelial cells and trophectoderm during early pregnancy (Bowen et al., 1996). The ewe does not appear to limit receptivity to implantation by modifying temporal and spatial patterns of integrin expression as the integrin subunits alpha(v), alpha(4), alpha(5), beta(1), beta(3), and beta(5) are constitutively expressed on conceptus trophectoderm and at the apical surface of uterine luminal epithelium and glandular epithelium in both cyclic and pregnant ewes. The constitutive expression of these subunits could contribute to the apical assembly of several osteopontin receptors. Therefore the authors conclude that osteopontin might act as a bridge between integrins heterodimers expressed by trophectoderm and uterine luminal epithelium and thereby being responsible for initial conceptus attachment (Johnson et al., 2001). Throughout the bovine estrous cycle the luminal epithelium was found to express integrin subunits beta(1), alpha(3), alpha(6), and integrin alpha(v)beta(3). Of these integrins, alpha(6) and alpha(v)beta(3) exhibited estrous cycle dependent expression. Alpha(v)beta(3) showed a reduced subepithelial stromal staining during diestrus which differs from the situation in women, where alpha(v)beta(3) is considered to be a marker of uterine receptivity (Kimmins and MacLaren, 1999). Furthermore, the mRNA of integrin beta(4) was shown to be upregulated at diestrus as compared to estrus (Bauersachs et al., 2005). Implantation associated changes in bovine uterine expression of integrins were investigated by McIntyre and coworkers (2002). The uterine epithelium constitutively expressed the alpha(1), alpha(3), and alpha(6) integrin subunits during early pregnancy, but the alpha(1) subunit was down-regulated as the luminal epithelium was modified by trophoblast cell migration.

The cycle dependent expression of integrins led to the assumption that their expression is modulated by steroid hormones and, indeed, in the murine uterus the alpha(4)beta(1) integrin is induced by estradiol and down-regulated by progesterone during implantation (Basak et al., 2002). Further evidence for the involvement of integrins in the implantation process was drawn from functional blockage experiments. Functional blocking of the alpha(v)beta(3) integrin leads to a reduced number of implantation sites in mice and in rabbits (Illera et al., 2000; Illera et al.,

2003). In addition, intrauterine blockade of alpha(4) subunit by a specific antibody resulted in implantation failure in mice (Basak et al., 2002).

Selectins

Selectins are carbohydrate-binding proteins that recognize specific oligosaccharide structures. In the vasculature they mediate interactions between leukocytes and endothelium to permit cell adhesion under flow (Alberts et al., 2004). To determine whether selectin ligands are present at appropriate times and locations to function in blastocyst adhesion to the uterus, Genbavec and coworkers (2003) studied their expression at the maternal-fetal interface in women using specific antibodies. During the luteal phase of the menstrual cycle staining was much stronger, particularly on the luminal epithelium, than throughout the follicular phase. Immunoblot analysis confirmed upregulation of selectin ligands as the window of human receptivity opens. They further examined whether the embryo expresses L-selectin. After hatching of the embryo from the zona pellucida, strong immunoreactivity was observed in association with the trophectoderm over the entire embryo surface. The authors next investigated the physiological importance of the interactions between L-selectin and its oligosaccharide ligands. They coated polystyrene latex beads with an oligosaccharide that binds to L-selectin and observed that the beads bound avidly to trophoblast cells under conditions that mimic those in the uterus. Additionally, isolated trophoblasts bound preferentially to uterine epithelial cells from endometrial tissue harvested during the receptive, but not the nonreceptive, period.

Glycosylation-dependent cell adhesion molecule 1

In the ovine uterus the expression of Glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1) was detected (Spencer et al., 1999). GlyCAM-1 is a sulfated glycoprotein that functions as carbohydrate ligand for L-selectin in the vasculature. It is secreted by the endothelium and mediates leukocyte-endothelial cell adhesion (Lasky et al., 1992; Rosen, 1993). In pregnant ewes, GlyCAM-1 in the luminal epithelium and stratum compactum glandular epithelium was low on days 11 and 13, increased on day 15, and was abundant on days 17 and 19. Immunoreactive GlyCAM-1 was also detected in the conceptus trophectoderm on days 13-19. The presence and pattern of expression of GlyCAM-1 in pregnant sheep endometria and conceptuses suggest a role in conceptus-maternal interaction during the peri-implantation period, presumably by acting as a selectin ligand.

2.1.4. The role of uterine secretions during the pre-implantation period

In contrast to rodents and primates, where the embryo attaches almost immediately to the uterine epithelium upon entering the uterus, domestic animals have a prolonged pre-implantation period upon arrival of the embryo in the uterus. During this period of time, the embryo is free floating in the uterine lumen and its survival, development and nourishment is dependent upon the secretion of the so called histotrophs from the uterine glands. The histotrophs are a complete mixture of enzymes, growth factors, cytokines, hormones, transport proteins and other substances (Bazer and Roberts, 1983) and evidence that endometrial gland secretions are required for conceptus development were obtained from observations of the ovine uterine gland knockout (UGKO) model. Ovine endometrial gland development is a postnatal event that can be inhibited by chronic exposure of ewe lambs to a synthetic progestin from birth to puberty. As adults these neonatally progestin-treated ewes lack endometrial glands and display an uterine gland knockout phenotype (Allison et al., 2000). When mature UGKO ewes were repeatedly bred to fertile rams, no pregnancies were observed by ultrasound on day 25. Transfer of day 7 blastocysts form normal superovulated ewes resulted in the establishment of a pregnancy in control ewes but not in UGKO ewes. When UGKO ewes were bred with fertile rams and the uteri were flushed on day 14, either no conceptus or severely growth retarded concepti were obtained. Histological analyses revealed that endometrial gland density was directly related to conceptus survival and developmental stage. Collectively, these results demonstrate that endometrial glands and, by interference, their secretions are required for peri-implantation conceptus survival and development (Gray et al., 2001). Furthermore, it has been shown that the expression of mucin 1, integrin alpha(v), alpha(5), beta(3), and beta(5) is not altered in UGKO ewes, whereas osteopontin and glycosylation-dependent cell adhesion molecule one (Gly-CAM1) are absent in uterine flushings from pregnant UGKO ewes. This further supports the hypothesis that UGKO are infertile due to the absence of uterine gland secretions rather than due the to altered expression of adhesion molecules (Gray et al., 2002).

There are hints that progesterone is the primary hormone regulating accumulation of fluid, protein, calcium, and specific endometrial proteins in the uterine lumen during gestation (Moffatt et al., 1987). The two best characterized glandular epithelium secretory products are the uterine milk protein and osteopontin.

Uterine milk protein

As shown by Moffatt and coworkers (1987), the uterine milk protein (UTMP) is the major component of uterine secretions obtained from pregnant ewes. On day 5 of gestation they ligated the uterine horn contralateral to the ovary bearing the corpus luteum to create a nongravid, ligated uterine horn and to confine the conceptus to the remaining nonligated uterine body and horn. Uterine fluids were obtained by aspirating the ligated uterine horn during later stages of pregnancy and UTMP was shown to be the predominant protein. Several authors have the expression of UTMP suggested to be regulated by progesterone: Amounts of UTMP in uterine flushings of ovariectomized ewes were greatly enhanced by subcutaneous administration of progesterone for 10 respectively 30 days when compared with the control group and immunhistochemical analysis indicated that the major site of UTMP localization was the glandular epithelium. In ovariectomized cows enhanced amounts of UTMP were not observed until after 30 days of progesterone treatment (Leslie and Hansen, 1991). Similar observations were made by Ing and coworkers (1989): progesterone treatment of ovariectomized ewes resulted in low levels of UTMP in uterine flushings, whereas increasing levels were observed after 14 and 30 days of progesterone administration. Production of a protein similar to UTMP was also noted in the uterus of a pregnant cow. Treatment of ovariectomized ewes with estrogens does not lead to enhanced presence of UTMP in uterine secretion (Moffatt et al., 1987). The findings of Bauersachs and coworkers (2005), however, contradict the common view that the expression of UTMP is directly stimulated by progesterone. In a global gene expression analysis, comparing bovine intercaruncular endometrium at two stages of the estrous cycle, end of the day of standing heat and day 12 of the estrous cycle, UTMP was found to be upregulated 150-fold at estrus.

Not only progesterone, respectively estrogen seems to influence the expression of UTMP, also administration of placental lactogen and growth hormone increases UTMP mRNA levels in the endometrium of ovariectomized ewes (Spencer et al., 1996). There is further evidence that lactogenic hormones play a role in the regulation of UTMP, as UTMP mRNA expression is correlated with placental lactogen production by the trophectoderm and state of superficial gland differentiation during pregnancy, which is driven by lactogenic hormones as well (Stewart et al., 2000).

Osteopontin

Osteopontin (OPN) is an acidic member of the small integrin-binding ligand N-linked glycoprotein (SIBLING) family of extracellular matrix proteins that undergoes extensive posttranslational modification (Johnson et al., 2003a). Osteopontin contains a Gly-Arg-Gly-Asp-Ser (GRGDS) sequence that binds to cell surface integrins to promote cell-cell attachment and it has been demonstrated that alpha(v)beta(5), alpha(v)beta(3), and alpha(v)beta(5) serve as receptors for osteopontin (Liaw et al., 1995; Hu et al., 1995; Bayless et al., 1998). Osteopontin is present in uterine flushings from pregnant ewes during the peri-implantation period (Johnson et al., 1999c) and immunoreactive osteopontin was shown to be localized to luminal and glandular epithelia, and to trophectoderm of day 19 conceptuses. Further, the alpha(v) and beta(3) integrins were detected on day 19 endometrium and conceptuses in the same study. This accumulation of osteopontin protein on endometrial luminal epithelium and conceptus trophectoderm led to the postulation that osteopontin induces adhesion between luminal epithelium and trophectoderm essential for implantation and placentation (Johnson et al., 1999b). In the rabbit uterus a dramamtic increase in osteopontin expression could be observed on days 4-7 of pregnancy, corresponding to the rise in circulating progesterone and the time of initial embryo attachment in this species and the mRNA was most prominently expressed on the endometrial epithelium. Induction of endometrial osteopontin expression was observed in unmated rabbits treated with progesterone and was prevented by cotreatment with an antiprogestin. Estradiol-17beta had no effect on osteopontin expression by itself (Apparao et al., 2003).

2.1.5. Remodeling of the ECM at implantation

The extracellular matrix (ECM) is a complex structural entity surrounding and supporting cells that are found within mammalian tissues. The ECM is often referred to as the connective tissue and is composed of 3 major classes of biomolecules: structural proteins, such as collagen and elastin, spezialized proteins, e.g. fibrillin, fibronectin, and laminin, and proteoglycans (Alberts et al., 2004). Remodeling of the endometrial extracellular matrix, mediated by an altered balance of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), is indispensable for successful implantation and placentation, and has been well described in humans and rodents (Farrar and Carson, 1992; Clark et al., 1993; Das

et al., 1997; Hurst and Palmay, 1999). Defects in the distribution of certain ECM glycoproteins have been implicated in implantation failure in women suffering from unexplained infertility (Bilalis et al., 1996) and functional studies using MMP inhibitors showed, that administration of these inhibitors retards decidual remodeling and growth during murine implantation (Alexander et al., 1996).

Although the bovine placenta is defined as epitheliochorial placenta, and trophoblast invasiveness is limited to fusion of migrating binucleate cells with uterine epithelial cells, extracellular matrix remodeling is a crucial event during the process of embryonic implantation. A change in the distribution pattern of ECM components, namely types I and IV collagen, laminin and fibronectin is evident by day 14 of gestation, as their immunoreactivity remarkably declines. Interestingly, immunoreactivity is reconstituted by day 30 of gestation, as implantation is completed (Yamada et al., 2002). In the caprine endometrium similar observations were made: as implantation proceeded, immunolabeling of the ECM proteins type I and IV collagen and laminin became undetectable in the endometrium adherent to the trophoblast. In the uterine compartments not in contact with the trophoblast, immunostaining remained unaltered, which suggests a local control of protease activity within the endometrium (Guillomot, 1999). The expression of MMPs and TIMPs has been studied at the trophoblast-uterine interface in the ewe. Messenger RNA for TIMP-1 and TIMP-2 is expressed in the endometrium throughout the estrous cycle and early pregnancy. Both TIMP-1 and TIMP-2 mRNA expression increases at day 12 to day 20. Uterine flushings from both non-pregnant and pregnant ewes on day 16 after estrus contain proMMP-2 and proMMP-9, but concentrations are higher in pregnancy. The production of MMPs and TIMPs in the endometrium and the changes associated with implantation suggest that they contribute to the marked endometrial remodeling associated with early placentation (Salamonsen et al., 1995).

2.2. Maternal recognition of pregnancy and the pivotal role of IFNτ

The bovine species exhibits an aseasonal polyestric sexual cycle with an average length of 21 days and the day of standing heat is designated as day 0 of the estrous cycle (Rüsse and Sinowatz, 1998). During the time course of each sexual cycle a dominant follicle is raised, and after ovulation the corpus luteum cyclicum is formed, which produces progesterone during the luteal phase of the estrous cycle. In the absence of a fertilized oocyte, an uterine-dependent luteolytic mechanism comes into

effect, resulting in the regression of the corpus luteum cyclicum approximately on day 16 of the sexual cycle (Döcke, 1994). The luteolytic factor produced by the endometrium is $PGF_{2\alpha}$, and binding of luteal oxytocin to its endometrial receptor is assumed to initiate pulsatile release of $PGF_{2\alpha}$ (Flint and Sheldrick, 1983). Hence, for successful establishment of a pregnancy, the embryo has to signal its presence to the uterine environment in order to abrogate the luteolytic mechanism described above. The maintenance of the corpus luteum ensures ongoing secretion of progesterone, which is a prerequisite for early embryonic development, implantation, placentation, and overall the successful outcome of gestation. Roger Short referred to this embryo-maternal cross-talk in 1969 as the process of “maternal recognition of pregnancy”.

The factors produced by the embryo to signal its presence vary considerably within different species, and in ruminants IFNτ, which has been shown to closely resemble the interferon-alpha family of antiviral proteins (Imakawa et al., 1987; Stewart et al., 1987; Stewart et al., 1989), especially the interferon alphas of class II (Charpigny et al., 1988; Charlier et al., 1989), is assumed to play a pivotal role in the context of maternal recognition of pregnancy. The trophoblast interferons were discovered in 1987 through the purification of ovine blastocyst secreted proteins with antiluteolytic properties. Their biological function was assessed, among other assays, by administration into the uterus of cyclic sheep and cows, which resulted in the prolongation of the estrous cycle (Knickerbocker et al., 1986; Stewart et al., 1987; Vallet et al., 1988; Meyer et al., 1995). Despite its clear role as reproductive hormone, IFNτ has the typical antiviral, antiproliferative, and immunmodulatory activities of other type I IFNs, but unlike, it is not virally inducible (Roberts, 1996).

Interferon-tau is synthesized and secreted by mononuclear cells of the bovine conceptus trophectoderm between days 15 to 26 postconception, with maximal secretion occurring around day 18 (Bazer et al., 1997). It acts in a paracrine manner on the endometrium to establish and maintain pregnancy by suppressing the normal pattern of pulsatile release of uterine $PFG_{2\alpha}$ leading to luteolysis at the end of the estrous cycle. Several studies in the ewe have shown that IFNτ suppresses cyclic increases of the estrogen receptor gene transcription and thereby prevents estrogen induced increases in oxytocin receptor gene expression, which normally occurs in cyclic ewes undergoing luteolysis (Knickerbocker et al., 1986; Spencer et al., 1995). The situation in the cow differs a little, since the expression of the oxytocin receptor

gene, but not the estrogen receptor gene, is altered due to the presence of a conceptus, implicating that the bovine conceptus exerts a direct effect on endometrial oxytocin receptor gene expression (Robinson et al., 1999). Even though one may assume, IFNτ does not act to stabilize or prevent autologous downregulation of the progesterone receptor mRNA and protein expression in the endometrium, which normally occurs towards the end of the estrous cycle (Spencer et al., 1995).

Furthermore, IFNτ acts on the endometrial luminal epithelium, the glandular epithelium and the stroma to regulate the expression of a number of IFN-stimulated genes (ISGs) which are hypothesized to play roles in endometrial differentiation and conceptus implantation (Thatcher et al., 1995). One of the most intensely investigated gene product induced by IFNτ, is the *interferon stimulated gene 15* (ISG15) also called ISG17 or ubiquitin cross reactive protein (UCRP), due to its crossreactivity with antibodies against ubiquitin (Austin et al., 1996). Johnson et al. (1999a) showed by Northern Blotting that ISG15 mRNA is present in endometrial tissue samples of day 18 pregnant cows, but is absent in the endometrium from non-pregnant control animals. *In situ* hybridization localized the mRNA to the stromal and glandular epithelial cells of pregnant cows. Several experiments have shown that IFNτ treatment of endometrial explants is able to induce the expression of both ISG15 mRNA and protein after a certain period of time (Staggs et al., 1998; Johnson et al., 1999a). Further, Western blot analysis revealed elevated levels of ISG15 protein (ISG15 conjugates) on day 17 of pregnancy when compared with the estrous cycle. ISG15 protein remained elevated from days 18 to 23 and levels declined sharply from days 23 to 45 and on day 50 of pregnancy no difference could be observed between pregnant and non-pregnant cows (Austin et al., 2004). The ubiquitin crossreactive protein conjugates to endometrial cytosolic proteins and thereby may regulate uterine proteins important for maintenance of early pregnancy (Johnson et al., 1998). Since the ISG15 gene contains an interferon response element (ISRE) (Perry et al., 1999), one can assume that it is only upregulated in ruminants, where conceptus derived IFNτ is believed to be responsible for establishment of pregnancy. But nevertheless, upregulation of the ubiquitin homolog seems to be a universal response to the embryo, as it is upregulated in many different species, including humans, baboons, cows, sheep, swine, and mice (Chwetzoff and d'Andrea, 1997; Bebington et al., 1999; Austin et al., 2003).

Granulocyte-macrophage colony-stimulating factor (GM-CSF), known to promote survival and growth of embryos, is expressed by the ruminant endometrium during early pregnancy and in response to intrauterine infusions of IFNτ (Teixeira et al., 1997; Emond et al., 2004). Separate culture of endometrial epithelial and stromal cells showed that high basal production of GM-CSF by epithelial cells is not regulated by recombinant ovine IFNτ. In contrast GM-CSF gene expression was readily stimulated in stromal cells (Emond et al., 2000). *Granulocyte chemotatic protein-2*, a member of the alpha-chemokine family is also released by cultured endometrial cells in response to IFNτ (Teixeira et al., 1997; Staggs et al., 1998). Transcripts of the *1-8 family* (1-8 and LeuU 13/9-27) of interferon inducible genes are elevated in endometrial tissue samples from pregnant cows when compared with non-pregnant animals. The mRNA is mainly localized to the glandular epithelium and to a lesser degree to the luminal epithelium, stroma and myometrium. In endometrial cells, treatment with recombinant IFNτ upregulated the gene expression of 1-8 family members (Pru et al., 2001). Pru and coworkers hypothesize that these proteins act as novel E2-like enzymes and therefore facilitate ligation of ISG15 to cytosolic proteins. *Mx*, a functional GTPase, is an IFN-induced antiviral protein. Its expression is directly induced in response to viral infections (Muller-Doblies et al., 2002) as well as during early pregnancy in both endometrium and myometrium (Ott et al., 1998; Hicks et al., 2003), where steady-state levels of Mx mRNA increase 10-fold above levels observed in cyclic cows by day 15 to 18.

On days 15 and 18 of bovine gestation, the *2´-5´-oligoadenylate synthase* activity was greater in endometrial surface epithelium, glandular epithelium, and stromal cells, when compared with the corresponding day of the estrous cycle. Additionally, in cultured bovine uterine cells a dose dependent stimulation of 2´-5´-oligoadenylate synthase by IFNτ could be observed (Schmitt et al., 1993).

The results of a study conducted by Spencer et al. (1998) suggest that IFNτ acts directly on the luminal and superficial glandular epithelium to during ovine pregnancy to sequentially induce *IRF-1* and then *IRF-2* gene expression. The induction of these genes is correlated temporally with the absence of the estrogen receptor as well as the oyxtocin receptor. The ovine estrogen receptor gene may contain an IFNτ responsive element that binds negative-acting, IFNτ inducible transcription factors, such as IRF-2, which silences transcription of the estrogen receptor gene in the endometrial epithelium during maternal recognition of pregnancy, thereby preventing

the luteolysis of the corpus luteum. Additionally, IRF-2 is made responsible for the phenomen that in the pregnant uterus interferon stimulated genes are induced or upregulated in the endometrial stroma and glandular epithelium, but expression of these genes is not detectable in the endometrial luminal epithelium or superficial glandular epithelium because expression of IRF-2 in the pregnant uterus is only detectable in the luminal epithelium and superficial glandular epithelium (Choi et al., 2001). Thus, IFNτ supports the maintenance of a pregnancy via multiple mechanisms

2.3. Mechanism of action of interferon tau on endometrial cells

All type I interferons, including IFNτ, exert their action through binding to classical type I IFN receptors which consist of two transmembrane subunits, IFNAR1 and IFNAR2 (Li and Roberts, 1994). Immunhistochemistry revealed that both polypeptide subunits are expressed in the ovine endometrium and that the major site of expression is the luminal and superficial glandular epithelium. The caruncular stromal cells also express IFNAR1 and IFNAR2 subunits, whereas the glandular epithelial cells near the myometrium are negative for both polypeptides. To localize the binding sites of IFNτ, immunhistochemistry with an antiserum against IFNτ was performed on uterine sections from day 14 non-pregnant ewes whose uteri had previously been infused with IFNτ. Staining was concentrated on the LE and superficial GE cells, and was absent from the deeper regions of the glands and from the stromal tissues, indicating that the LE and the superficial GE are the main targets for IFNτ (Rosenfeld et al., 2002).

The intracellular domains of both receptor subunits are associated with cytoplasmatic tyrosine kinases (Janus kinases, JAKs); IFNAR1 is associated with Tyk2 and IFNAR2 is associated with Jak1. Binding of IFNτ to its receptor leads to activation of the JAKs, which in turn phosphorylate each other leading to subsequent phosphorylation of receptor tyrosine residues. Once the receptor subunits are phosphorylated, signal transducers and activators of transcription (STAT) proteins are bound to the phosphotyrosine residues via their SH2 domain. After docking of the STAT proteins to the receptor subunits they become tyrosine phosphorylated by the JAKs with subsequent dissociation of the phosphorylated STATs. The phosphorylated STATs are now able to form homo- and heterodimers which are shuttled into the nucleus where they influence the expression of certain genes (Alberts et al., 2004) (see Fig. 1). Of particular interest is the STAT1-2 homodimer

because it associates with the interferon regulatory factor (IRF) protein 9, also known as p48 or ISGF3G, to form the interferon-stimulated gene factor 3 (ISGF3) transcription factor complex (Fu et al., 1990; Schindler et al., 1992; Veals et al., 1992). The ISGF3 complex is translocated into the nucleus, where the IRF9 component acts as a sequence specific DNA-binding protein and confers recognition upon the activated STAT1-2 homodimer (Levy et al., 1989; Veals et al., 1993; Horvath et al., 1996; Martinez-Moczygemba et al., 1997). The contribution of IRF9 as an adapter for the STAT1-2 heterodimer represents a unique feature of the ISGF3 complex, as other STAT-signaling pathways involve STAT homodimers that bind directly to DNA. The ISGF3 complex binds to interferon-stimulated response elements (ISREs), resulting in the expression of interferon stimulated genes (ISGs) (Fu et al., 1990) such as ISG15 (Kessler et al., 1988; Zhao et al., 2004) and OAS (Floyd-Smith et al., 1999; Zhao et al., 2004). STAT1 homodimers (γ –activated factor, GAF) increase the expression of genes through gamma activated sequences (GAS) elements in the promotor region of genes, such as the interferon regulatory factor 1 (IRF-1) gene (Pine et al., 1994). The interferon regulatory factor 1 in turn activates IRF-2, a transcriptional repressor which is proposed to block directly or indirectly the expression of the oxytocin receptor, thereby preventing the uterine-dependent luteolytic mechanism and ensuring the establishment of pregnancy (Bazer et al., 1997).

The signal transduction cascade has been studied more detailed using an ovine endometrial cell line (Stewart et al., 2001). Within 30 min of IFNτ stimulation, STAT1, -2, -3, 5a/b, and 6 become tyrosine phosphorylated and translocated to the nucleus. However, in response to stimulation with IFNτ for longer than 30 min, STAT1 and -2 remain tyrosine phosphorylated, whereas STAT3, -5a/b, and -6 are rapidly dephosphorylated. Because STAT1 and STAT2 are persistently tyrosine phsophorylated it is likely that only these two STAT proteins mediate the long-term effects of IFNτ on target gene expression relevant for establishing of pregnancy. Whether STAT3, -5a/b, and -6 form dimers in response to IFNτ and regulate gene expression is not known yet.

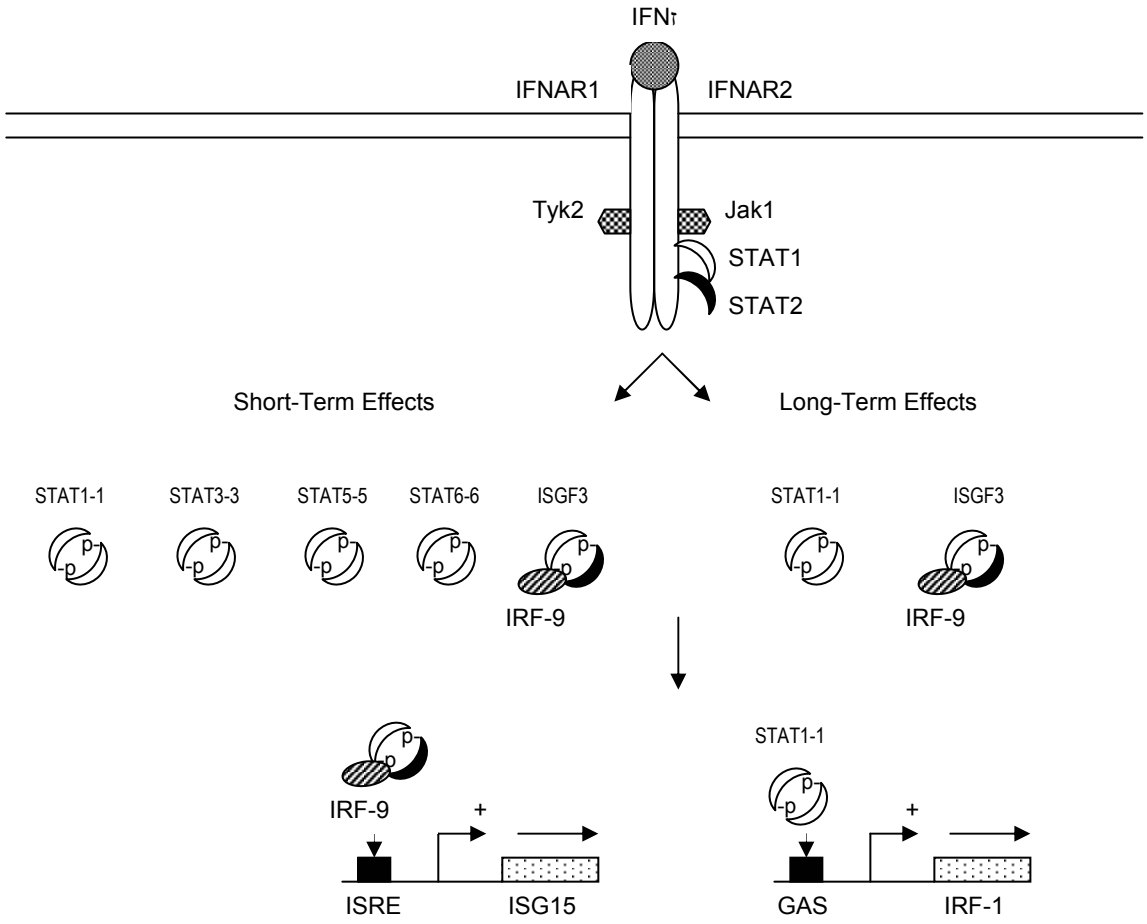


Fig. 1: Signal transduction cascade of IFNτ

For further explanation see text.

2.4. Holistic approaches for the analysis of the transcriptome

Nearly each cell contains a complete genome, but there is a distinct pattern of gene activity for every single cell type and also for different physiological stages of a cell or tissue. Biological processes, such as the embryo-maternal cross-talk, are associated with differential gene expression patterns resulting in characteristic cell and stage specific protein repertoires in the corresponding tissues. Thus, a powerful approach to analyze the regulation and the nature of a biological process at the molecular level is to characterize genes that vary in expression levels of their mRNAs during this process.

Besides targeting specific genes, the global description of transcriptional activity is of particular interest. Techniques for the analysis of small sets of candidate genes include for example quantitative RT-PCR, *in situ* hybridization and Northern blotting. For the systematic identification of differentially expressed genes techniques like

differential display PCR (DD-PCR) (Liang and Pardee, 1992), serial analysis of gene expression (SAGE) (Velculescu et al., 1995), hybridization to gene arrays of various formats (Chee et al., 1996) or suppression subtractive hybridization (SSH) (Diatchenko et al., 1996) can be udsed. Suppression subtractive hybridization (SSH), a highly efficient method for the generation of subtracted cDNA libraries and the screening of such libraries by cDNA array hybridization is now described in detail.

SSH combines subtractive hybridization and the so-called suppressor PCR. Subtraction of common sequences within two populations of cDNAs is achieved by subtractive hybridization, which involves hybridization of cDNAs from one population to cDNAs of the second population and subsequent separation of the unhybridized fraction from hybridized, common sequences. First both mRNA populations are converted into cDNA. The cDNA population that contains specific, differentially expressed transcripts of interest is referred to as “tester” and the reference cDNA is reffered to as “driver”. Both cDNA populations are digested with a restriction enzyme that yields blunt ends. The tester cDNA is then subdivided into two portions, and each portion is ligated with a different adapter composed of a long and a short oligonucleotide. The 5´-ends of the adapters do not have a phosphate group, so that only the long oligonucleotide of the adapter is ligated to the 5´-end of each cDNA. The driver cDNA sample is left without adapters. Subsequently two rounds of hybridization are performed. In the first round, an excess of driver cDNA is added to each sample of the tester cDNA resulting in the formation of the molecules ‘a’, ‘b’, ‘c’, and ‘d’ in each sample (see Fig. 2). The type ‘a’ molecules represent differentially expressed sequences that are only present in the tester cDNA population. The concentration of high- and low abundance sequences is equalized among the single strand type ‘a’ molecules because reannealing forming the type ‘b’ molecules is faster for the more abundant molecules due to the second order kinetics of hybridization (a process referred to as normalization). Those cDNAs that are not differentially expressed within the two populations form the type ‘c’ molecules. The type ‘d’ molecules represent sequences that are only present in the driver cDNA population. For the second round of hybridization the two hybridization samples are mixed together. Now, only the remaining equalized and subtracted single stranded tester cDNAs (type ‘a’ molecules) can hybridize and form new type ‘e’ molecules. These new hybrids are double stranded tester molecules with different ends, which correspond to the sequences of the two different adapters. The entire population is

then subjected to PCR. The type ‘a’ and ‘d’ molecules are missing primer annealing sites and thus cannot be amplified. Most type ‘b’ molecules form a panhandle-like structure because they possess the same adapter on each end. This panhandle-like structure prevents their exponential amplification, a phenomen referred to as suppressor PCR effect. Type ‘c’ molecules only have one primer annealing site and can only be amplified linearly. Only type ‘e’ molecules, which possess two different adapters, can be amplified exponentially. These are the equalized, differentially expressed sequences. The PCR products can then be utilized to establish a subtracted cDNA library.

The major disadvantage of subtracted cDNA libraries is the presence of background clones representing non-differentially expressed cDNA species in the subtracted library. Therefore, a further step is necessary to identify the differentially expressed cDNA fragments within a subtracted library. This can be accomplished by transferring a certain number of individual cDNA clones from the subtracted library onto a nylon membrane and subsequent array hybridization with labeled cDNA probes derived from both cDNA populations. Those cDNA clones that show a difference in signal intensity are differentially expressed and hence subjected to further analyses, e.g. DNA sequencing, and bioinformatic analyses.

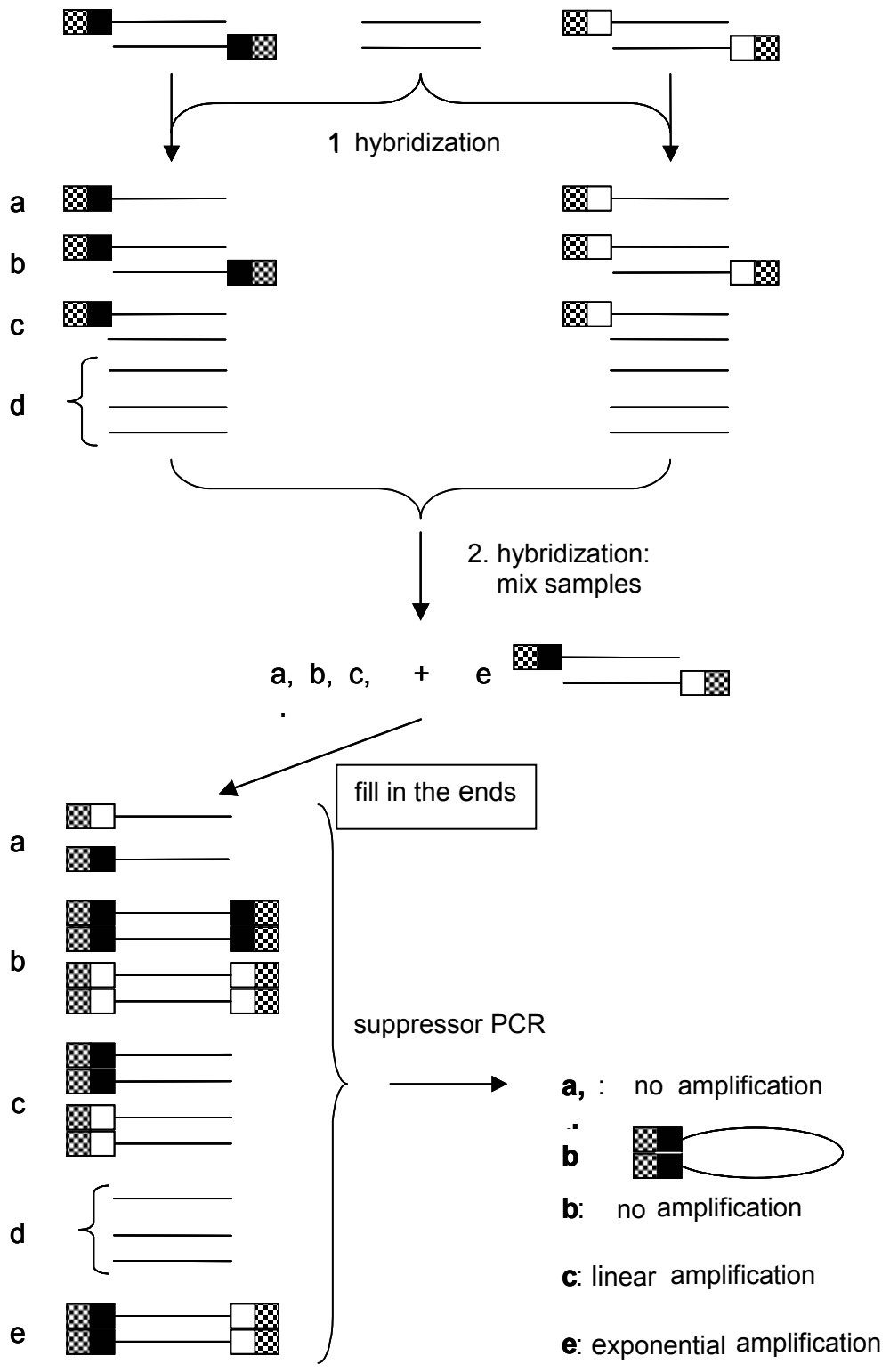


Fig. 2: Scheme of the SSH method according to Diatchenko et al. (1996).

Solid lines represent the *RsaI* digested driver or tester cDNA. The boxes represent the outer and inner parts of adapter 1 and adapter 2, respectively.

2.5. Global gene expression analyses of female reproductive tissues

As already mentioned above (2.4), global gene expression profiling is a powerful method to gain deeper insight into the molecular basics of biological processes and a number of studies of this kind dealing with female reproductive tissues have been performed so far in several species.

In humans several studies using commercially available microarrays were done investigating gene expression changes occurring in the endometrium during the menstrual cycle. In two studies, the proliferative phase of the menstrual cycle was compared with the phase coincident with the “window of implantation” (Kao et al., 2002; Carson et al., 2002) and in another three studies gene expression differences between the early secretory phase (2-4 days after the LH-surge) and mid-luteal phase (days 7-9 after the LH-surge) were analyzed (Martin et al., 2002; Riesewijk et al., 2003; Borthwick et al., 2003). Endometrial gene expression of the proliferative phase versus the secretory phase of the sexual cycle was also compared in the rhesus monkey (Ace and Okulicz, 2004) and in the mouse (Tan et al., 2003).

Tanaka et al. (2000) applied a mouse developmental cDNA microarray to elucidate divergence of embryo-derived mid-gestation placenta and embryonic gene expression profiles.

In order to identify progesterone-regulated pathways in the uterus during implantation, mice were treated at day 3 of pregnancy with either RU486 (a progesterone receptor antagonist) or a vehicle (Cheon et al., 2002). Tissues were collected on day 4 of pregnancy and conducted to microarray analysis to determine the difference in gene expression. To gain knowledge about genes whose expression is regulated by estrogens, Andrade and others (2002) compared endometrial mRNA profiles of ovariectomized virgin rats either treated with conjugated equine estrogens or a placebo.

Bauersachs and coworkers (2003) used a combination of subtracted cDNA libraries and array hybridization to compare gene expression profiles of bovine epithelial cells from the ipsilateral oviduct versus the contralateral oviduct at day 3.5 of the estrous cycle. In an additional study they evaluated the differerence in gene expression profiles of epithelial cells from the ipsilateral oviduct at estrus and diestrus (Bauersachs et al., 2004). They furthermore performed a holistic screen of transcriptome changes in bovine intercaruncular endometrium at the day of standing heat and day 12 of the estrous cycle (Bauersachs et al., 2005).

Reese et al. (2001) compared mRNA profiles of implantation and interimplantation sites in mice at day 4 of pregnancy. They further compared the uterine gene expression profile of ovariectomized progesterone-treated, delayed implanting mice to that of mice in which delayed implantation was terminated by estrogen activation. Recently, suppression subtractive hybridization and an array of oligonucleotides, respectively was applied to determine genes involved in the early process of embryonic implantation in the rhesus monkey (Sun et al., 2004) and in mice (Yoshioka et al., 2000). Until now, only one approach was undertaken in the bovine species to characterize gene expression profiles in the bovine endometrium during pregnancy. Ishiwata et al. (2003) collected endometrial (caruncular and intercaruncular) and placental (cotyledonary and intercotyledonary) tissue samples on days 0 and 10 of the estrus cycle and days 30, 60, 100, and 245 of gestation to establish a normalized cDNA library. For microarray analysis endometrial and placental tissue were obtained on day 13 of the estrous cycle and days 56, 59, and 64 of gestation.

So far, no study was conducted to investigate endometrial gene expression changes occurring in the endometrium during the pre-implantation period due to the presence of a conceptus in the bovine species.

2.6. The usage of monozygotic twin pairs as experimental animals

When performing gene expression studies which utilize tissue samples obtained from different individuals, it has to be considered that monitored differences in gene expression levels are partly due to a different genetic background of individuals, rather than due to the influence of a certain condition investigated (e.g. the presence of a conceptus in the uterine lumen). Hence, an experimental strategy which eliminates the influence of a different genetic background of individuals on the results of gene expression studies would be of great benefit. Such an experimental design is the exploitation of monozygotic twin uniformity, which arises from the fact that monozygotic twin pairs are genetically identical. Only few global gene expression studies, exploring the potential usefulness of this approach have been performed so far (Evans et al., 1999; Mak et al., 2004).

Embryo splitting has been described to be a viable method for producing monozygotic twins (Niemann and Meinecke, 1993). Clones produced by embryo splitting are completely genetically identical with respect to nuclear as well as

mitochondrial DNA, whereas clones produced by nuclear transfer exhibit a various degree of mitochondrial heterogeneity (Evans et al. 1999). Consequently, the usage of monozygotic twin pairs as experimental model is a unique possibility to eliminate the influence of a different gentic background of individuals on the results of gene expression analyses.

3. Materials and Methods

3.1. Materials

3.1.1. Chemicals

Agarose (15510-027)	Invitrogen, Karlsruhe
Agarose (Typ VII Low Gelling)	Sigma, Deisenhofen
Agarose Seakem LE	Biozym, Hess. Oldendorf
ammonium acetat	Merck, Darmstadt
ampicillin	Boehringer, Mannheim
bromphenolblue	Serva, Heidelberg
chloroform	Riedel-de Hâen, Deisenhofen
DTT (100 mM)	Invitrogen, Karlsruhe
EDTA	Merck, Darmstadt
ethanol	Roth, Karlsruhe
ethidium bromide (10mg/ml)	Roth, Karlsruhe
glycerol	Fluka, Buchs, Switzerland
isopropanol	Roth, Karlsruhe
magnesium chloride ($MgCl_2$)	Merck, Darmstadt
mineral oil (Nujol)	Perkin Elmer, Köln
SDS ultrapure	ICN, Irvine, CA, USA
sodium acetate	Merck, Darmstadt
sodiumchloride	Merck, Darmstadt
sodium hydroxide	Merck, Darmstadt
Tris-(hydroxymethyl)-amino-methan (Tris)	Roth, Karlsruhe
Tris-HCl (pH 8.4)	Roth, Karlsruhe

3.1.2. Enzymes and other reagents

α-[33P]-dCTP (2500 Ci/mmol)	Amersham Bioscience, Freiburg
Advantage2 cDNA Polymerase Mix	BD Clontech, Heidelberg
AscI (10 U/µl)	New England Biolabs, Beverly, USA
Better Buffer	Web Scientific, Cheshire, UK
BigDye® Terminator v1.1/v3.1	Applied Biosystems, Langen

Sequencing Buffer (5X)	
BSA (10 mg/ml)	New England Biolabs, Beverly, USA
BssHII (4U/µl)	New England Biolabs, Beverly, USA
buffer L	Roche Diagnostics, Mannheim
dephosphorylization buffer (10x)	Roche Diagnostics, Mannheim
DNA ligase (E. coli) (10 U/µl)	Invitrogen, Karlsruhe
DNA polymerase I (E. coli) (10 U/µl)	Invitrogen, Karlsruhe
dNTP	Roche Diagnostics, Mannheim
DYEnamic ET Terminator	Amersham Bioscience, Freiburg
Cylce Sequencing Kit	
E. coli SURE©	Stratagene, Heidelberg
Estrumate®	Essex Tierarznei, München
FIREPol	AGT biosynthesis, Merzhausen
first-strand buffer (5x)	Invitrogen, Karlsruhe
High Prime DNA Labeling Kit	Roche Diagnostics, Mannheim
Human C0T-1 DNA (1 mg/ml)	Invitrogen, Karlsruhe
ligasebuffer (10x) for T4-ligase	New England Biolabs, Beverly, USA
Low Gelling Type VII Agarose	Sigma, Deisenhofen
NEBuffer3	New England Biolabs, Beverly, USA
NEBuffer4	New England Biolabs, Beverly, USA
pBSIISK$^-$MO (208 ng/µl)	Stratagene, Heidelberg
PCR buffer (10x)	BD, Clontech, Heidelberg
phenol-chloroform-isoamylalcohol (25:24:1, TE saturated, pH 7.5)	Merck, Darmstadt
QIA prep Spin Miniprep Kit	Quiagen, Hilden
RNase, DNase-free (0.5 mg/ml)	Roche Diagnostics, Mannheim
RNaseH (E. coli) (2 U/µl)	Invitrogen, Karlsruhe
RNase-Inhbitor (40 U/µl)	Invitrogen, Karlsruhe
RNAlater™	Ambion, Huntigton, UK
RsaI (10 U/µl)	Roche Diagnostics, Mannheim
second-strand buffer (5x)	Invitrogen, Karlsruhe
Shrimps-AP (1 U/µl)	Roche Diagnostics, Mannheim
Superscript III (200 U/µl)	Invitrogen, Karlsruhe
T4-DNA Polymerase (1 U/µl)	Roche Diagnostics, Mannheim

T4-DNA-Ligase (2000 U/µl)	New England Biolabs, Beverly, USA
Trizol®Reagent	Invitrogen, Karlsruhe

3.1.3. Apparatuses

3100-Avant Genetic Analyzer	Applied Biosystems, Langen
agarose gel electrophoresis chamber	Harnischmacher, Fröndenberg
electronic dispensing system EDOS 5222	Eppendorf, Hamburg
E. coli-Pulser®	BioRad, Hercules, Ca, USA
Eagle Eye® II	Stratagene, Heidelberg
DU® 640 Spectrophotometer	Beckman, München
heater with magnetic stirrer	IKA Labortechnik, Staufen
HERAhybrid 12, hybridiaztion oven	Kendro Laboratory Products, Langenselbold
Horizon® 58	Invitrogen, Karlsruhe
incubator WTB	Binder, Tuttlingen
incubator shaker KF-4	Infors, Bottmingen
microwave Electronic M 708	Miele, Gütersloh
multi channel pipette	Eppendorf, Hamburg
Omnigrid Accent Microarrayer	Gene Machines, San Carlos, USA
oven	Heraeus, München
Pioetus®	Hirschmann Laborgeräte,
Pipetman®	Gilson, Bad Camberg
precise balance	Sartorius, Göttingen
shaker IKA-Vibrax®	IKA Labortechnik, Staufen
Solid pins (SSP015) für Omnigrid Acc.	Telechem International, Suunyvale, USA
Storm 860	Amersham Bioscience, Freiburg
tissue homogenizer (DIAX 900)	ART Labortechnik, Mühlheim
Vortex-Genie 2	Scientifics Industries, Bohemia, USA
water bath	Memmert, Schwabach
XL-1500 UV-Crosslinker	Spectronics Corp., New York, USA
Thermocyclers	
PerkinElmer Cetus, 480	Perkin Elmer, Köln
T1 Thermocycler T3	Biometra, Göttingen

Centrifuges

Centrifuge (5417R)	Eppendorf, Hamburg
GS-15R	Beckman, München
Sorvall® RC 5C	Kendro Laboratory Products, Langenselbold

3.1.4. Other Materials

384-Well Storage Plate	Abgene, Hamburg
adhesive PCR Foils Seal	ABgene, Hamburg
AIDA Image Analyzer, Version 4.00	Raytest, Straubenhardt
Bibliosphere software,Version 5.02	GenomatiX, Munich
centrifuge tubes (15ml)	Josef Peske oHG, Aindling
cryotube	Nunc, Wiesbaden
electroporation cuvette	BioRad, Hercules, CA, USA
gel blotting paper (GB002, GB005)	Schleicher & Schuell, Dassel
glass hybridization bottles	VWR, Darmstadt
imaging plate BAS-IP SR 2025	Fujifilm, Kanagawa, Japan
MF-Millipore Membrane Filter (0.25 µm)	Millipore, Billerica, Mass., USA
MicroSpin™ G-25 Columns	Amersham Bioscience, Freiburg
MicroSpin™ S200-HR	Amersham Bioscience, Freiburg
Miltex® GP (0.22µm)	Milipore, Carrigtwohill, Ireland
Nytran® SuperCharge	Schleicher & Schuell, Dassel
PCR reaction tubes (0.2, 0.5 ml)	Perkin Elmer, Köln
ProbeQuant™ G-50 Micro Columns	Amersham Bioscience, Freiburg
QIAquick Gel Extraction Kit	Quiagen, Hilden
reaction tubes „safe lock“ (2, 1,5, 0,5, 0,2 ml)	Eppendorf, Hamburg
Thermo-Fast®, 96, Low Profile	AbGene, Hamburg
tube with cap, 84x30 mm	Sarstedt, Nümbrecht

3.1.5. Media and Solutions

better buffer special

1 µl	Better Buffer
1 µl	BigDye® Terminator v1.1/v3.1 Sequencing Buffer (5X)
1 µl	deionized water

PBS (10x)

Solution A:

250 mM	Na_2HPO_4
1.25 mM	NaCl

Solution B:

250 mM	Na_2HPO_4
1.25 mM	NaCl

Solution B is added to solution A until pH = 7.5 is reached.

TE buffer

10 mM	Tris-HCl, (pH 8,0)
1 mM	EDTA

TBE electrophoresis buffer (10x)

108 g	Tris-Base
55 g	boric acid
40 ml	0,5 M EDTA, (pH 8,0)

fill up with deionized water at 1000 ml

SOB-medium

2 %	trypton
0.5 %	yeast extract
0.05 %	NaCl
10 mM	$MgCl_2$
10 mM	$MgSO_4$

SOC-medium

2 %	trypton
0.5 %	yeast extract
0.05 %	NaCl
10 mM	$MgCl_2$
10 mM	$MgSO_4$
0.4 %	Glucose

loading buffer for agarose gel elctrophoresis (2.5x)

4 M	urea
5 g	saccharose
0.5 M	EDTA (pH 8,0)
0.1 %	bromphenolblue

dilution buffer for SSH

20 mM	HEPES (pH 8,3)
50 mM	NaCl
0.2 mM	EDTA

LB (luria broth)-medium (low salt)

1 %	Trypton
0,5 %	yeast extract
0.5 %	NaCl

Adjust pH at 7 - 7.5 with 0.1 % 2 M NaOH.

LB-agar plates

1,2 %	agar
1 %	trypton
0,5 %	yeast extract
0.5 %	NaCl

Adjust pH at 7 – 7.5 with 0.1 % 2 M NaOH; ad ampicillin to a final concentration of 100 µg/ml.

hybridization buffer for SSH (4x)

200 mM	HEPES (pH 8,3)
2 M	NaCl
40 %	PEG 8000
0,08 mM	EDTA

reaction buffer (10x) for FIREPol®

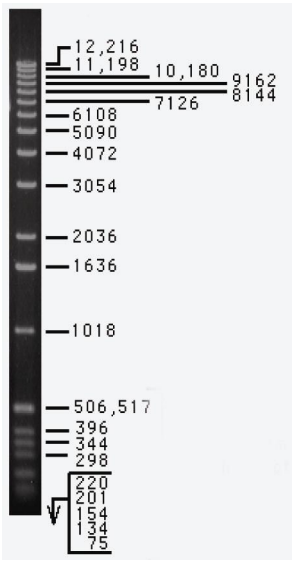
200 mM	Tris-HCl (pH (8.55)
160 mM	$(NH_4)_2SO_4$
20 mM	$MgCl_2$

3.1.6. Oligonucleotides

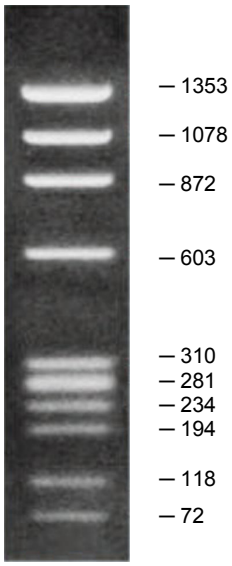
cDNA primer 1:	3'-AACTGCGGCCGCGTACAGCT$_{20}$VN (V=A, C or G)-5'
cDNA primer 2:	3'-GAGAT$_{20}$VN-5'
adapter 1:	5'-GTAATACGACTCACTATAGGGCTGGAGCGCGCCGC AGGGCAGTG-3'
adapter 1 reverse:	3'-GGGCGTCCCGTCAC-5´
adapter 2:	5'-GTAATACGACTCACTATAGGGCAGGGCGTGGTGC GCGCTGCTGG-3'
adapter 2 reverse:	5'-CCAGCAGCGCGCAG-3'
PCR primer 1:	5'-GTAATACGACTCACTATAGGGC-3'
nested primer 1:	5'-TCGAGCGCGCCGCAGGGCAGTG-3'
nested primer 2:	5'-AGGGCGTGGTGCGCGCTGCTGG-3'
NLT7:	3'-GCGTAATACGACTCACTATAGG
LT3:	3'-CTCACTAAAGGGAACAAAAGCTG
T7ex:	3'-GTAATACGACTCACTATAGGGC
PM0:	3'-CCAAATATCTCTCCCACCAGC

3.1.7. DNA molecular weight markers

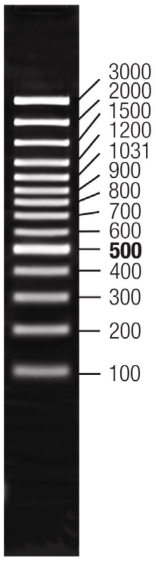
1 kb DNA Ladder (15615-016)	Invitrogen, Karlsruhe
DNA Molecular Weight Marker IX	Roche, Mannheim
GeneRuler™ 100 bp DNA Ladder Plus	Fermentas



1 kb DNA Ladder (15615-016)



DNA Molecular Weight Marker IX



GeneRuler™ 100 bp DNA Ladder Plus

3.2. Methods

3.2.1. Animals and tissue collection

The estrous cycle of five monozygotic twin pairs (Simmental cows, non-lactating), generated by embryo splitting, which had given birth to an average of 1.7 calves previously, was synchronized by a single intramuscular injection of 500 µg Cloprostenol (Estrumate®, Essex Tierarznei, München) at diestrus. Animals were observed for sexual behavior (i.e. toleration, sweating, vaginal mucus) to determine standing heat, which occurred around 60 h after Estrumate® injection. Seven days after standing heat, two *in vitro* produced bovine blastocysts (day 7 after *in vitro* fertilization) in 200 µl culture medium were transferred into the ipsilateral uterine horn of one twin of each pair. *In vitro* production of embryos was performed as described previously (Stojkovic et al., 1999) with minor modifications. The corresponding twin received a sham-transfer of the same amount of transfer medium without embryos as a control. On Day 18 of pregnancy, or of the estrous cycle, respectively, the animals were slaughtered. Blood samples for determination of serum progesterone levels were drawn just before slaughter. All animals displayed high progesterone values (mean 3.7 ng/ml), which did not differ significantly between both groups, indicating the presence of an endocrinologically active corpus luteum. The uterine horns were opened longitudinally with a scissors and intercaruncular endometrial tissue samples from defined uterine regions (for further details see fig. 3) were dissected using a scalpel. Pregnancy was confirmed by the presence of an apparently normal conceptus in the uterine lumen. The tissue samples were transferred into tubes containing 4 ml of RNAlater™ within 23 minutes after slaughter, stored overnight at 4°C and then at -20°C until further processing. For gene expression analyses only those tissues samples of pregnant animals, which had been in contact with an embryo were used, i.e. the middle and caudal sections of the uterine horn ipsilateral to the ovary bearing the corpus luteum. From control animals the same uterine sections as for the corresponding twin were analyzed. All experiments with animals were carried out with permission from the local veterinary authorities.

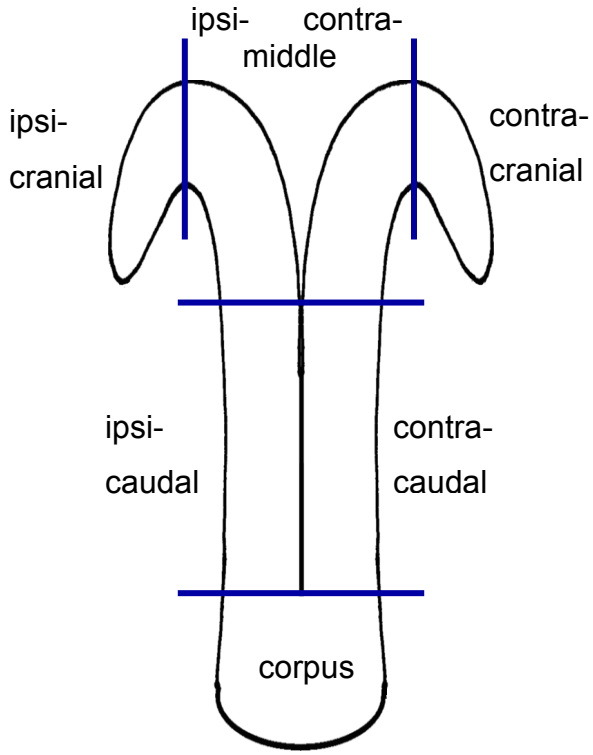


Fig. 3: Definition of uterine regions for tissue collection

Ipsi referrs to the uterine horn ipsilateral to the ovary bearing the corpus luteum, contra referrs to the uterine horn contralateral to the ovary bearing the corpus luteum.

3.2.2. Standard methods used in molecular biology

3.2.2.1. Isolation of total RNA from bovine endometrial cells

Total RNA was isolated using Trizol©Reagent, a monophasic solution of phenol and guanidine isothiocyanate, according to the manufacturer's instructions. The tissue samples were homogenized in 1 ml Trizol©Reagent per 50-100 mg of tissue which leads to the denaturing of proteins (mainly due to the action of guanidine isothiocyanate). The homogenized samples were then incubated for 5 min at rt to permit complete dissociation of nucleoprotein complexes. Next, 0.2 ml chloroform per 1 ml Trizol©Reagent were added, the sample tubes shaken vigorously by hand for 15 seconds and afterwards incubated at 24°C for 5 min. Centrifugation of the samples at 12,000 x g at 6°C for 15 minutes led to separation of the mixture in a lower phenol-chloroform phase, an interphase and an upper aqueous phase. The organic phenol-

chloroform phase contains the proteins, whereas the RNA exclusively remains in the aqueous phase. The aqueous phase was therefore transferred into a fresh tube. The RNA was precipitated from the aqueous phase by addition of 0.5 ml isopropanol per 1ml Trizol©Reagent. After incubation for 10 min at 24°C, the samples were centrifuged at 12,000 x g at 6°C for 10 min which led to formation of a sediment containing the RNA. The supernatant was then removed and the RNA sediment washed with 75% ethanol by adding 1 ml ethanol per 1 ml Trizol©Reagent and centrifugation at 7,500 x g at 6°C for 5 min. The supernatant was removed and the RNA sediment air dried. Then the RNA was dissolved in 100 µl deionized water per 1 ml Trizol©Reagent.

One microliter of the RNA solution was diluted 70-fold in 69 µl TE buffer to determine the concentration of RNA by UV-spectrometry and a 250 ng/µl dilution of the RNA was prepared to check the quality by agarose gel electrophoresis using a 0.8 % 1 x TBE gel.

Afterwards the RNA samples were divided into several aliquots each comprising 50 µg of total RNA. Deionized water was added to yield a total volume of 150 µl. Then 15 µl NaOAc and 15 µl isopropanol were added and after incubation for 10 min at rt the samples were stored until further processing at -20°C.

3.2.2.2. Agarose gel electrophoresis

Agarose gel electrophoresis is a method that separates nucleic acids according to their molecular weight. During the process of electrophoresis, the nucleic acids are forced to move through the pores of an agarose gel due to the application of electrical current. DNA fragments are visualized by staining with ethidium bromide, a fluorescent dye which intercalates between bases of DNA and RNA.

To cast a gel, agarose powder was mixed with water to the desired concentration and heated in a microwave oven until completely melted. After cooling the solution to about 60°C, 10 x TBE buffer and ethidium bromide were added (final concentration of 0.5 µg/ml). The solution was then poured into an electrophoresis chamber containing a sample comb and allowed to solidify at room temperature. Prior to analyzing RNA samples, the electrophoresis chamber was treated with 1 % SDS. Samples containing DNA or RNA (mixed with 2.5 x loading buffer) were then pipetted into the slots of the gel. The gel was then covered with TBE electrophoresis buffer, containing ethidium bromide in a final concentration of 0.5 µg/ml, and the electric current was

applied (3 V/cm for 18 min followed by 5 V/cm for 30 min respectively for up to 2.5 h). The agarose gels were prepared of standard agarose unless otherwise specified. All gels were documented using the Eagle Eye® II.

3.2.2.3. Precipitation of DNA

DNA precipitation is a method to purify and/or concentrate DNA or RNA. The precipitation was performed in an alcohol/water mixture in the presence of a high concentration of inorganic salt (NaOAc or NH_4OAc) and involved the following steps:

Sodium chloride precipitation

1. Addition of 1/10 volume 3 M NaOAc (pH 5.2) and 1 volume isopropanol.
2. Thorough mixture and subsequent incubation at room temperature for 30 min, if required, storage at -20°C until further processing.
3. Microcentrifugation for 15 min at 12,000 x g – 25,000 x g and 6°C (RNA), respectively 18°C (DNA).
4. Removal of the supernatant.
5. Washing of the pellet with 500 µl 75% ethanol to desalt the DNA/RNA and centrifugation as described above.
6. Aspiration of the liquid and air drying of the pellet.
7. Resuspendation of the pellet to the required concentration in water or appropriate buffer.

Ammonium acetate precipitation

1. Addition of 1 volume 5 M NH_4OAc
2. Thorough mixture and subsequent incubation for 15 min on ice
3. Microcentrifugation for 15 min at 25,000 x g and 4°C.
4. Removal of the supernatant.
5. Addition of 2.5 volume 2.5 M NH_4OAc.
6. Microcentrifugation for 5 min at 25,000 x g and 4°C.
7. Removal of the supernatant.
8. Washing of the pellet with 500 µl 75% ethanol to desalt the DNA/RNA and centrifugation as above.
9. Aspiration of the liquid and air drying of the pellet.

10. Resuspendation of the pellet to the required concentration in water or appropriate buffer.

3.2.2.4. Phenol/chloroform extraction

Phenol/chloroform extraction is a technique used to remove proteins from nucleic acids and was performed as follows:

1. Addition of an equal volume of phenol/chloroform/isoamylalcohol (25:24:1, TE saturated, pH 7.5) and thorough mixture.
2. Centrifugation at 25,000 x g for 2 min (this led to phase separation in an upper aqueous phase, which contains the nucleic acids and a lower organic phase).
3. Transfer the aqueous phase to a new reaction tube.
4. To remove traces of phenol, add an equal volume of chloroform to the aqueous phase, mix, and centrifuge as above.
5. Remove the aqueous layer to a new reaction tube.
6. Isopropanol precipitation of the DNA as described in 3.2.2.3.

3.2.3. Generation of a subtracted cDNA library

RNA samples obtained from the twin pair 405/406 were chosen to prepare a subtracted library, enriched for genes upregulated in the bovine endometrium due to the presence of a conceptus. Animal 405 received two *in vitro* produced embryos, animal 406 served as control. The production of the subtracted library was done according to the suppression subtractive hybridization (SSH) method, which has been described in detail previously (2.4). Suppression subtractive hybridization was first described by Diatchenko and coworkers (1996) and later on modified by Bauersachs and coworkers (2003; 2004).

A second library was constructed using a purchased subtracted cDNA from the vertis AG, Freising (for details see 3.2.4).

3.2.3.1. Synthesis of cDNA for subtractive hybridization

First-strand synthesis

Synthesis of cDNA was started with 50 µg total RNA (corresponding to approximately 0.5-2.5 µg mRNA) which had been stored as isopropanol/NaOAc precipitation at −20°C. The RNA samples were incubated for 10 min at rt and then centrifuged at

12,000 x g for 15 minutes at 6°C. The supernatant was removed and the remaining RNA was sediment was washed by addition of 500 µl ethanol and an additional centrifugation at 7,500 x g for 5 min at 6°C. The supernatant was discarded and the RNA sediment air dried and afterwards dissolved in 13.5 µl deionized water. Then 0.5 µl cDNA primer 1 (100 pmol/µl) was added and the samples were heat denatured at 80°C for 10 min to dissolve secondary structures of the RNA. After heat denaturing, samples were chilled using an ice/ethanol bath and the following reactors were added for first-strand synthesis:

0.5 µl	RNase-Inhibitor (40 U/µl)
5.0 µl	first-strand buffer (5x)
2.5 µl	DTT (100 mM)
1.0 µl	dNTP-Mix (10 mmol/l dATP, dGTP, dTTP, dCTP respectively)
2.0 µl	Superscript III (200 U/µl)

The samples were incubated for 10 min at 37°C, followed by incubation at 45°C for 80 min. Superscript III is a reverse transcriptase that synthesizes a complementary DNA strand from single-stranded RNA. The enzyme shows no RNaseH activity so full length cDNA is generated. RNase Inhibitor was added to prevent degradation of RNA by ubiquitously present RNases.

Second-strand synthesis

Synthesis of the second strand was performed immediately after first-strand synthesis had been accomplished. The samples were placed on ice and the following reactors were added:

86 µl	deionized water
30 µl	second-strand buffer (5x)
3 µl	dNTP-Mix (10 mmol/l dATP, dGTP, dTTP, dCTP respectively)
1 µl	DNA ligase (*E. coli*) (10 U/µl)
4 µl	DNA polymerase I (*E. coli*) (10 U/µl)
1 µl	RNaseH (*E. coli*) (2 U/µl)

The reaction was performed at 16°C for 2.5 hours. Addition of a primer was not necessary because RNaseH is an endoribonuclease which specifically degrades the RNA strand of an RNA-DNA hybrid. The remaining pieces of RNA can be used by the DNA polymerase I as primers to start synthesis of the second strand of cDNA.

After completion of the second-strand synthesis the enzymes were heat denatured at 75°C for 10 min. Ribosomal and other residual RNAs were digested by addition of

3 µl RNase (0.5 mg/ml) for 90 min at 37°C. The double stranded cDNA was then incubated with 3.6 µl dNTP mix (10 mmol/l dATP, dGTP, dTTP, dCTP respectively) and 10 µl T4 DNA polymerase (1 U/µl) for 5 min at 16°C to yield blunt ends. To inactivate the T4-DNA polymerase, 10 µl EDTA (0.5 M) was added and the samples were incubated for 15 min at 75°C.

To remove proteins, a phenol/chloroform extraction with subsequent DNA precipitation was performed as described in 3.2.2.4. The DNA sediment was dissolved in 10 µl TE buffer and 34 µl deionized water. One-point-five microliter (1/30) DNA solution was removed to check the efficiency of cDNA synthesis by agarose gel electrophoresis, performed after the following step, which involved digestion of the cDNA using a restriction enzyme.

Because longer cDNA fragments do disturb the efficiency of suppressor PCR, the cDNA was digested with *RsaI*, a restriction enzyme with a four-base recognition sequence, to yield shorter cDNA fragments. Hence the following reactors were added:

0.5 µl	BSA (10mg/ml)
5.0 µl	buffer L
2.0 µl	*RsaI* (10 U/µl)

The digestion was performed at 37°C and after 60 min an additional microliter of *RsaI* was added and the digestion was continued for 60 min; the enzyme was afterwards inactivated at 65°C for 20 min.

The digested cDNA was precipitated (3.2.2.3) and the DNA sediment was dissolved in 5.5 µl 0.5 x TE buffer. For agarose gel electrophoresis 0.183 µl (1/30) DNA were taken out of the DNA solution.

To check the efficiency of cDNA synthesis and the success of digestion with the restriction enzyme *RsaI*, an agarose gel electrophoresis (0.8% gel) was performed using DNA probes derived from the same sample before and after digestion with *RsaI*.

3.2.3.2. Ligation of tester cDNA with the adapters

Before subtractive hybridization the tester cDNA (derived from animal 405) needed to be ligated with two different adapters, adapter 1 / adapter 1 reverse and adapter 3 / adapter 3 reverse in two separate reactions. Additionally, a molecular

weight marker (DNA Molecular Weight Marker IX, M-IX) was also ligated with the adapters. The latter reaction served as control.

First of all two different reactions with the following composition were prepared (each reaction was prepared three times):

	premix adapter 1/1r	premix adapter 3/3r
deionized water	3.5 µl	3.5 µl
ligase buffer (10x)	0.5 µl	0.5 µl
adapter 1 (40 pmol/µl)	0.5 µl	-
adapter 1 reverse (40 pmol/µl)	0.5 µl	-
adapter 3 (40 pmol/µl)	-	0.5 µl
adapter 3 reverse (40 pmol/µl)	-	0.5 µl

The six samples were placed into water tempered at 72°C, which was subsequently allowed to cool down at 10.5°C in order to yield double stranded adapters. Now 6 reactions were prepared as follows:

	405 1/1r	405 3/3r	M-IX 1/1r	M-IX 3/3r	M-IX 1/1r	M-IX 3/3r
cDNA 405	2 µl	2 µl	-	-	-	-
BM-IX	-	-	2 µl	2 µl	2 µl	2 µl
T4-DNA-ligase (2000 U/µl)	0.2 µl	0.2 µl	0.2 µl	0.2 µl	0.2 µl	0.2 µl
ligase buffer (10x)	0.5 µl	0.5 µl	0.5 µl	0.5 µl	0.5 µl	0.5 µl
deionized water	2.3 µl	2.3 µl	2.3 µl	2.3 µl	2.3 µl	2.3 µl
premix adapter 1/1r	5 µl	-	5 µl	-	5 µl	-
premix adapter 3/3r	-	5 µl	-	5 µl	-	5 µl
RsaI (10U/µl)	0.1 µl	0.1 µl	0.1 µl	0.1 µl	-	-

Ligation was performed overnight at 16°C. To serve as additional control, a seventh sample (K-405) was created, by mixing one microliter 405 1 / 1r with one microliter 405 3 / 3r prior to performing the ligation reaction. The next morning the samples were incubated at 70°C for 20 min to achieve heat inactivation of the enzymes.

One microliter of K-405 was submitted to PCR. Therefore, the following reactors were added to the template:

2.5 µl PCR buffer (10x)

19.5 µl	deionized water
0.5 µl	dNTP-Mix (10 mmol/l dATP, dGTP, dTTP, dCTP respectively)
1.0 µl	PCR-primer 1 (10 pmol/µl)
0.5 µl	Advantage2 DNA Polymerase Mix

The polymerase chain reaction was performed in a Perkin Elmer 480 Cetus thermocycler with the following parameters: 75°C for 5 min; 30 cycles at 94°C for 30 sec, 66°C for 30 sec, and 72°C for 90 sec; 72°C for 3 min.
Agarose gel electrophoresis was applied to verify the effiency of the adapter ligation and of the control PCR. A 2% gel prepared out of small DNA agarose was used.

3.2.3.3. Subtractive Hybridization

First step of subtractive hybridization
Prior to the first step of subtractive hybridization the DNA was precipitated to remove residual proteins that might hinder successful subtractive hybridization. One-pint-five microliter driver cDNA (derived from animal 406) was added to the sample containing tester cDNA ligated to adapter1 and adapter 1 reverse respectively ligated to adapter 3 and adapter 3 reverse (1.5 µl each). This was equivalent to a 30-fold excess of driver cDNA. The samples were mixed and then a DNA precipitation (3.2.2.3) was carried out. After dissolving the sediment in 2.5 µl 0.1 x TE buffer, 0.5 µl 25 mM $MgCl_2$ and 1 µl hybridization buffer for SSH (4x) were added. Subtractive hybridization was performed using the following parameters: 4°C for 9:35 h, 98°C for 1.5 min, cooling down from 98°C to 68°C in 20 min and finally 68°C for 10 h.

Second step of subtractive hybridization
To initiate the second step of subtractive hybridization the two samples of the first step were mixed and allowed to hybridize at 68°C. After 24 hours the hybridization was stopped by adding 200 µl dilution buffer and incubation of the sample at 72°C for 7 min.

Suppressor PCR
To selectively amplify those double stranded cDNA molecules that contain different adapters on each side, suppressor PCR was performed. As template, 2 µl of the hybridization reaction were used and the following reactors were added:

2.5 µl	PCR buffer (10x)

19.5 µl	deionized water
0.5 µl	dNTP-Mix (10mmol/l dATP, dGTP, dTTP, dCTP respectively)
1.0 µl	PCR primer 1
0.5 µl	Advantage2 DNA Polymerase Mix

Primary PCR was performed with the following parameters: 75°C for 5 min; 29 cycles at (94°C for 30 sec, 66°C for 30 sec, 72°C for 1.5 min), 72°C for 3 min. The PCR product was diluted 10-fold with TE buffer and 1 µl of the solution was subsequently used as template for secondary PCR. Secondary PCR was performed using nested primers thereby reducing the possibility to obtain unspecific PCR products. The following reactors were added:

2.5 µl	PCR-buffer (10x)
18.5 µl	deionized water
0.5 µl	dNTP-Mix (10mmol/l dATP, dGTP, dTTP, dCTP respectively)
1.0 µl	nested primer 1 (10 pmol/µl)
1.0 µl	nested primer 2 (10 pmol/µl)
0.5 µl	Advantage2 Polymerase Mix

The following parameters were employed: 96°C for 1 min, 12 cycles at (94°C for 30 sec, 68°C for 30 sec, 72°C for 1.5 min), 72°C for 3 min. All PCR reactions are done in a Perkin Elmer Cetus thermal cycler.

After PCR reaction was completed the PCR product was analyzed by 1 % agarose gel electrophoresis.

To eliminate Taq polymerase made use of for PCR, a phenol/chloroform extraction was performed (3.2.2.4). The DNA sediment was dissolved in 5 µl TE buffer and 20 µl water.

3.2.3.4. Ligation with vector DNA

The next steps involved ligation of the PCR products with vector DNA and subsequent introduction of the ligation reaction into bacteria. The plasmid pBSIIK$^-$MO, a derivative of pBluescript II SK$^-$, served as vector DNA.

cDNA preparation

First of all the PCR products were digested with *BssHII*. The recognition site of this restriction enzyme (“GCGCGC”) is located inside the adapter sequence and the resulting sticky ends are compatible with the *AscI* recognition site located in the

vector. Digestion was performed by addition of 3 µl NEBuffer3 and 2 µl *BssHII* (4 U/µl) and subsequent incubation at 50°C for 2 h, followed by heat inactivation of the restriction enzyme at 75°C for 20 min.

In the next step, cDNA fragments counting less than 100 to 200 base pairs had to be removed. Those short fragments are on the one hand primer oligonucleotides used as primers for suppressor PCR and emerge on the other hand from the former digestion with *BssHII* and resemble the outer part of the adapters. Removal was achieved by a preparative agarose gel electrophoresis as follows: ten microliter loading buffer for agarose gel electrophoresis were added to the PCR products obtained from suppressor PCR, which were then loaded onto a 1% low melting agarose gel. After electrophoresis, the gel below 100 to 200 base pairs according to the molecular weight marker was cut off and discarded. The remaining gel, containing the cDNA longer than 100 to 200 base pairs was transferred back to the electrophoresis chamber and electrophoresis was done again but with inverse polarity. Electrophoresis was stopped before the cDNA arrived at the slots of the gel. Then the cDNA band was cut off and cDNA was isolated from the gel using the QIAquick Gel Extraction Kit according to the manufacturer's recommendations. The principle of this kit is a silicia gel membrane, which binds DNA in high-salt buffer. Elution of the DNA is performed using low-salt buffer or water. The purification procedure removes the agarose from the DNA sample. The purified DNA was analyzed by 1% agarose gel electrophoresis.

Vector preparation

Throughout the next step, the vector DNA was digested with *AscI* to yield sticky ends. These sticky ends are compatible with the ends resulting from the *BssHII* digestion of the cDNA. Digestion was performed under the following conditions:

6 µl	pBSIIK$^-$MO (208 ng/µl)
3 µl	NEBuffer4
20 µl	deionized water
0.3 µl	BSA (10 mg/ml)
1 µl	AscI (10 U/µl)

The sample was incubated at 37°C. After 60 min 1 µl *AscI* (10 U/µl) was added and the sample was incubated at 37°C for additional 60 min. Afterwards the enzyme was heat inactivated at 65°C for 20 min.

To avoid self-ligation of the plasmid DNA, the phosphate group of the 5´ end of the vector needed to be removed. This was achieved by addition of 5 µl Shrimps-AP (1 U/µl) and 4 µl dephosphorylization buffer (10x) and subsequent incubation at 37°C for 20 min. The enzyme was inactivated at 70°C for 20 min.

Ligation reaction and introduction into E. coli SURE©

During the ligation reaction a ratio of cDNA to plasmid DNA of 1:2 is required. The average length and concentration of cDNA or plasmid DNA, respectively, was estimated on the basis of the gel electrophoresis photos and the required amounts of cDNA or plasmid DNA were calculated. Two microliter plasmid DNA (25 ng/µl), with an average length of 300 bp and 0.66 µl cDNA (5 ng/µl), with an average length of 400 bp, were mixed and incubated at 65°C for 5 min and afterwards chilled on ice. Then 0.2 µl T4-DNA-Ligase (2000 U/µl), 1 µl ligase buffer (10x) and 5.8 µl deionized water were added and the reaction was performed at 16°C for 4 hours followed by heat inactivation at 70°C for 20 min.

The ligation reaction was introduced directly into *E. coli* SURE® electroporation-competent cells by electroporation. One microliter ligation reaction was added to 50 µl *E. coli* SURE©, which had been thawed on ice, and incubated for 2 min on ice. The cell-DNA mixture was then transferred into a chilled electroporation cuvette and electroporation was performed at 12.5 kv/cm field strength using an *E. coli* Pulser©. Afterwards the bacteria were immediately transferred into a 1.5 ml reaction tube containing 840 µl chilled SOC medium and incubated at 37°C for 30 min.

One hundred microliter SOB medium were added to 5 µl respectively 15 µl of the bacteria and plated. After addition of 150 µl glycerol (final concentration 15%), the remaining cells were transferred to a cryotube, frozen on dry ice and stored at -80°C until further processing. The agar plates were incubated over night at 37°C. The next morning, the number of colonies grown over night was counted and the average number of colonies present in the subtracted library was calculated.

Isolation of plasmid DNA

The colonies were washed off from the agar plate using 10 ml LB-medium. The suspension was then transferred into a 15 ml centrifuge tube. Centrifugation at 8,400 x g for 5 min at 4°C led to the formation of a sediment containing the bacteria. The supernatant was discarded and the plasmid DNA was isolated using the QIA prep Spin Miniprep Kit according to the manufacturer’s instruction.

3.2.4. cDNA library construction from subtracted cDNA produced by the vertis Biotechnologie AG

Subtracted cDNA enriched for cDNAs of genes upregulated due to the presence of a conceptus during the peri-implantation period was purchased from the vertis AG (Freising). Subtraction was performed as previously described by Ros and coworkwers (2004) using the same RNA samples as used for construction of the library according the SSH method in our laboratory.

cDNA synthesis was started using 3.5 µg total RNA. First-strand synthesis was performed using different oligo(dT)-*Not* I-primer for the preparation of the driver and tester cDNA and M-MLV-RNase H^- Reverse Transcriptase. The linker primer used for synthesis of the tester cDNA contained a *Not I* recognition site. Second-strand synthesis was performed using different "random linker primer" for tester and driver cDNA preparation and the Klenow DNA-polymerase. The resulting cDNA was then amplified with long and accurate PCR (LA-PCR). For subtraction, single stranded (ss) cDNA was prepared from the tester (sense strand) and driver (antisense strand) cDNA. A 10-fold excess of antisense ss-driver was then hybridized with the sense-tester. Reassociated tester/driver ds-DNA was separated from the remaining ss-tester cDNA (subtracted cDNA) by passing the mixture over a hydroxylapatite column. After hydroxylapatite chromatography, the ss-tester cDNA was amplified with the tester-specific primer using LA-PCR. A second round of subtraction was performed.

Cloning of the cDNA was performed in our laboratory as described in 3.2.3.4. The only difference consisted in the preparation of the vector DNA. Instead of digestion with *AscI* the vector DNA was first digested with *Eco* RI to generate *Eco* RI overhangs at the 5´-ends followed by digestion with Not I.

3.2.5. Preparation of cDNA arrays

Amplification of cDNA inserts via PCR

A part of the subtracted libraries was plated onto agar plates composed of Luria broth medium and ampicillin. Due to the fact that the agar plates contain ampicillin, only bacteria which are ampicillin resistant, like bacteria containing the plasmid $pBSIIK^-$ MO, were able to grow on these plates. After incubation of the plates overnight at

37°C, 3072, respectively 1536 clones were randomly picked from the SSH and vertis library, and grown overnight in 100 µl Luria broth medium in 96-well microtiter plates. Two microliter bacterial suspensions were diluted 20-fold in TE buffer and subsequently incubated at 96°C for 20 min to lyse the bacterial cells. Two microliter of this solution was used as template to amplify the cDNA insertions via PCR in 96-well cycle plates. Each PCR reaction was performed using the following reactors:

0.2 µl	FIREPol®
0.4 µl	NLT7 primer (15 pmol/ml)
0.4 µl	LT3 primer (15 pmol/ml)
0.4 µl	dNTP-Mix (10 mmol/l dATP, dGTP, dTTP, dCTP respectively)
14.6 µl	deionized water
2 µl	reaction buffer (10x) for FIREPol®

and the following PCR parameters: 96°C for 2 min, 25 cycles at (94°C for 25 sec, 64°C for 25 sec, 72°C for 90 s), 15 cycles at (94°C for 25 sec, 62°C for 25 sec, 72°C for 90 sec) and 72°C for 300 sec. All PCR reactions were done in a T1 Thermocycler T3 and all PCR products were analyzed by 0.8% agarose gel electrophoresis.

Spotting the PCR products onto nylon membranes

In the next step, the PCR products were spotted onto positively charged nylon membranes (Nytran® SuperCharge). Prior to the spotting procedure, the nylon membranes were rinsed for 5 min in water, 5 min in ethanol (100%) and afterwards air dried in order to remove any potential dirt.

Fifteen microliter of the PCR reactions were transferred to 384 well microtiter plates containing 15 µl of 2-fold spotting buffer with the following composition:

40 mmol/l	Tris-HCl (pH 8.4)
2 M	NaCl
2 mM	EDTA
0.001%	bromphenolblue

Now the PCR products were spotted onto nylon membranes using an Omnigrid Accent microarrayer and solid pins (0.015 inch in diameter). PCR products (n=1536) were spotted within an area of 20 x 50 mm. Spotting was performed six times for each PCR product on the same position to achieve sufficient and equal application. Up to fifty arrays, each containing 1536 cDNA fragments can be produced simultaneously. After spotting was completed, the spotted DNA was denatured on the arrays by incubation on filter paper soaked with 0.5 N NaOH for 20 min at room

temperature. DNA was fixed by baking at 80°C for 30 min and UV crosslinking (120 mJ/cm^2) leading to hydrophobic and covalent linkage, respectively.

3.2.6. Array hybridization with ^{33}P-labeled cDNA probes

RNA of all 10 animals (pregnant n=5, non-pregnant n=5) was converted to double stranded cDNA which was subsequently labeled with ^{33}P-dCTP and subjected to array hybridization.

Synthesis of cDNA

Fifty microgram total RNA was subjected to cDNA synthesis. Prior to conversion into cDNA the RNA was precipitated using NH_4OAc (3.2.2.3). After dissolving the RNA sediment in 13.5 µl deionized water, 0.5 µl cDNA primer 2 (100 pmol/µl) was added and the samples were heat denatured at 80°C for 10 min. After heat denaturing the samples were chilled in an ice/ethanol bath. For first-strand synthesis the following reactors were added:

2 µl	Superscript III (200 U/µl)
0.5 µl	RNase-Inhibitor (40 U/µl)
1 µl	dNTP-Mix (10 mmol/l dATP, dGTP, dTTP, dCTP, respectively)
2.5 µl	DTT (100 mM)
5 µl	first-strand buffer (5x)

After incubation at 37°C for 10 min to allow annealing of the primer, reverse transcription was performed at 45°C for 80 min.

Synthesis of the second strand was performed at 16°C for 2.5 hours using the following reactors:

86 µl	deionized water
30 µl	second strand buffer (5x)
3 µl	dNTP-Mix (10mmol/l dATP, dGTP, dTTP, dCTP respectively)
1 µl	*E. coli* DNA-Ligase (*E. coli*)(10 U/µl)
4 µl	DNA-Polymerase I (*E. coli*) (10 U/µl)
1 µl	RNaseH (*E. coli*) (2 U/µl)

After synthesis of double stranded cDNA was completed, the enzymes were heat inactivated at 75°C for 15 min. Residual RNA was digested at 37°C for 90 min using 3 µl RNase (0.5 mg/ml).

Microspin™ S200-HR Columns were used to remove residual primers according to the manufacturer's instructions.
The purified DNA was then precipitated using NaOAc and isopropanol (3.2.2.3) and the sediment was dissolved in 20 µl 0.5-fold TE.
The quality of the cDNA was checked by 0.8 % agarose gel electrophoresis.

Radioactive labeling of the cDNA

^{33}P-labeled cDNA probes were generated from double stranded cDNA corresponding to 7.5 to 15 µg total RNA using the High Prime DNA Labeling Kit. The method of random primed DNA labeling, which was originally developed by Feinberg and Vogelstein (Feinberg and Vogelstein, 1983; Feinberg and Vogelstein, 1984), is based on the hybridization of oligonucleotides of all possible sequences to the denatured DNA to be labeled. The complementary DNA strand is synthesized by Klenow polymerase using the 3`OH termini of the random oligonucleotides as primers. Modified deoyxribonuclease-triphosphates (i.e. α[33P]dCTP) present in the reaction are incorporated into the newly synthesized complementary DNA strand.
The cDNA was heat denatured at 96°C for 10 min and then chilled on ice. The labeling reaction was performed at 37°C for at least 4 hours using the following reactors:

4 µl	High Prime DNA Labeling Mix (5x)
90 µCi	[α-^{33}P]dCTP
0.375 µl	dNTP-Mix (0.3 mmol/l dCTP and 20 mmol/l dATP, dGTP, dTTP, respectively)

The reaction was heat inactivated at 65°C for 20 min. Then 50 µl TE and 30 µl human C0T-1 DNA were added. Human C0T-1 DNA mainly comprises repetitive DNA sequences and was used to suppress cross-hybridization to repetitive sequences during the following process of hybridzation. The reactions were then purified using ProbeQuant™ G-50 Micro Columns according to the manufacturer's instructions, to remove unincorporated nucleotides and primers from the labeling reaction. Labeling efficiency was estimated by comparing the impulse per seconds (ips) of the purified labeling reaction and the column residue. The optimal ratio of labeling reaction to column residue is 3:1.

Prehybridization of arrays

Prehybridization was done for up to 6 arrays together in a glass hybridization bottle of 15 cm length and involved the following steps:

2 x 10 min 10 ml 1 x PBS / 10% SDS at 65°C
1 x 1 h 10 ml 1 x PBS / 10% SDS at 65°C
1 x 10 min 10 ml 1 x PBS / 10% SDS at 85°C
2 x 10 min 10 ml 0.1 x PBS / 1% SDS at 85°C
3 x 10 min 10 ml 1 x PBS / 10% SDS at 65°C

SDS is an anionic detergent, which can bind via its hydrophilic tails to the nylon membrane. This blocking procedure was necessary, because otherwise DNA of the hybridization probe would bind in a non-specific manner to free binding sites of the membrane during array hybridization.

Hybridization

Hybridization was performed in plastic vials containing 3 ml hybridization solution (1 x PBS/10% SDS and labeled probe) for 45 hours at 65°C. Hybridization probes were denatured for 15 min at 96°C immediately before adding to the hybridization solution. After hybridization, 6 arrays were put together in a 15 cm glass hybridization bottle and washed as follows to remove non hybridized cDNA fragments:

3 x 5 min 10 ml 1 x PBS/10% SDS at 65°C
3 x 10 min 10 ml 1 x PBS/10% SDS at 65°C
3 x 10 min 10 ml 0.1 x PBS/1% SDS at 65°C
2 x 10 min 10 ml 1 x PBS/1% SDS/2 mM EDTA at room temperature

Filters were dried at 80°C for 20 min and then exposed to an imaging plate BAS-SR 2025. This imaging plate has a flexible polyester base coated with highly dispersed barium fluorohalide phosphor crystals. When a radioactively labeled sample is exposed, the energy from that sample is transferred to the phosphor crystals and stored as trapped electrons. After optimal exposition length the imaging plate is scanned with a phosphor imager, Storm 860. Scanning the exposed imaging plate releases the trapped electrons resulting in the emission of light at about 400 nm. This process is known as photo-stimulated luminescence. The blue light emitted is collected to produce the digitized image.

3.2.7. Analysis of array data

Array analysis was done using AIDA Image Analyzer software (Version 4.00). Background, i.e. fluorescence due to unspecific binding of cDNA, was subtracted with the function “weighted image regions”. For further analysis raw data were exported to Microsoft Excel and normalized to the median signal intensity of all cDNA clones which showed a difference in signal intensity of less than 2-fold. Normalization means to adjust microarray data for effects which arise from variation in the technology rather than from biological differences between the RNA samples. Pair-wise comparison (pregnant-control) was performed within twin pairs, thus eliminating changes in gene expression due to a different genetic background of individuals. Complementary cDNA clones, which exhibit a difference in signal intensity of 2-fold or more in four out of five twin pairs were assumed to be upregulated at the pre-implantation period and subjected to further analysis, i.e. DNA sequencing. To test significance of expression differences a paired Student’s t-test was performed and the coefficient of variation (CV) of the expression ratios between pregnant and non-pregnant animals was calculated. Due to the nature of subtracted libraries for many genes (37) more than one cDNA fragment was present on the arrays and for these genes the mean of the signal ratios was calculated.

3.2.8. Sequencing of cDNAs with differential hybridization signals and data analysis

Those cDNA clones which showed a difference in signal intensity of 2-fold or more in at least four out of five twin pairs were sequenced directly from spotting solutions by automated DNA sequencing (3100-Avant Genetic Analyzer). The DNA sequencing (i.e. the determination of the precise sequence of nucleotides in a sample of DNA) was carried out by the AG Blum (AG Dr. H. Blum, LAFUGA, Gene Center, Munich). Prior to sequencing, a microdialysis was performed to remove hampering salts, nucleotides and PCR primers from the spotting solution. A petri dish was filled with 40 ml 0.25 x TE buffer, the dialysate. A MF-Millipore membrane filter (0.025 µm) was then floated on the surface of the dialysate and 2.5 µl spotting solution were deposited on the membrane. A tight fitting lid was placed on the petri dish to prevent evaporation. After 1 h the desalted sample was recovered and transferred into a reaction tube containing 1 µl PM0- or T7ex- primer (3.5 pmol/µl) and 3.5 µl deionized

water. Then 1 µl DYEnamic ET Terminator Cycle Sequencing Kit and 3 µl better buffer special were added and sequence reaction was performed using the following parameters: 96°C for 120 s and 35 cycles at (96°C for 30 s, 54°C for 30 sec, 60°C for 30 s). In this sequence reaction, the template was supplied with a mixture of deoxynucleotides (dATP, dCTP, dGTP, dTTP) in ample quantities, a mixture of dideoxynucleotides that lack the 3`-OH, each present in limiting quantities and each labeled with a different fluorescencent dye and a thermostable polymerase. Because all four deoxynucleotides are present, chain elongation proceeds normally until, by chance, DNA polymerase inserts a dideoxnucleotide instead of the normal deoxynucleotides which leads to chain termination.

Resulting sequences were compared with public sequence databases using the basic local alignment search tool at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/blast/blast.cgi). cDNAs without similar entries in the “ref” or “nr” database subsets were in addition compared with the “est” database or the raw version of the bovine genome (http://pre.ensembl.org/Multi/blastview?species=Bos_taurus). Based on the data for the human orthologous genes simplified Gene Ontologies were built of the data obtained with the Gene Ontology filter function of the Bibliosphere software in order to categorize the genes regarding their molecular function or the biological processes they are involved in, respectively. Resulting data were supplemented with additional information from Entrez Gene (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene), and from the literature. Bibliosphere was further used to identify relationships between the identified genes based on co-citations in NCBI Pubmed. Co-citations were limited to “sentence level”, i.e. two genes are co-cited in the same sentence.

3.2.9. Real-time RT-PCR

One microgram of each sample of total RNA was reverse transcribed in a total volume of 60 µl, containing 1x buffer (Promega, Madison, USA), 0.5 mM dNTPs (Roche, Mannheim), 2.5 µM hexamer primers (Gibco BRL, Grand Island, USA), and 200 U M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (Promega, Madison, USA). Primers were designed to amplify specific fragments referring to selected regulated genes:

C1R (for 5´-AGGGGATAGTGGAGGGGTC;

rev 5´-GGACATTGGTGTAAAACCCG [117 bp]),

C1S (for 5´-AACAGGAGTGGGTCATCCAG;
rev 5´-CGGCTGTGTTGGTCTTTCAG [149 bp]),
DTX3L (for 5´-AAAGGATGTCTTGAGCCAAGC;
rev 5´-CTCCAGAAATGACAACCTTGC 39 bp]),
HSXIAPAF1 (for 5´-GAGGAGGCTCTGAGCTTGC;
rev 5´-GCAGAGAAAGATGTCCGTCC [143 bp]),
IFITM3 (for 5´-CGTGTGGTCCCTGTTCAAC;
rev 5´-CCATCTTCCGGTCCCTAGAC [95 bp]),
ISG15 (for 5´-GACAGCAGGGAGGTGCTG;
rev 5´-ACCCTTGTCGTTCCTCACC [203 bp]),
SERPING1 (for 5´-ACCAACCTCAGGATCAGGC;
rev 5´-CTATCTTCCACTTGGCGCTC [97 bp])
UBE1L (for 5´-GTGTTCATACCGCACGTGAC,
rev 5´-GGTTGTGGCAGGAATGTACC [105 bp])
UTMP (for 5´-ATATCATCTTCTCCCCCATGG;
rev 5´-GTGCACATCCAACAGTTTGG [126 bp]), and the
Ubiquitin mRNA as a housekeeping gene (for 5´-AGATCCAGGATAAGGAAGGCAT;
rev 5´-GCTCCACCTCCAGGGTGAT [198 bp]) (referring to Neuvians et al., 2003). All amplified PCR fragments were sequenced with forward and reverse primers (3100-Avant Genetic Analyzer, Applied Biosystems, Langen) to verify the resulting PCR product. Thereafter the specific melting point of the amplified product served as verification of the product identity (Ulbrich et al., 2004). For each of the following real-time PCR reactions, 1 µl of cDNA was used to amplify specific target genes. Quantitative real-time PCR reactions using the LightCycler® DNA Master SYBR Green I protocol (Roche, Mannheim) were performed as described previously (Ulbrich et al., 2004). In each PCR reaction 17 ng/µl cDNA were introduced and amplified in a 10 µl reaction mixture (3 mM $MgCl_2$, 0.4 µM primer forward and reverse each, 1x Light Cycler DNA Master SYBR Green I, Roche, Mannheim, Germany) using a real-time LightCycler instrument (Roche, Mannheim). The annealing temperature was 60°C for all reactions. To ensure an accurate quantification, a high temperature fluorescence measurement was undertaken in a fourth segment of the PCR (*C1R* and *UTMP* 80°C, *C1S* and *IFITM3* 81°C, *DTX3L* and *HSXIAPAF1* 75°C, *ISG15* 87°C, *SERPING1* 83°C, *UBE1L* 82°C and *Ubiquitin* 78°C, respectively. The cycle number (CP) required achieving a definite SYBR Green fluorescence signal

was calculated by the second derivative maximum method (LightCycler software version 3.5.28). The CP is correlated inversely with the logarithm of the initial template concentration. As negative controls, water instead of cDNA was used.
All Real-time RT-PCR experiments were kindly carried out by Dr. S.E. Ulbrich (Physiology-Weihenstephan, Technical University of Munich).

3.2.10. Data Analysis of Real-time RT-PCR

The cycle number (CP) required to achieve a definite SYBR Green fluorescence signal was calculated by the second derivative maximum method (LightCycler software version 3.5.28) (Ulbrich et al., 2004). The CP is correlated inversely with the logarithm of the initial template concentration. To verify the equal relative quantity of the reverse transcribed cDNA PCR for the housekeeping gene Ubiquitin mRNA was carried out. The CP determined for the target genes were normalized against Ubiquitin (ΔCP). Differences between pregnant (ET) and control animals are stated as ΔΔCP (Livak and Schmittgen, 2001). From the ΔΔCP of every twin pair the fold-change was calculated. Significance of differences between groups was tested using a paired Student's t-test.

3.2.11. *In situ* Hybridization

Formalin-fixed (3.7 %), paraffin-embedded samples were used to localize mRNA transcripts for *C1S*, *C1R*, *DTX3L*, *HSXIAPAF1*, *IFITM3*, *SERPING1*, and *UBE1L* within bovine uterine tissue samples (caudal section of the ipsilateral horn) collected at day 18 of gestation. Buffers (50 mM Tris-buffered saline [TBS] and 0.1 M sodium phosphate buffer) were adjusted to a pH of 7.4 unless otherwise noted. All solutions for *in situ* hybridization were prepared using DEPC-treated water and glassware sterilized at 200 °C. In addition, all steps prior to and during hybridization were conducted under RNase-free conditions. Sections were deparaffinized with xylene (3 x 10 min), immersed in isopropanol (2 x 5 min) and then allowed to air dry. Dried sections were submerged in 2 x saline sodium citrate (SSC, pH 7.0) and preheated in a Bain-Marie water bath (80 °C) for 10 min followed by gradual cooling off for 20 min at room temperature. Slides were then washed in distilled water (2 x 5 min), TBS (2 x 5 min) and permeabilized for 20 min with 0.05% proteinase E (VWR, Ismaning, Germany) in TBS at room temperature. Sections were relocated in TBS (2 x 5 min)

followed by distilled water (2 x 5 min) and post-fixed for 10 min in 4 % paraformaldehyde/PBS (pH 7.4). After washing in PBS (2 x 5 min) and distilled water, slides were dehydrated in an ascending graded series of ethanol and air-dried. Hybridization was carried out by overlaying the dried sections with 40 µl of the corresponding biotinylated oligonucleotide probe (100 pmol/µl), diluted 1:20 in *in situ* hybridization solution (DAKO, Munich), and incubating them in a humidified chamber (using cover slips to prevent drying-out) at 38 °C overnight. RNase-free hybridization solution (DAKO, Munich) contained 60 % formamide, 5 x SSC, hybridization accelerator, RNase inhibitor, and blocking reagents. Subsequently, slides were washed in 2 x SSC (2 x 15 min, preheated to 38 °C), distilled water (2 x 5 min) and TBS (2 x 5 min). Detection of hybridized probes was performed using HRP-labeled ABC kit reagents developed by DAB (DAKO, Munich) according to the manufacturer's instructions. Negative controls were done omitting the oligonucleotide probe and hybridization with sense oligonucleotide probes (complementary sequences of the antisense oligonucleotides). The sequences of the antisense oligonucleotides were as follows:

C1R: 5´-GGACATTGGTGTAAAACCCG,

C1S: 5´-CTGGATGACCCACTCCTGTT,

DTX3L: 5´-GCTTGGCTCAAGACATCCTTT,

HSXIAPAF1: 5´-GCAGAGAAAGATGTCCGTCC,

IFITM3: 5´-CCATCTTCCGGTCCCTAGA,

SERPING1: 5´ CTATCTTCCACTTGGCGCTC, and

UBE1L: 5´-GTCACGTGCGGTATGAACAC.

All *in situ* hybridization experiments were kindly carried out by M. Vermehren and Prof F. Sinowatz (Institute of Veterinary Anatomy, Histology and Embryology, Ludwig-Maximilians University).

4. Results

To gain deeper insight into the molecular mechanism underlying the embryo-maternal cross-talk two subtracted cDNA libraries enriched for genes upregulated in the bovine endometrium due to the presence of a conceptus were analyzed using array hybridization. One cDNA library was constructed according the SSH method (Diatchenko et al., 1996) as described previously by Bauersachs and coworkers (2003), with minor modifications, a second cDNA library was constructed of subtracted cDNA purchased from the vertis Biotechnologie AG. For both approaches intercaruncular endometrial tissue samples obtained from one monozygotic twin pair were utilized. Array hybridization was performed using RNA samples obtained from five monozygotic twin pairs. The use of monozygotic twins is a unique possibility to eliminate genetic variability as a factor potentially causing differences in transcriptome profiles. The expression of nine selected genes was confirmed by real-time RT-PCR. Furthermore, the localization of mRNA expression within the endometrium was determined by *in situ* hybridization for seven selected genes. The experimental strategy is illustrated fig. 4.

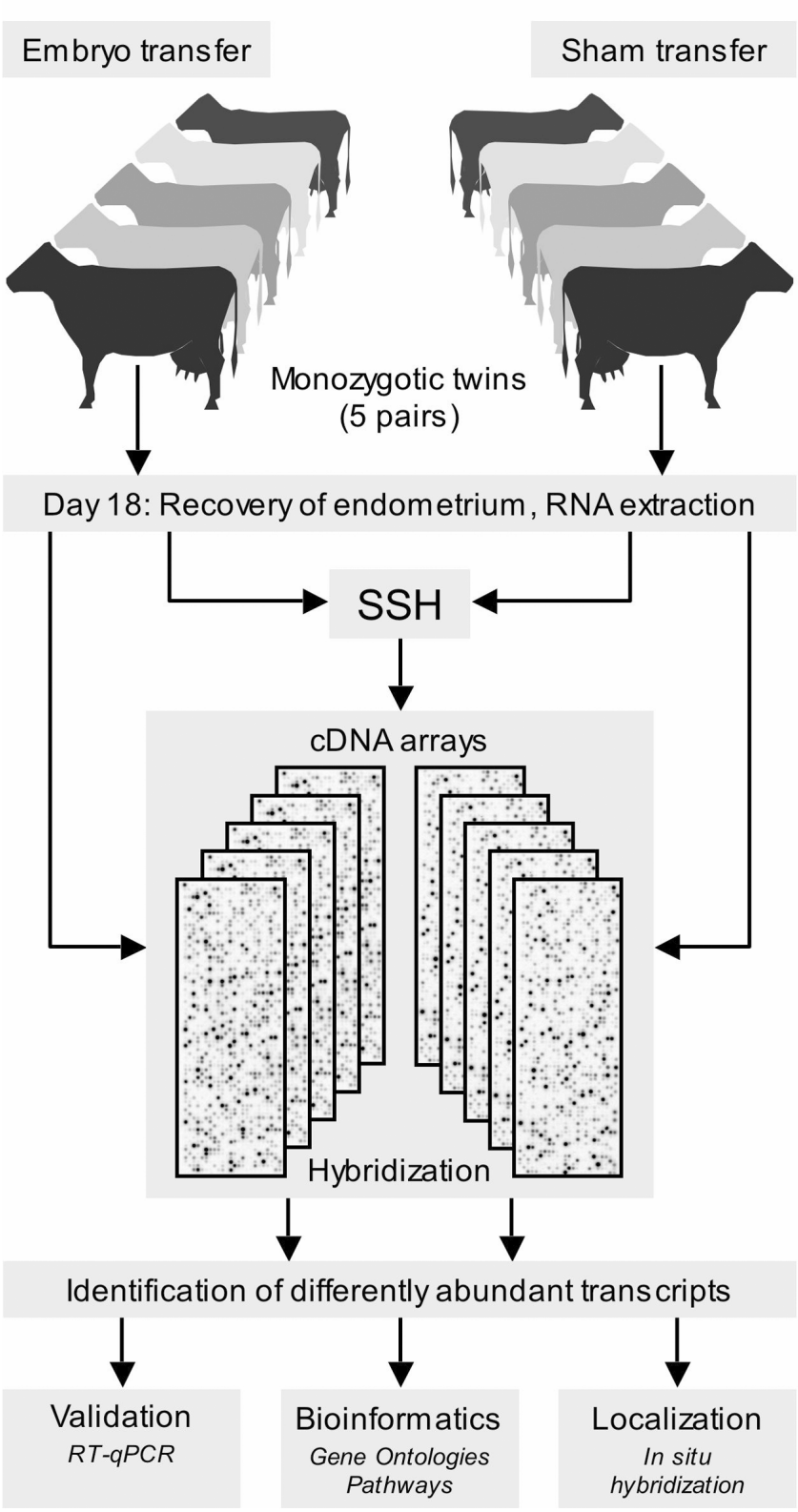


Fig. 4: Overview of the experimental design for the identification of specific transcriptome patterns at day 18 of pregnancy. The use of monozygotic twins eliminates genetic variability as a factor potentially causing differences in transcriptome profiles.

4.1. Isolation of total RNA from bovine endometrial cells

Total RNA was isolated from intercaruncular endometrial tissue samples obtained from five monozygotic twin pairs. A 250 ng/µl dilution of the RNA was prepared to check the quality by agarose gel electrophoresis using a 0.8 % 1 x TBE gel. Fig. 5 shows such an agarose gel electrophoresis of total RNA isolated from bovine endometrial cells. The isolated RNA was of consistent high quality. The two bands of intense fluorescence represent the 28 S ribosomal RNA (at approximately 1636 bp) and 18 S ribosomal RNA (at approximately 1018 bp). The band representing the 28 S rRNA shows a more distinct fluorescence than the band representing the 18 S rRNA. This is an indicator for the integrity of the total RNA. The mRNA is separated in the form of an even smear.

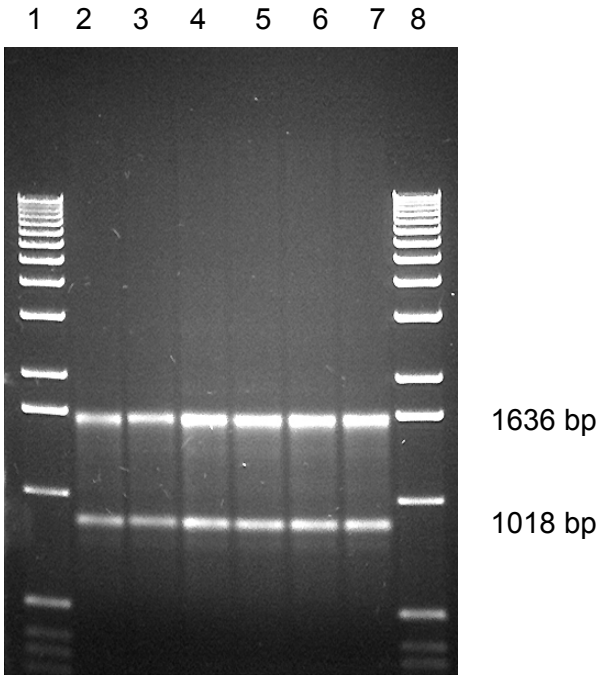


Fig. 5: Total RNA isolated from bovine endometrial cells

Each of the lanes 2 - 7 comprises 250 ng isolated RNA from bovine endometrial cells. The two bands of intense fluorescence represent the 28 S rRNA (at approximately 1636 bp) and 18 S rRNA (at approximately 1018 bp). Lanes 1 and 8 comprise the DNA molecular weight marker 1 kb DNA Ladder.

4.2. Generation of subtracted cDNA libraries

To enrich cDNA fragments of genes upregulated in the bovine endometrium due to the presence of a conceptus two subtracted cDNA libraries were produced. One cDNA library was constructed according the SSH method (Diatchenko et al. 1996) as described previously by Bauersachs and coworkers (2003), with minor modifications. The second cDNA library was constructed of subtracted cDNA generated by the vertis Biotechnologie AG. For both approaches intercaruncular endometrial tissue samples obtained from one monozygotic twin pair were utilized.

4.2.1. Synthesis of cNDA for subtractive hybridization

cDNA for subtractive hybridization was synthesized from total RNA isolated from the monozygotic twin pair 405/406. Because longer cDNA fragments do disturb the efficiency of suppressor PCR, the synthesized cDNA was digested with *RsaI*, a restriction enzyme with a four-base recognition sequence, to yield shorter cDNA fragments. To check the efficiency of cDNA synthesis and the success of digestion with the restriction enzyme *RsaI*, an agarose gel electrophoresis (0.8% gel) was performed using DNA probes derived from the same sample before and after digestion with *RsaI*. Fig. 6 shows such an agarose gel electrophoresis. Lanes 2 and 4 comprise full length cDNA, lanes 3 and 5 comprise cDNA after digestion with *RsaI.* The full lenght cDNA is separated as an even smear reaching from approximately 200 base pairs to more than 10,000 base pairs. There are no discrete bands visible, meaning that only the mRNA was amplified by the poly(A)-specific cDNA primer 1. The cDNA after digestion with *RsaI* shows a shorter average length, indicating that the digestion with *RSAI* was successful.

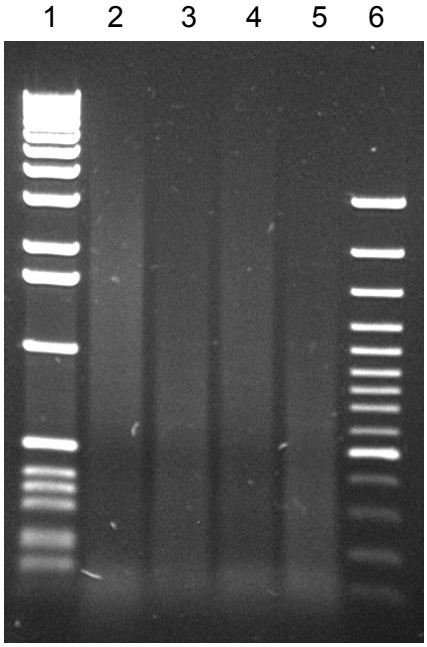


Fig. 6: Synthesis of cDNA and digestion with *RsaI*

Lanes 2 and 4 comprise full length cDNA; lanes 3 and 5 comprise cDNA after digestion with *RsaI.* Lane 1 comprises the DNA molecular weight marker 1 kb DNA Ladder (15615-016) and lane 6 comprises the DNA molecular weight marker GeneRuler™ 100 bp DNA Ladder Plus

4.2.2. Ligation of the tester cDNA with the adapters

Before subtractive hybridization the tester cDNA (derived from animal 405) needed to be ligated with two different adapters, adapter 1 / adapter 1 reverse and adapter 3 / adapter 3 reverse in two separate reactions. Additionally, a molecular weight marker (DNA Molecular Weight Marker IX, M-IX) was also ligated with the adapters. The latter reaction served as control. Additionally a control PCR was performed. As template a mixture of the two different ligation reactions (tester cDNA with adapter 1 / adapter 1 reverse and adapter 3 / adapter 3 reverse) was used.
Agarose gel electrophoresis was applied to verify the effiency of the adapter ligation and of the control PCR. A 2% gel prepared out of small DNA agarose was used (see fig. 7). Lanes 2 and 3 comprise the DNA Molecular Weight Marker IX, lanes 4 and 5 comprise the DNA Molecular Weight Marker IX ligated with the adapters. It is obvious that ligation of the DNA Molecular Weight Marker IX with the adapters was doing well, as a distinct shift in the average length of the DNA Molecular Weight Marker IX can be seen. The small bands at the end of the gel represent excessive adapter

molecules which have not been ligated. The control PCR was also successful, since the intense smear of lines 6 and 7 represents the PCR product.

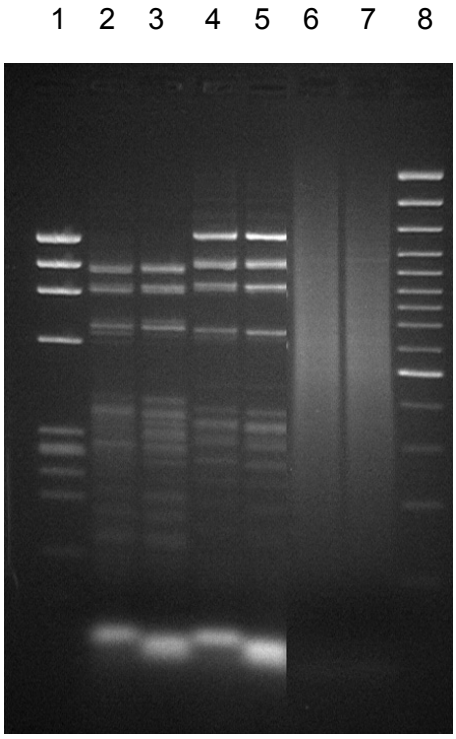


Fig. 7: Ligation of tester cDNA with the adapters

Lanes 2 and 3 comprise the DNA Molecular Weight Marker IX, lanes 4 and 5 comprise the DNA Molecular Weight Marker IX ligated with the adapters. Lanes 7 and 8 comprise the PCR product obtained by the control PCR. Lane 1 comprises the DNA molecular weight marker DNA Molecular Weight Marker IX and lane 9 comprises the DNA molecular weight marker GeneRuler™ 100 bp DNA Ladder Plus.

4.2.3. Suppressor PCR

To selectively amplify those double stranded cDNA molecules that contain different adapters on each side after subtractive hybridization, suppressor PCR was performed. As template, 2 µl of the hybridization reaction were used. After PCR reaction was completed the PCR product was analyzed by 1 % agarose gel electrophoresis (see fig. 8). Lanes 2 – 5 comprise the PCR products obtained from primary PCR, lanes 6 – 9 comprise the PCR products obtained from secondary PCR. All PCR products are separated as a smear. The products obtained from secondary PCR show a more intense fluorescence than those obtained from primary PCR. The

PCR products acquired from secondary PCR contain some, more or less, distinct bands.

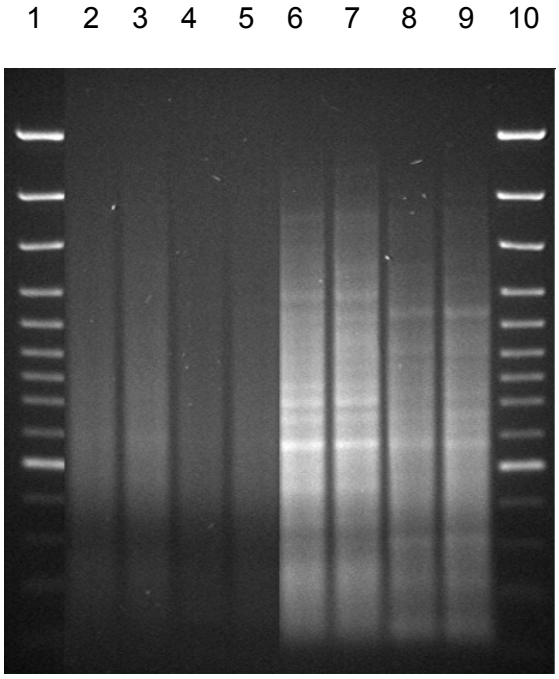


Fig. 8: Suppressor PCR

Lanes 2 – 5 comprise the PCR products obtained from primary PCR, lanes 8 – 11 comprise the PCR products obtained from secondary PCR. Lanes 1 and 11 comprise the DNA molecular weight marker GeneRuler™ 100 bp DNA Ladder Plus.

4.2.4. Ligation with vector DNA and introduction into *E.coli* Sure®

The PCR products obtained by suppressor PCR were ligated with vector DNA and the ligation reaction was subsequently introduced into bacteria.

First of all the PCR products were digested with *BssHII*. After digestion with the restriction enzyme, cDNA fragments counting less than 100 to 200 base pairs were removed by preparative agarose gel electrophoresis. The cDNA was then isolated from the gel using the QIAquick Gel Extraction Kit. An aliquot of the purified cDNA was analyzed by 1% agarose gel electrophoresis (see fig. 9). Lanes 2 and 3 comprise an aliquot of the cDNA isolated from the preparative agarose gel. The cDNA is separated as an even smear, indicating that the isolation from the agarose gel was complete.

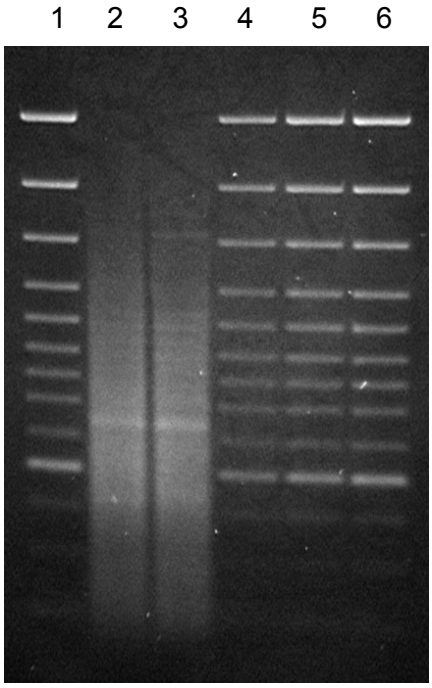


Fig. 9: Analysis of cDNA after isolation from agarose gel

Lanes 2 and 3 comprise the cDNA isolated from agarose gel, lanes 1 and 4 – 6 comprise the DNA molecular weight marker GeneRuler™ 100 bp DNA Ladder Plus.

The prepared cDNA was then ligated with the vector and the ligation reaction was introduced directly into *E. coli* SURE® electroporation-competent cells by electroporation. A definite part of the bacteria was then plated onto agar plates which were incubated over night. The next morning the number of colonies grown over night was counted and the average number of colonies present in the subtracted library was calculated (96,000 colonies at all).

An aliquot of the plasmid DNA was analyzed by (1%) agarose gel electrophoresis in comparison to the plasmid DNA of the empty vector in order to check the effiency of the ligation reaction (see fig. 10). Lane 2 comprises plasmid DNA of the empty vector, lanes 3 and 4 comprise plasmid DNA ligated with cDNA. The plasmid DNA ligated with the cDNA shows an greater average lenght than the DNA of the empty plasmid vector, indicating that the ligation reaction was successful.

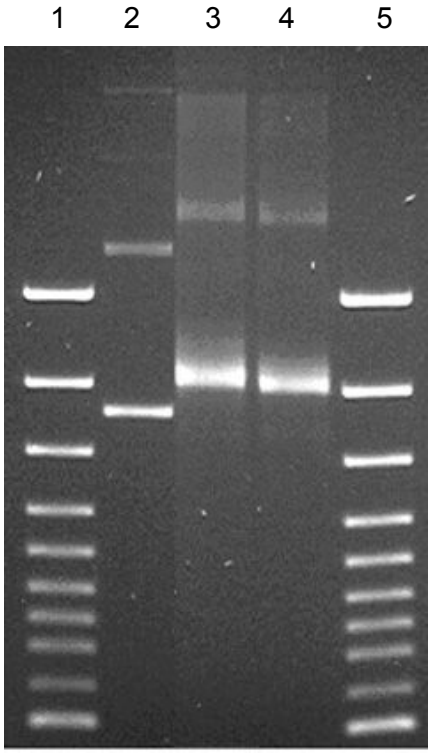


Fig. 10: Analysis of plasmid DNA

Lane 2 comprises plasmid DNA of the empty vector, lanes 3 and 4 comprise plasmid DNA ligated with cDNA. Lanes 1 and 5 comprise the DNA molecular weight marker GeneRuler™ 100 bp DNA Ladder Plus.

4.3. Preparation of cDNA arrays

A total of 4608 individual clones, 3072 from the “SSH library” and 1536 from the “vertis library” were picked and their cDNA inserts were amplified via PCR and analyzed by agarose gel electrophoresis. The amplified cDNA fragments were used to prepare three cDNA arrays, two “SSH arrays” and one “vertis array”, each containing 1536 PCR products.

Fig. 11 shows an agarose gel electrophoresis of the PCR products obtained by amplification of the cDNA inserts via PCR. In all 96 reactions a specific PCR product, represented by a distinct band, could be obtained.

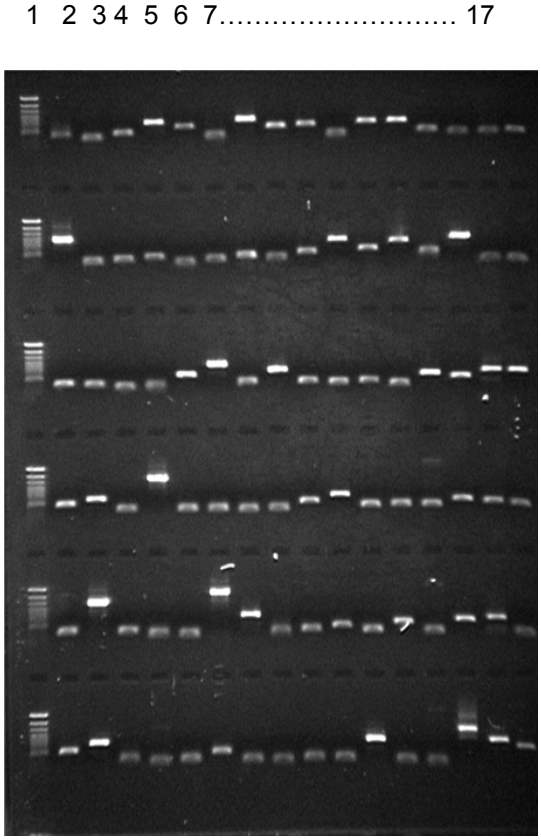


Fig. 11: Amplification of cDNA inserts via PCR

Lanes 2 – 17 comprises the PCR products resulting from amplification of cDNA fragments via PCR, lanes 1 comprise the DNA molecular weight marker GeneRuler™ 100 bp DNA Ladder Plus.

4.4. cDNA synthesis for array hybridization

RNA from intercaruncular endometrial tissue samples of the caudal part of the ipsilateral uterine horn of all 10 animals (pregnant n=5, non-pregnant n=5) was converted to double stranded cDNA. The quality of the cDNA was checked by 0.8 % agarose gel electrophoresis (see fig. 12). The synthesized cDNA is separated as a smear.

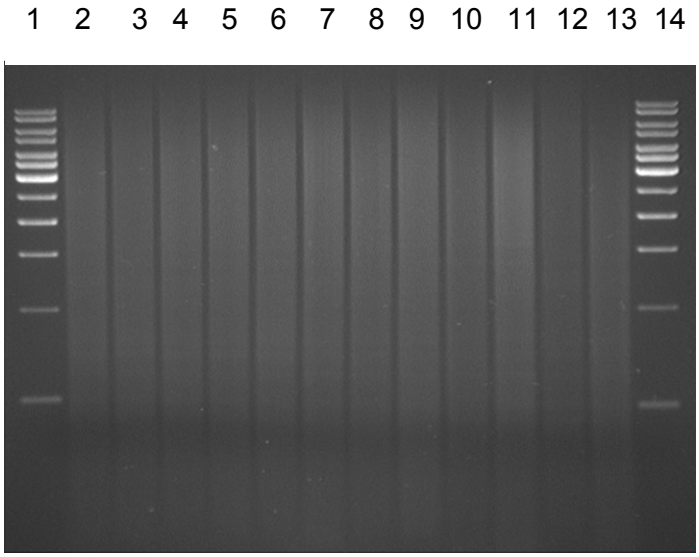


Fig. 12: cDNA synthesis for array hybridization

Lanes 2 – 13 comprise cDNA, lanes 1 and 14 comprise the DNA molecular weight marker GeneRuler™ 100 bp DNA Ladder Plus.

4.5. Array hybridization

The cDNA synthesized for array hybridization was labeled with ^{33}P-dCTP and subjected to array hybridization. Relative gene expression levels pregnant versus non-pregnant were determined for each individual cDNA fragment by pair wise comparison within twin pairs, thus eliminating changes in gene expression due to a different genetic background of individuals. A total of 376 cDNA fragments displayed a difference in signal intensity of 2-fold or more in at least four out of five twin pairs (see fig. 13). These cDNA fragments were assumed to be upregulated in the endometrium at the pre-implantation period.

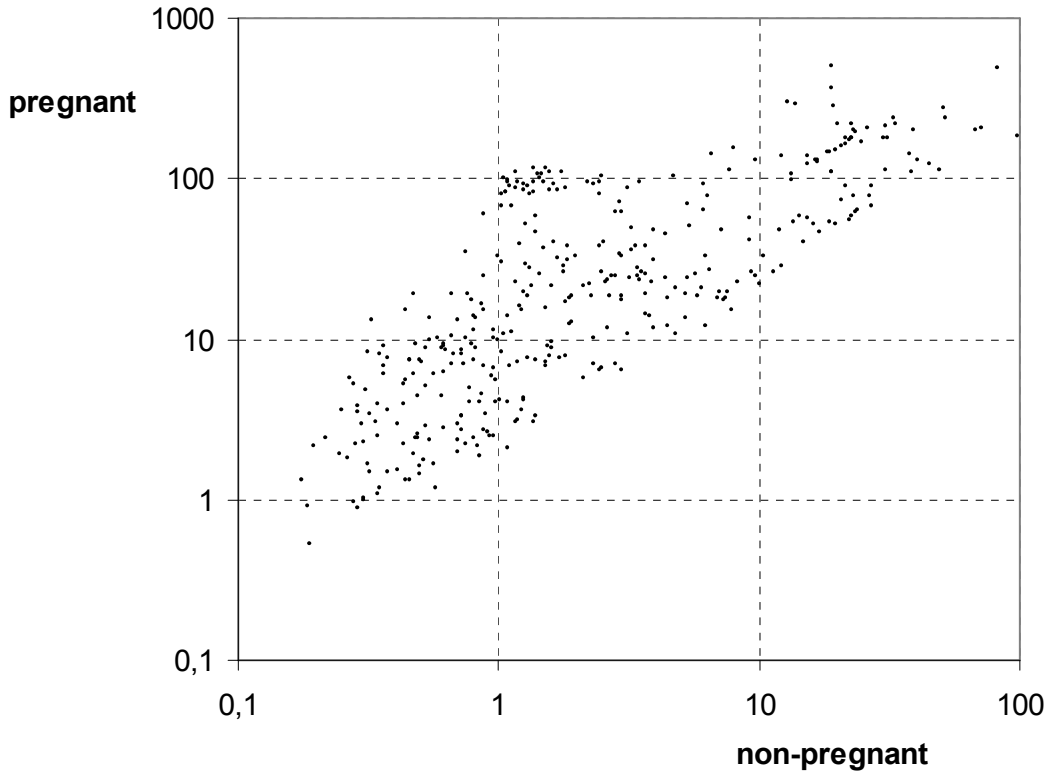
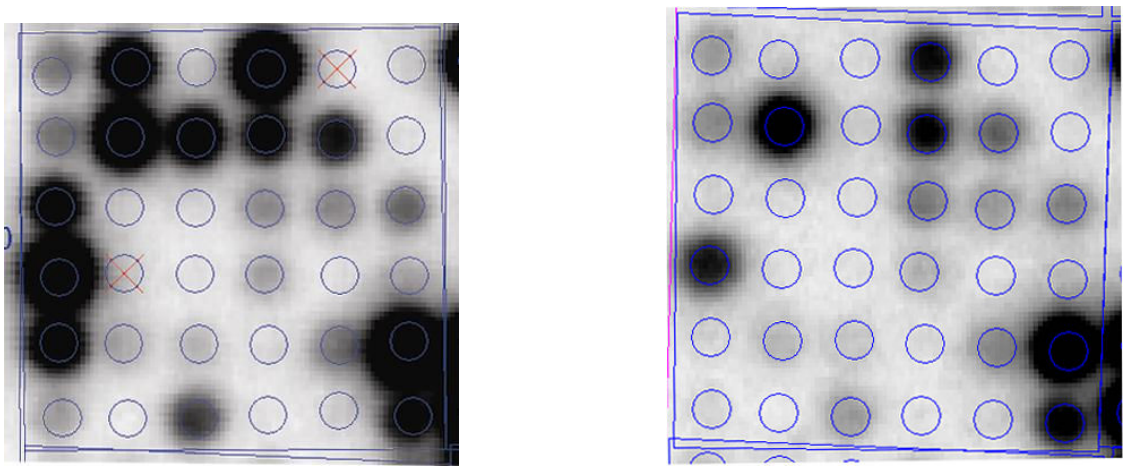


Fig. 13: Scatter Plot of mean normalized signal intensitites of all upregulated cDNA fragments (≥ 2-fold) in at least four out of five twin pairs



pregnant **non-pregnant**

Fig. 14: Corresponding sections of a microarray hybridized with two different cDNA probes: Obvious difference in signal intensity

4.6. Sequence analysis of differentially expressed cDNA framents

Those cDNA fragments displaying a difference in signal intensity of two-fold or more in at least four out of five twin pairs (i.e. 376 fragments) were subjected to sequence analysis, which revealed 87 different genes or mRNAs. For 37 genes more than one cDNA fragment was found and for these genes the mean of all signal ratios was calculated. The genes most abundantly represented on the cDNA arrays were *ISG15* (n=48), *BST2* (n=28), *KIAA 1404* (n=25), *IFITM1* (n=17), *IFITM3* (n=17), and *OAS1* (n=16). Eighty genes corresponded to genes with known or inferred function, either the bovine gene or the human orthologue. For 7 genes a match with bovine expressed sequence tags was obtained only. Table 1 lists the results of the sequence analysis. The common name, the Gene ID, the GenBank accession number, whether stimulated by type I interferons, the results of Gene Ontology classification, the expression ratio, the t-test p-value, and the coefficient of variation (CV) is shown.

Fifteen genes displayed a difference in signal intensity of two-fold or more in only four out of five twin pairs (i.e. *CNDP2*, *CTGF*, *CTMP*, *GRIM19*, *HLA-A*, *HLA-C*, *IFIT2*, *IGFBP-2*, *MTMR6*, *MYO6*, *IDH1*, *PENK*, *RNH*, *SAMD9*, and *SLC15A3*). These genes were consistently represented by one cDNA fragment on the array and the signal ratio for the fifth twin pair ranged from 1.1 to 1.96. For three of these genes the t-test p-value was slightly higher than 0.05 (i.e. *IFIT2*, *HLA-C*, and *SAMD9*), but they were still assumed to be upregulated and included in further analyses. There was only one more gene, i.e. *LY6G6C*, exhibiting a p-value of more than 0.05. The mean coefficient of variation of expression ratio between pregnant and non-pregnant was 41%.

Table 1: Genes that show higher mRNA expression levels in bovine endometrium of pregnant animals

Bovine cDNA/gene or homologue	Gene name	Gene ID	H. s. Gene ID	GenBank acc. number	stimulated by type I IFN	Gene Ontologies Biological Process	Gene Ontologies Molecular Function	Gene Ontologies Cellular Component	ratio	p-value	CV [%]
B.t. beta-2-microglobulin	B2M	280729	567	NM_173893	x	immune response	antigen presentation	_	3.0	0.002	7
H.s. bone marrow stromal cell antigen 2	BST2	684	684	NM_004335	x	cell proliferation; cell-cell signaling; development; humoral immune response	signal transducer activity	integral to plasma membrane	18.1	0.002	34
H.s. complement component 1, r	C1R	715	715	NM_001733	x	complement activation, classical pathway; immune response	calcium ion binding; complement component C1r activity; hydrolase activity	extracellular	4.0	<0.001	34
H.s. complement component 1, s	C1S	716	716	NM_001734	x	complement activation, classical pathway; proteolysis and peptidolysis	calcium ion binding; complement component C1s activity; hydrolase activity	extracellular	3.8	0.005	9
H.s. DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	DDX58	23586	23586	NM_014314	x	_	ATP binding; helicase activity; hydrolase activity; nucleic acid binding; nucleotide binding	_	10.7	0.003	23
DKFZP564J0863 protein	DKFZP564J0863	25923	25923	CK982804	x	immune response	GTP binding, GTPase activity	_	6.4	0.020	26
H.s. interferon, alpha-inducible protein (clone IFI-6-16)	G1P3	2537	2537	NM_002038	x	immune response	_	_	20.5	<0.001	19
H.s. guanylate binding protein 1	GBP1	2633	2633	NM_002053	x	immune response	GTP binding; GTPase activity	_	5.3	0.034	56
H.s. guanylate binding protein 3	GBP3	2633	2635	NM_018284	x	immune response	GTP binding; GTPase activity	_	7.2	0.041	64
H.s. guanylate binding protein 4	GBP4	115361	115361	NM_052941	x	immune response	GTP binding; GTPase activity	_	7.5	0.011	40
H.s. guanylate binding protein 5	GBP5	115362	115362	NM_052942	x	immune response	GTP binding; GTPase activity	_	3.4	0.006	44
B.t. cell death-regulatory protein GRIM19	GRIM19	338084	51079	NM_176672	x	apoptosis; negative regulation of cell growth and protein biosynthesis	ATP binding; NADH dehydrogenase activity; oxidoreductase activity	integral to membrane; mitochondrial electron transport chain	2.4	0.022	31
Bovine MHC class I BoLA gene	HLA-A	533142	3105	M21044	x	antigen presentation; antigen processing, endogenous antigen via MHC class I; immune response	MHC class I receptor activity	integral to plasma membrane	2.6	0.003	26
B.t. mRNA for MHC class I heavy chain	HLA-C	533049	3107	AB008613	x	antigen presentation	_	_	2.7	0.065	44
H.s. XIAP associated factor-1	HSXIAPAF1	54739	54739	NM_199139	x	_	regulation of apoptosis	_	9.7	0.004	29

Bovine cDNA/gene or homologue	Gene name	Gene ID	H. s. Gene ID	GenBank acc. number	stimulated by type I IFN	Gene Ontologies Biological Process	Gene Ontologies Molecular Function	Gene Ontologies Cellular Component	ratio	p-value	CV [%]
H.s. interferon, gamma-inducible protein 16	IFI16	3428	3428	NM_005531	x	cell proliferation; monocyte differentiation; regulation of transcription	double-stranded DNA binding; transcriptional repressor activity	nucleus	15.2	0.005	94
H.s. interferon induced with helicase C domain 1	IFIH1	64135	64135	NM_022168	x	Dna Replication, Recombination, And Repair(LI)	_	_	12.3	0.003	35
H.s. interferon-induced protein with tetratricopeptide repeats 1	IFIT1	3434	3434	NM_001548	x	immune response	Posttranslational Modification, Chaperones, Signal Transduction Mechanisms(LI), Dna Replication		16.3	0.049	65
H.s. interferon-induced protein with tetratricopeptide repeats 2	IFIT2	3433	3433	NM_001547	x	immune response	Cell Cycle Control, Cell Division, Chromosome Partitioning, Posttranslational Modification, Protein Turnover, Chaperones(LI)	_	4.2	0.064	56
H.s. interferon-induced protein with tetratricopeptide repeats 3	IFIT3	3437	3437	NM_001549	x	immune response	Carbohydrate Transport And Metabolism, Posttranslational Modification, Protein Turnover, Chaperones, Signal Transduction Mechanisms(LI)	_	14.8	0.017	57
H.s. interferon-induced protein with tetratricopeptide repeats 5	IFIT5	24138	24138	NM_012420	x	immune response	Posttranslational Modification, Protein Turnover, Chaperones, Signal Transduction Mechanisms(LI)	_	10.9	0.014	35
B.t. interferon induced transmembrane protein 1 (9-27)	IFITM1	353510	8519	NM_174551	x	cell surface receptor linked signal transduction; negative regulation of cell proliferation	receptor signaling protein activity	integral to membrane; plasma membrane	8.5	0.006	24
B.t. interferon induced transmembrane protein 3 (1-8U)	IFITM3	282255	10410	NM_181867	x	immune response; response to biotic stimulus	_	integral to membrane	6.3	0.003	7
H.s. 28kD interferon responsive protein	IFRG28	64108	64108	NM_022147	x	_	_	integral to membrane	4.7	<0.001	56
H.s. interferon regulatory factor 1	IRF1	3659	3659	NM_002198	x	negative regulation of cell cycle; transcription from Pol II promoter	transcription factor activity	nucleus	3.0	0.004	27
Sus scrofa inflammatory response protein 6	IRG6 (RSAD2)	396752	91543	NM_213817	x	_	catalytic activity; iron ion binding	_	18.4	0.010	52
TPA: B.t. mRNA for putative ISG12(a) protein	ISG12a (IFI27)	507138	3429	BN000214	x	_	_	_	11.9	0.011	48
B.t. interferon-stimulated protein, 15 kDa	ISG15 (G1P2)	281871	9636	NM_174366	x	cell-cell signaling; immune response	protein binding	cytoplasm; extracellular space	41.4	0.001	37

Bovine cDNA/gene or homologue	Gene name	Gene ID	H. s. Gene ID	GenBank acc. number	stimulated by type I IFN	Gene Ontologies Biological Process	Gene Ontologies Molecular Function	Gene Ontologies Cellular Component	ratio	p-value	CV [%]
H.s. lymphocyte antigen 6 complex, G6C	LY6G6C	80740	80740	NM_025261	x	_	_	_	97.6	0.052	148
B.t. myxovirus (influenza) resistance 1	MX1	280872	4599	NM_173940	x	immune response; induction of apoptosis; signal transduction	GTP binding; GTPase activity	cytoplasm	11.2	0.012	25
B.t. myxovirus (influenza virus) resistance 2	MX2	280873	4600	NM_173941	x	immune response	GTP binding; GTPase activity	cytoplasm; nucleus	19.4	0.011	57
B.t. 2',5'-oligoadenylate synthetase 1, 40/46kDa	OAS1	347699	4938	NM_178108	x	immune response; nucleotide and nucleic acid metabolism; response to virus	ATP binding; RNA binding; nucleotidyltransferase activity	cytoplasm	20.4	<0.001	40
H.s. 2'-5'-oligoadenylate synthetase 2, 69/71kDa	OAS2	4939	4939	NM_016817	x	nucleobase, nucleoside, nucleotide and nucleic acid metabolism	ATP binding; RNA binding; nucleotidyltransferase activity	membrane; microsome	4.1	0.025	77
B.t. serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor), member 1	SERPING1	281035	710	NM_174821	x	inflammatory response; regulation of lipid metabolism	DNA binding; chymotrypsin inhibitor activity; serine-type endopeptidase inhibitor activity	extracellular; intracellular	2.6	0.040	23
H.s. SP110 nuclear body protein	SP110	3431	3431	NM_080424	x	regulation of transcription, DNA-dependent	hematopoietin/interferon-class (D200-domain) cytokine receptor signal transducer activity, transcription factor activity	nucleus	5.8	0.017	45
PREDICTED: B.t. similar to SP140 nuclear body protein isoform 1 (LOC508830)	SP140	508830	11262	XM_585664	x	defense response, regulation of transcription, DNA-dependent	metal ion binding, transcription factor activity	nuclear membrane, nucleoplasm, nucleus	2.7	0.005	4,00
H.s. signal transducer and activator of transcription 1, 91kDa	STAT1	6772	6772	NM_007315	x	regulation of transcription; tyrosine phosphorylation of STAT protein	cytokine receptor signal transducer transcription factor activity	cytoplasm; nucleus	4.6	0.013	18
B.t. ubiquitin E1-like enzyme	UBE1L	497204	7318	NM_001012284	x	ubiquitin cycle	ATP binding;ubiquitin activating enzyme activity; ubiquitin conjugating enzyme activity	_	11.3	0.005	34
H.s. Rho guanine nucleotide exchange factor (GEF) 12	ARHGEF12	23365	23365	NM_015313		cell growth; GTPase activator activity; IGF-1 receptor interacting molecule	_	_	6.0	0.019	28
H.s. ATPase, Na+/K+ transporting, beta 2 polypeptide	ATP1B2	482	482	NM_001678		sodium:potassium-exchanging ATPase activity	_	_	15.5	<0.001	27
H.s. BAT2 domain containing 1	BAT2D1	23215	23215	NM_015172		_	_	_	20.1	0.010	43
B.t. complement component C4	C4A	280678	720	BTU16749		complement activation	complement component C4 activity	extracellular	8.3	0.008	32

Bovine cDNA/gene or homologue	Gene name	Gene ID	H. s. Gene ID	GenBank acc. number	stimulated by type I IFN	Gene Ontologies Biological Process	Gene Ontologies Molecular Function	Gene Ontologies Cellular Component	ratio	p-value	CV [%]
H.s. chromosome 5 open reading frame 18	C5orf18	7905	7905	NM_005669		_	_	integral to membrane	2.8	0.034	28
H.sapiens C-type lectin domain family 4, member F	CLEC4F	165530	165530	NM_173535		endocytosis	receptor activity, sugar binding	integral to membrane	4.1	0.031	36
H.s. CNDP dipeptidase 2 (metallopeptidase M20 family)	CNDP2	55748	55748	NM_018235		proteolysis and peptidolysis	metallopeptidase activity	_	2.3	0.033	43
B.t. connective tissue growth factor	CTGF	281103	1490	NM_174030		DNA metabolism; cell adhesion; cell growth; regulation of cell growth	heparin binding; insulin-like growth factor binding; protein binding	extracellular matrix (sensu Metazoa); plasma membrane; soluble fraction	3.2	0.027	56
H.s. C-terminal modulator protein	CTMP	117145	117145	NM_053055		_	catalytic activity	_	3.1	0.001	31
H.s. deltex 3-like (Drosophila) (DTX3L)	DTX3L	151636	151636	NM_138287		_	_	_	3.8	<0.001	30
B.t. eukaryotic translation initiation factor 4E	EIF4E	28175	1977	NM_174310		regulation of translation; translational initiation	RNA cap binding; translation initiation factor activity	eukaryotic translation initiation factor 4F complex	8.3	0.006	55
H.s. epithelial stromal interaction 1 (breast)	EPSTI1	94240	94240	NM_001002264		_	_	_	11.3	<0.001	37
B.t. fatty acid binding protein (heart) like	FABP3	281758	2170	NM_174313		_	_	_	10.7	0.016	63
B.t. glycosylphosphatidyl-inositol specific phospholipase D1	GPLD1	287025	2822	NM_174816		cell-matrix adhesion	glycosylphosphatidylinositol phospholipase D activity	extracellular; integrin complex	5.4	0.003	18
Mus musculus general transcription factor II I repeat domain-containing 1	Gtf2ird1	57080	9569	NM_020331		regulation of transcription, DNA-dependent	transcription factor activity	nucleus	19.5	0.005	82
Ovis aries cytosolic NADP-isocitrate dehydrogenase	IDH1	443257	3417	AY208678		_	_	_	2.7	0.044	39
B.t. trophoblast protein-1	TP-1	317698		M31557		cell cycle arrest; defense response; response to virus	interferon-alpha/beta receptor binding	extracellular	2.7	0.005	45
B.t. insulin-like growth factor binding protein 2	IGFBP2	282260	3485	NM_174555		cell growth and/or maintenance; regulation of cell growth	insulin-like growth factor binding	extracellular space	2.6	0.002	40
H.s. KIAA1404 protein	KIAA1404	57169	57169	NM_021035		_	_	_	5.2	0.003	39

Bovine cDNA/gene or homologue	Gene name	Gene ID	H. s. Gene ID	GenBank acc. number	stimulated by type I IFN	Gene Ontologies Biological Process	Gene Ontologies Molecular Function	Gene Ontologies Cellular Component	ratio	p-value	CV [%]
PREDICTED: H.s. KIAA0342 gene product; lupus brain antigen 1	LBA1	9881	9881	XM_047357		_	_	_	6.8	0.002	31
H.s. lipopolysaccharide binding protein	LBP	3929	3929	NM_004139		acute-phase response; defense response to bacteria; lipid transport	lipid binding	extracellular space; integral to membrane	3.5	0.037	46
H.s. likely ortholog of mouse D11lgp2	LGP2	79132	79132	NM_024119		DNA restriction	ATP binding; ATP-dependent helicase activity; nucleic acid binding	cytoplasm	4.5	0.001	19
PREDICTED: H.s. similar to LINE-1 reverse transcriptase homolog	LOC401623	401623	401623	XM_377072		inflammatory response; regulation of lipid metabolism	DNA binding; serine-type endopeptidase inhibitor activity	extracellular; intracellular	3.8	0.005	16
B.t. milk fat globule-EGF factor 8 protein	MFGE8	281913	4240	NM_176610		cell adhesion; fertilization (sensu Metazoa)	protein binding	lipid particle	6.9	0.031	28
H.s. matrix metalloproteinase 19	MMP19	4327	4327	NM_002429		angiogenesis; collagen catabolism; proteolysis and peptidolysis	hydrolase activity; metalloendopeptidase activity; zinc ion binding	extracellular matrix (sensu Metazoa)	3.9	0.008	5
H.s. membrane-spanning 4-domains, subfamily A, member 8B	MS4A8B	83661	83661	NM_031457		signal transduction	receptor activity	integral to membrane	5.8	0.011	96
H.s. myotubularin related protein 6	MTMR6	9107	9107	NM_004685		protein amino acid dephosphorylation	protein serine/threonine phosphatase activity; protein tyrosine phosphatase activity	cellular_component unknown	2.5	0.045	27
H.s. myosin VI	MYO6	4646	4646	NM_004999		perception of sound; striated muscle contraction	ATP binding; actin binding; calmodulin binding	unconventional myosin	2.2	0.012	20
H.s. neighbor of BRCA1 gene 2	NBR2	10230	10230	NM_005821		_	_	_	2.7	0.009	30
H.s. p8 protein (candidate of metastasis 1)	P8	26471	26471	NM_012385		protein transport	GTP binding	Golgi apparatus	2.7	<0.001	20
H.s. zinc finger CCCH type domain containing 1	PARP12	64761	64761	NM_022750		cell growth; induction of apoptosis	molecular_function unknown	nucleus	4.4	0.015	56
B.t. proenkephalin (PENK)	PENK	281387	5179	NM_174141		cell-cell signaling; neuropeptide signaling pathway; signal transduction	neuropeptide hormone activity; opioid peptide activity	soluble fraction	4.0	0.006	52
H.s. placenta-specific 8	PLAC8	51316	51316	NM_016619		_	_	_	8.6	0.009	55
Sus scrofa placenta expressed transcript protein	PLET	396570	–	NM_213744		_	_	_	9.6	0.014	70

Bovine cDNA/gene or homologue	Gene name	Gene ID	H. s. Gene ID	GenBank acc. number	stimulated by type I IFN	Gene Ontologies Biological Process	Gene Ontologies Molecular Function	Gene Ontologies Cellular Component	ratio	p-value	CV [%]
H.s. polymerase (RNA) II (DNA directed) polypeptide I, 14.5kDa	POLR2I	5438	5438	NM_006233		RNA elongation; DNA-dependent; transcription from Pol II promoter	DNA-directed RNA polymerase activity; transcription factor activity; transferase activity	DNA-directed RNA polymerase II, core complex; nucleus	21.3	0.031	42
H.s. RNA binding motif and ELMO domain 1	RBED1	84173	84173	NM_032213		apoptosis; phagocytosis		cytoskeleton	6.3	0.009	25
ribonuclease, RNase A family, 11	RNASE11	122651	122651	NM_145250		RNA degradation	endonuclease activity hydrolase activity nucleic acid binding	_	6.2	0.009	51
H.s. ribonuclease/angiogenin inhibitor	RNH1	6050	6050	NM_002939		RNA catabolism	ribonuclease inhibitor activity	_	2.5	0.046	54,00
H.s. sterile alpha motif domain containing 9	SAMD9	54809	54809	NM_017654		_	_	_	3.5	0.076	56
H.s. scotin	SCOTIN	51246	51246	NM_016479		positive regulation of I-kappaB kinase/NF-kappaB cascade	signal transducer activity	_	3.7	0.004	35
H.s. solute carrier family 15, member 3	SLC15A3	51296	51296	NM_016582		oligopeptide transport	transporter activity	membrane	5.4	0.009	78
B.t. tissue inhibitor of metalloproteinase 2	TIMP2	282093	7077	NM_174472		_	metalloendopeptidase inhibitor activity	extracellular matrix (sensu Metazoa)	3.8	0.003	30
B.t. uterine milk protein precursor	UTMP	286871	–	NM_174797		_	_	_	7.6	0.009	97
B.t. cDNA	–	–	–	AJ674490		_	_	_	8.5	0.026	49
B.t. cDNA	–	–	–	AJ677683		_	_	_	3.5	0.006	28
Bos sp. cDNA	–	–	–	AJ818354					2.4	0.012	11
B.t. cDNA	–	–	–	BE682545		_	_	_	15.0	<0.001	66
B.t. cDNA; UniGene Bt.9217	–	–	–	BM251742		_	_	_	9.2	0.014	43
B.t. cDNA	–	–	–	CB443652		_	_	_	3.1	0.009	28
B.t. cDNA	–	–	–	CK958692		_	_	_	12.3	0.009	52

[A] B.t. *Bos taurus*, H.s. *Homo sapiens*; [B] in parentheses *Homo sapiens* gene name if differing; [C] used for Gene Ontology analysis; [D] stimulated by type I interferones

4.7. Categorization of the identified genes regarding their function

The expression of 38 genes is known to be stimulated by type I interferons (see table 1), corresponding to almost one half of the identified genes whose function is known or inferred. To get an overview of the identified genes regarding their function a simplified Gene Ontology classification was built based on the human orthologous genes. The results are visualized in figures 15 - 17, whereat figure 15 gives an overwiev of the classification of the identified genes into functional categories, and figures 16 and 17 list in more detail the results of the categorization according to the molecular function of the identified genes or the biological process they are involved in, respectively.

The majority of genes was assigned either to one of the biological processes “regulation of gene expression”, “cell communication”, “cell growth, proliferation and differentiation”, or exhibits “cell adhesion molecule activity” or an “immune-related function”.

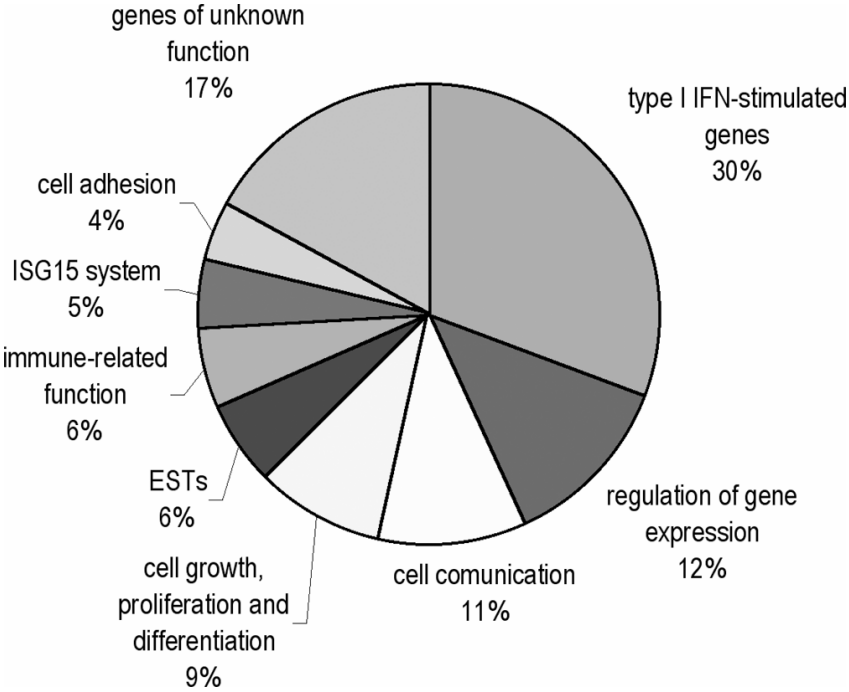


Fig. 15: Classification of the identified genes into functional categories. Multiple naming is possible.

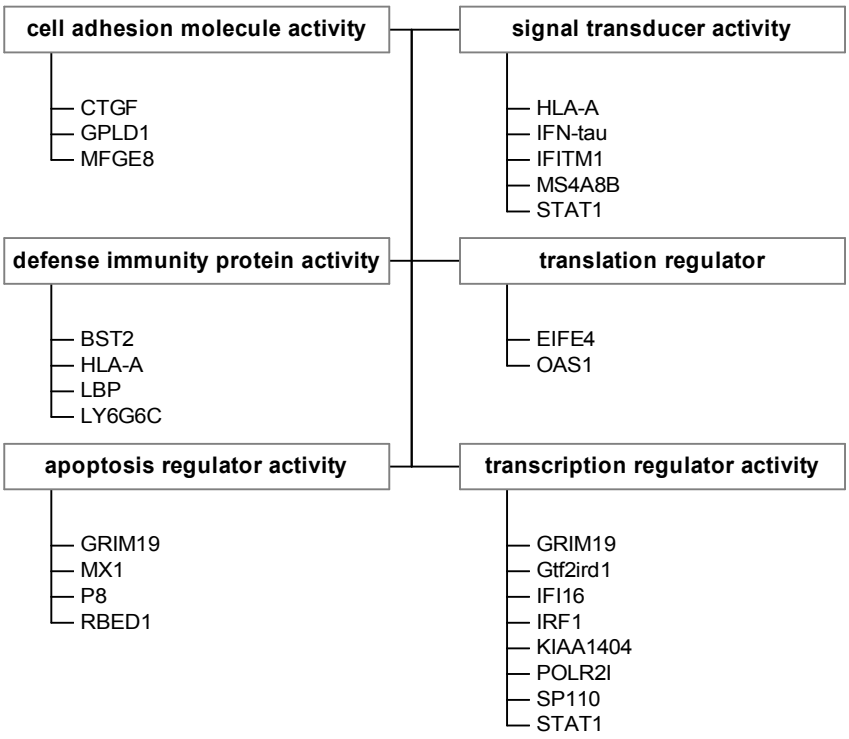


Fig. 16: Gene Ontology "Molecular Function"

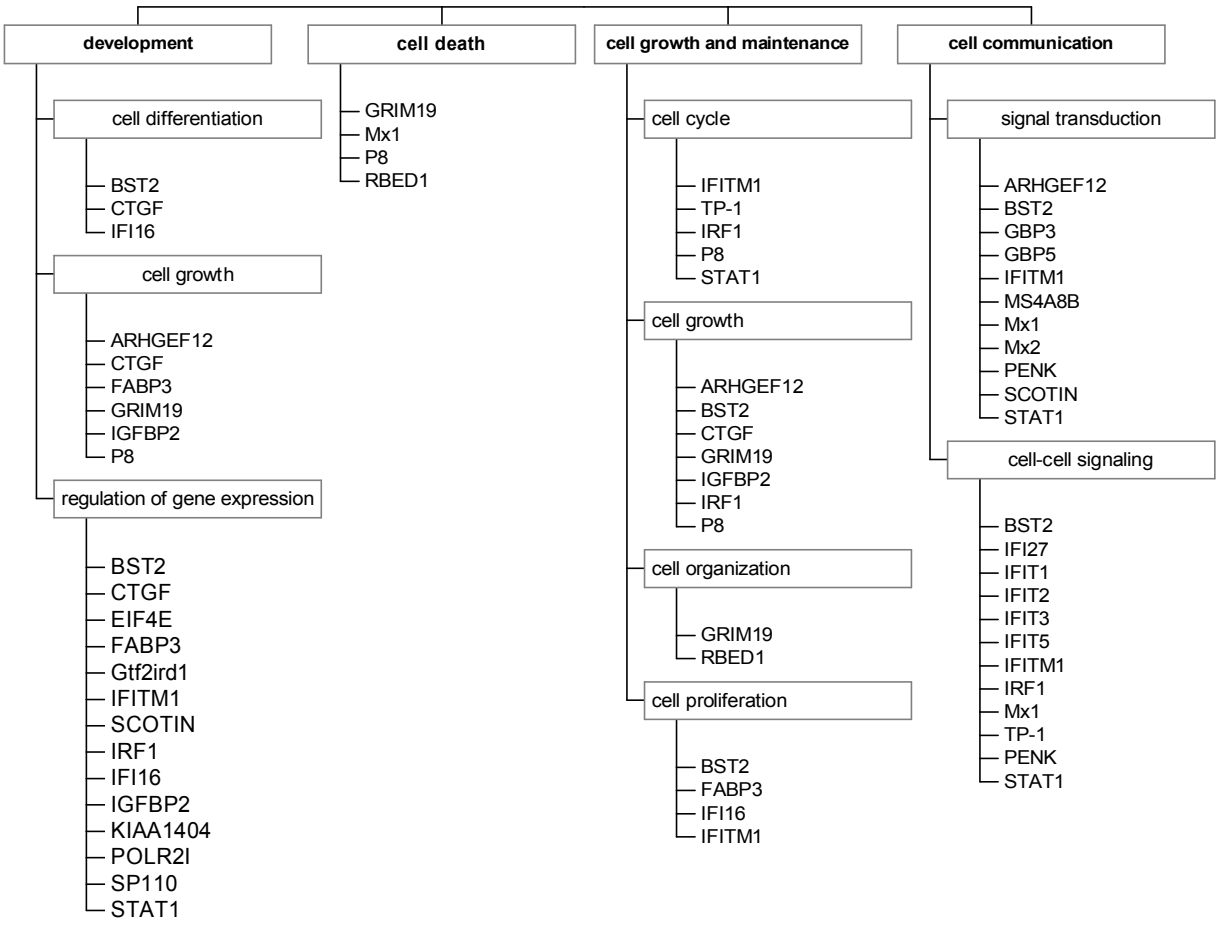


Fig. 17: Gene Ontology "Biological Process"

Table 2 lists in more detail important biological processes or functional groups and the corresponding genes with their fold upregulation. Besides many genes involved in signal transduction, there were seven genes coding for products with GTPase activity, five potential members of the ISG15ylation system, four members of the complement system (including the C1 inhibitor *SERPING1*), and also two genes involved in or related to the IGF system, respectively (*IGFBP2* and *CTGF*). With respect to the process “cell proliferation” three of the seven genes are described to negatively regulate this process. In contrast, three out of six apoptosis-related genes positively regulate cell death. The categories “proteolysis and peptidolysis”, “cell adhesion”, and “morphogenesis” contain some genes, which code for proteins that are particularly important for remodeling of the endometrium, like MMP19, TIMP2, and CTGF.

Table 2: Important biological processes or functional groups and the corresponding genes with their fold upregulation

Biological Process/Molecular Function	Gene name	Fold up
signal transduction	HLA-A	2.6
	HLA-C	2.7
	IFITM1	8.5
	MS4A8B	5.8
	MX1	11.2
	PENK	4.0
hematopoietin/interferon-class (D200-domain) cytokine receptor signal transducer activity	SP110	5.8
	STAT1	4.6
I-kappaB kinase/NF-kappaB cascade	BST2	18.1
	STAT1	4.6
	SCOTIN (+)	3.7
GTPase activator activity	ARHGEF12	6.0
GTPase activity	DKFZP564J0863	6.4
	GBP1	5.3
	GBP3	7.2
	GBP4	7.5
	GBP5	3.4
	MX1	11.2
	MX2	19.4
protein modification		
ISGylation	DTX3L	3.8
	IFITM1	8.5
	IFITM3	6.3
	ISG15 (G1P2)	41.4
	UBE1L	11.3
phosphorylation	MTMR6	2.5
ADP ribosylation	PARP12	4.4
cell communication	CTGF	3.2
	GPLD1	5.4
	MFGE8	6.9
	MS4A8B	5.8
	PENK	4.0
	SCOTIN	3.7
cell-cell signaling	BST2	18.1
	ISG15 (G1P2)	41.4
	PENK	4.0
regulation of transcription	GRIM19 (-)	2.4
	GTF2IRD1	19.5
	IFI16 (-)	15.2
	IRF1	3.0
	POLR2I	21.3
	SP110	5.8
	SP140	2.7
	STAT1	4.6
proteolysis and peptidolysis		
metallopeptidase activity	CNDP2	2.3
	MMP19	3.9
peptidase activity	C1R	4.0
	C1S	3.8
protease inhibitor activity	C4A	8.3
	SERPING1	2.6
	TIMP2	3.8

Biological Process/Molecular Function	Gene name	Fold up
	STAT1	4.6
cell adhesion	CLEC4F	4.1
	CTGF	3.2
	MFGE8	6.9
transport	ATP1B2	15.5
	FABP3	10.7
	GRIM19	2.4
	LBP	3.5
	NBR2	2.7
	SLC15A3	5.4
	STAT1	4.6
cell proliferation	BST2	18.1
	FABP3 (-)	10.7
	IFI16	15.2
	IFITM1 (-)	8.5
	IFITM3 (-)	6.3
	IRF1	3.0
	STAT1	4.6
cell cycle	IFITM1	8.5
	IFITM3	6.3
	IRF1 (-)	3.0
	STAT1	4.6
apoptosis	GRIM19 (+)	2.4
	HSXIAPAF1 (+)	9.7
	IFIH1	16.3
cell-matrix adhesion	GPLD1	5.4
	MX1 (+)	11.2
	P8	2.7
	STAT1	4.6
morphogenesis	CTGF	3.2
	IGFBP2	2.6
	MMP19	3.9
	P8	2.7
growth	P8	2.7
insulin-like growth factor binding	CTGF	3.2
	IGFBP2	2.6
complement activation	C1R	4.0
	C1S	3.8
	C4A	8.3
	SERPING1	2.6
helicase activity	DDX58	10.7
	IFIH1	12.3
	LGP2	4.5
reproduction	MFGE8	6.9
angiogenesis	MMP19	3.9

4.8. Comparison of the “vertis library” and the “SSH library”

A total of 376 cDNA fragments displayed a difference in signal intensity of 2-fold or more in at least four out of five twin pairs; 210 cDNA fragments were located on one of the two “SSH arrays”, the remaining 166 cDNA fragments were located on the “vertis array” (view table 3).

The analysis of the “SSH library” revealed 71 different genes or mRNAs, 35 different genes or mRNAs could be identified analyzing the “vertis library”. Table 3 gives an overview of the results obtained by array hybridization and sequence analysis.

	SSH library	vertis library
number of individual cDNA clones analyzed	3072	1536
number of cDNA clones showing a difference in signal intensity of 2-fold or more in at least 4 out 5 twin pairs	210	166
number of different cDNA clones	71	35

Table 3: Results obtained by array hybridization and sequence analysis

Altogether, the sequence analysis revealed a total of 87 different genes or mRNAs; 19 cDNAs were present in both libraries, 52 cDNAs were only present in the SSH library, respectively 16 cDNAs were only present in the vertis library (view fig. 18).

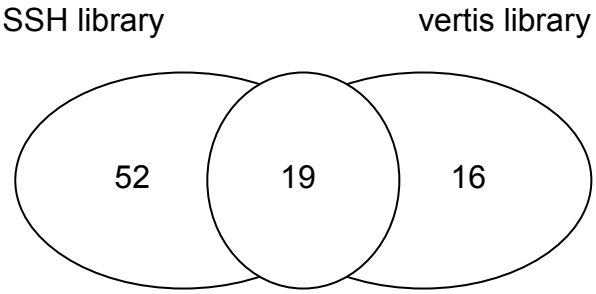


Fig. 18: Extent of overlap between the SSH library and the vertis library

Altogether 87 different cDNAs were identified. From these 87 cDNAs, 19 cDNAs were present in both libraries, 52 cDNAs were only present in the SSH library and 16 cDNAs were only present in the vertis library.

In general, cDNAs that were represented by three or more cDNA clones, either on the “SSH array” or “vertis array”, were present in both arrays. In contrast, cDNAs that were represented by one or two cDNA clones, either on the “SSH array” or “vertis

array", were only present on one array in the majority of cases. However, some cDNAs (i.e. *KIAA1404*, *ISG12*, *G1P3*, and *PENK*) were identified to be present only on one array, even though they were represented by five or more cDNA fragments (view table table 4).

	SSH library	vertis library
KIAA1404	-	15
OAS1	14	3
ISG12	13	-
G1P3	11	-
PENK	5	-

Table 4: cDNAs identified to be present in only one library, even though they were represented by five or more cDNA fragments on the array

The percentage of cDNA clones showing a difference in signal intensity of 2-fold or more in at least four out of five twin pairs was substantially higher in the vertis library than in the SSH library (10.8% versus 6.8%) but redundancy of the identified genes was also much higher in the vertis library. Thus, the number of differentially expressed genes in relation to the total number of analyzed cDNA clones was almost the same in both libraries (2.3% in the SSH library versus 2.2% in the vertis library)
Fig. 19 shows the high abundance of certain genes in the vertis library as compared to the SSH library. In the vertis library five genes represent more than 50 % of the differentially expressed cDNA clones, whereas such a predominance of certain genes is not observed in the SSH library.

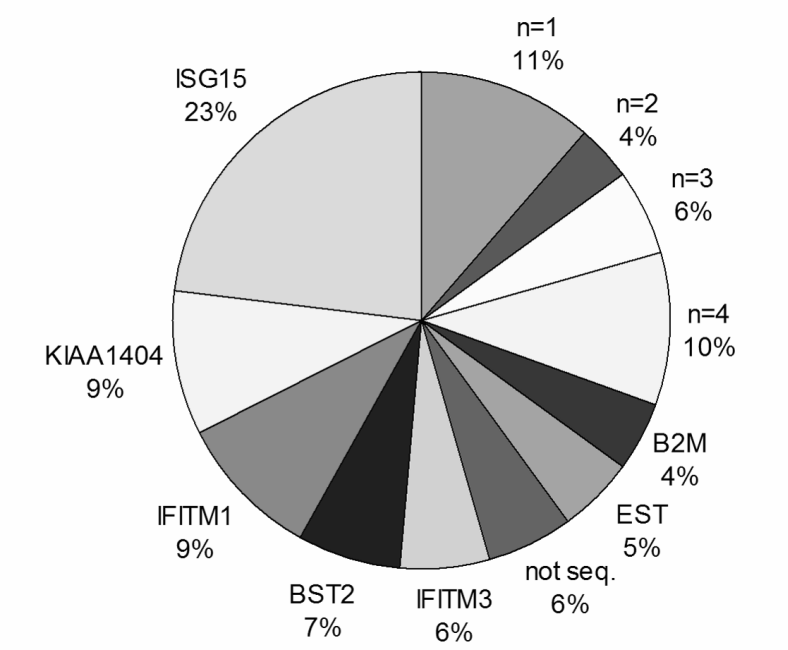


Fig. 19a: vertis library

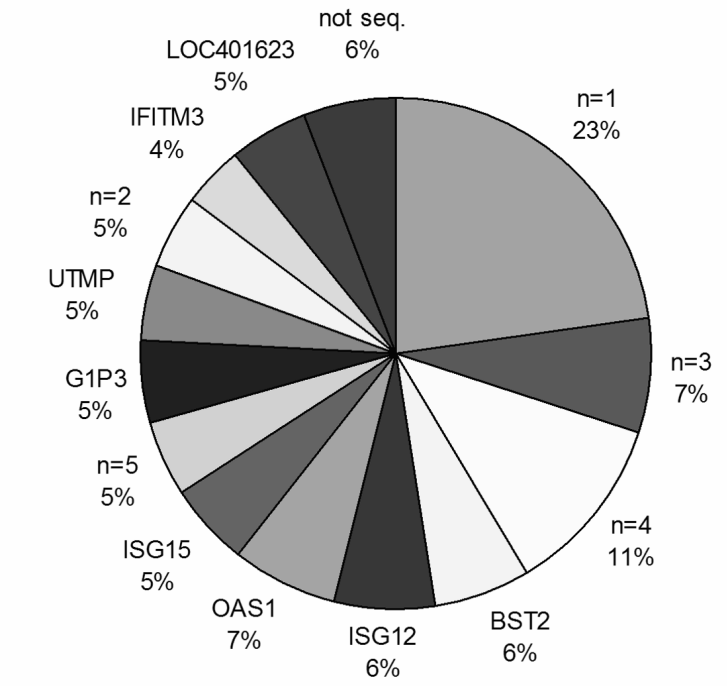


Fig. 19b: SSH library

Fig. 19: Proportional frequency distributions of the identified genes in relation to the total number of cDNA clones showing a difference in signal intensitiy of > 2.0 fold in at least four out of five twin pairs

(EST: expressed sequence tags; not seq.: no match with neither of the utilized databases; n=1, 2, 3, or 4: genes which were represented by 1, 2, 3, or 4 cDNA fragments on the array)

4.9. Validation and precise quantification of mRNA expression by quantitative real-time RT-PCR for selected genes

For nine selected genes (*C1S*, *C1R*, *DTX3L*, *HSXIAPAF1*, *IFITM3*, *ISG15*, *SERPING1*, *UBE1L* and *UTMP*) the mRNA expression in the bovine endometrium was quantified by the use of quantitative real-time RT-PCR (qPCR) to verify the results obtained by array hybridization and to perform more precise quantitative measurements for these genes. The same 10 RNA samples as for hybridization experiments were used. In addition to the fold-upregulation revealed by qPCR and by array hybridization table 5 shows the mean of the crossing points (CP), the mean of the CP normalized to the ubiquitin mRNA (ΔCP), and the expression difference in form of the ΔΔCP. On the basis of the CP the expression level of the mRNAs can be estimated (low CP = high and high CP = low expression). The abundance of all investigated transcripts was significantly higher in the pregnant stage. The highest expression ratio (186-fold) was found for the *ISG15* mRNA. The ratios found by qPCR were in most cases slightly higher and in some cases, e.g. *UTMP* and *ISG15* clearly higher compared to those obtained by array hybridization. The results are further visualized in fig. 20.

	CP Mean/SEM		ΔCP Mean/SEM		ΔΔCP Mean/ SEM	Fold up Mean/SEM		t-test p value	
Gene	Contr	ET	Contr	ET	ET	qPCR	Array	qPCR	Array
Ubiquitin	19.2 ±0.2	19.5 ±0.4	-	-	-	-	-	-	-
C1R	22.1 ±0.3	20.2 ±0.2	2.8 ±0.3	0.7 ±0.3	2.1 ±0.3	4.7 ±0.9	4 ±0.6	0.001	<0.001
C1S	23.4 ±0.4	21.3 ±0.6	4.1 ±0.4	1.9 ±0.4	2.3 ±0.4	5.6 ±1.4	3.8 ±0.2	0.003	0.005
DTX3L	23.7 ±0.3	20.9 ±0.7	4.5 ±0.4	1.5 ±0.3	3 ±0.3	8.8 ±1.6	3.8 ±0.5	<0.001	<0.001
HSXIAPAF1	22.7 ±0.3	19.5 ±0.6	3.4 ±0.4	0.1 ±0.3	3.3 ±0.4	12.1 ±3.3	9.7 ±1.3	<0.001	0.004
IFITM3	19.7 ±0.3	16.8 ±0.4	0.5 ±0.4	-2.6 ±0.1	3.1 ±0.4	9.8 ±2.6	6.3 ±0.2	<0.001	0.003
ISG15	30.4 ±0.5	23.1 ±0.5	11.2 ±0.4	3.7 ±0.3	7.5 ±0.2	185.6 ±27.7	41.4 ±6.8	<0.001	0.001
SERPING1	30.9 ±0.4	29 ±0.4	11.7 ±0.4	9.6 ±0.3	2.1 ±0.4	5.2 ±1.9	2.6 ±0.3	0.004	0.040
UBE1L	24.6 ±0.4	20.3 ±0.5	5.3 ±0.4	0.8 ±0.3	4.5 ±0.3	25.6 ±6.8	11.3 ±1.7	<0.001	0.005
UTMP	26.8 ±1.4	22.3 ±0.8	7.6 ±1.4	2.8 ±0.7	4.8 ±1.3	163.9 ±147.8	7.6 ±3.3	0.012	0.009

CP: crossing point; ΔCP: $CP-CP_{ubi}$; ΔΔCP: $\Delta CP_{ET} - \Delta CP_{Contr}$

Table 5: Validation of array results by quantitative real-time RT-PCR

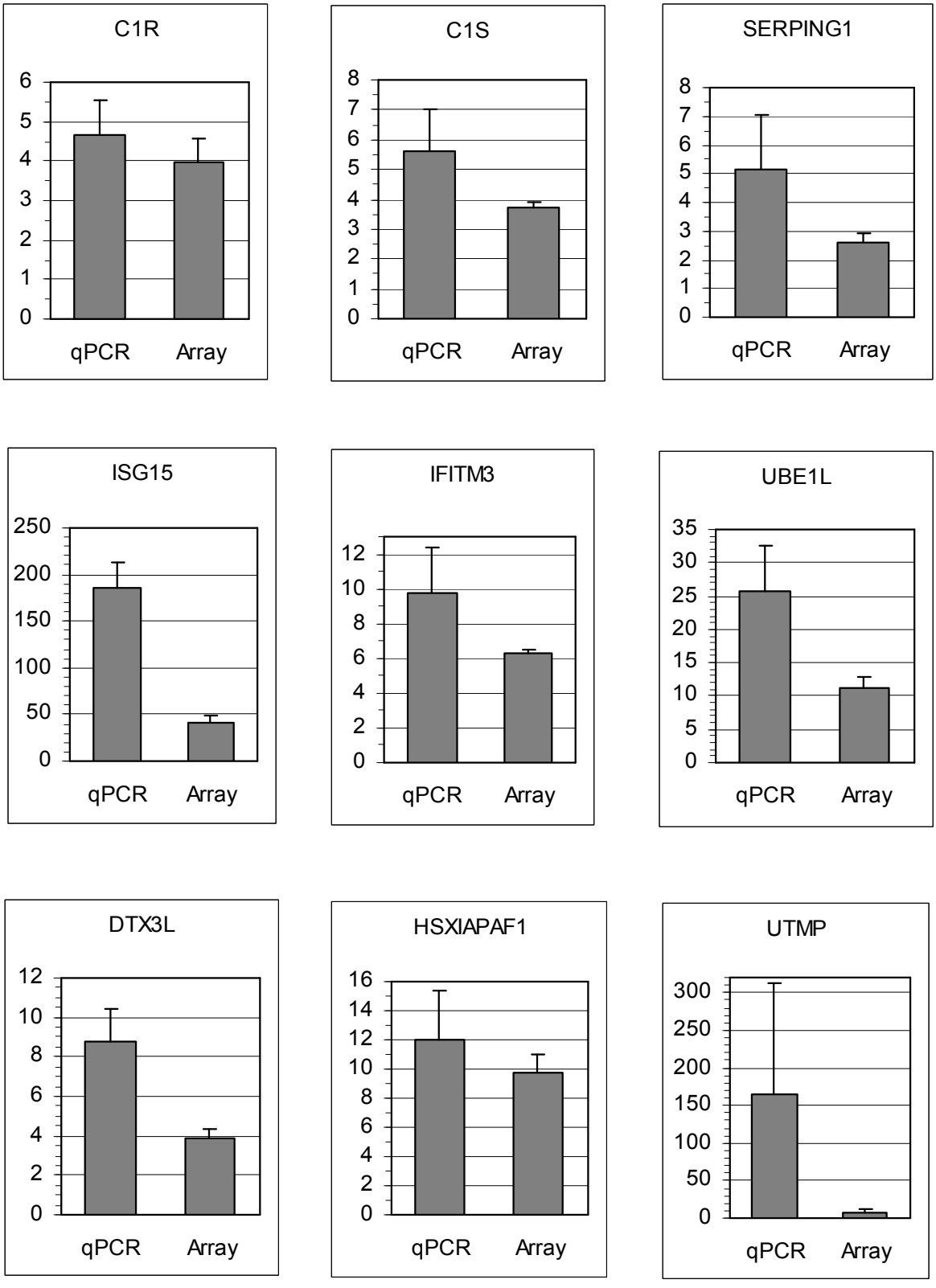


Fig. 20: Validation of array results by quantitative real-time RT-PCR. The mean fold-upregulation revealed by qPCR and by array hybridization is shown. The error bars represent the SEM.

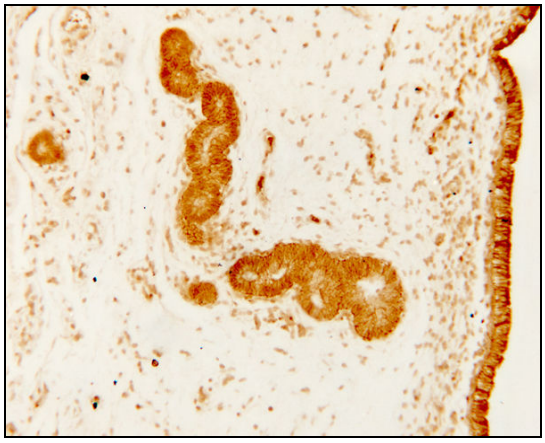
4.10. Localization of mRNA expression for selected genes by *in situ* hybridization

For seven selected genes (*C1S*, *C1R*, *DTX3L HSXIAPAF1*, *IFITM3*, *SERPING1*, and *UBE1L*) *in situ* hybridization with bovine endometrial tissue sections was performed to localize the mRNA expression in this complex tissue. A specific pattern of mRNA distribution was found for each of these genes (fig. 21 - 23). The hybridization signal was always confined to cells of the endometrium and was absent in the myometrium and the serosa. No specific signals were observed in sections hybridized with the sense strand or in sections incubated with buffer only instead of the oligonucleotide probe (not shown). Table 6 summarizes the results of the *in situ* hybridization experiments. The mRNAs for *DTX3L*, *IFITM3*, *HSXIAPAF1*, and *UBE1L*, which were selected due to their potential role in the ISG15ylation system showed a very similar expression pattern with strong signals in the luminal epithelium (except for *DTX3L*), in the superficial and deep uterine glands and weak expression in stromal cells. The three members of the complement system showed also a similar expression pattern. Co-localization was found in the luminal epithelium and the superficial glands. Specific expression of *SERPING1* mRNA was not detectable in stromal cells.

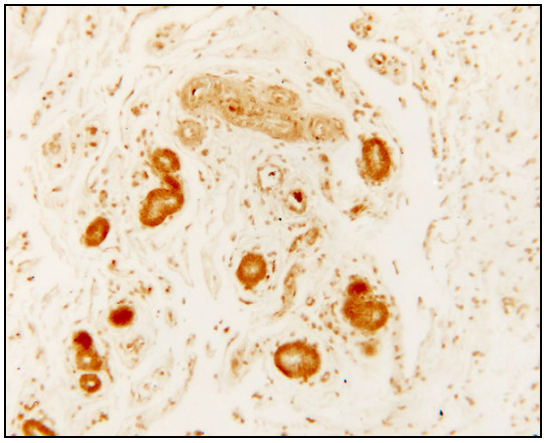
Gene	luminal epithelium	glandular epithelium		stromal cells
		superficial glands	deeper glands	
DTX3L	+	++	+	(+)
IFITM3	+++	++	++	+
UBE1L	+++	++	++	(+)
HSXIAPAF1	+++	+++	+++	(+)
C1R	+++	+++	+++	+
C1S	++	++	-	(+)
SERPING1	+	++	+	-

- no staining, (+) weak staining, + moderate staining, ++ distinct staining, +++ strong staining

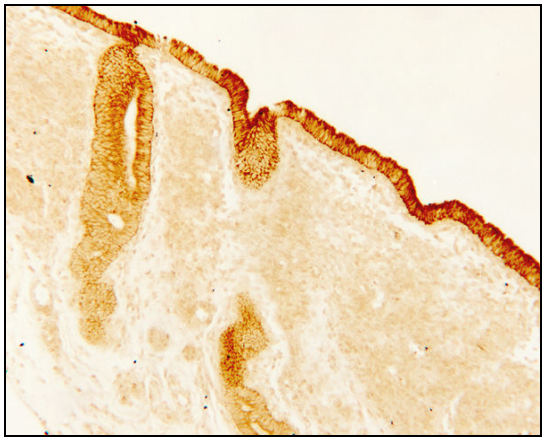
Table 6: Results of *in situ* hybridization with bovine endometrium



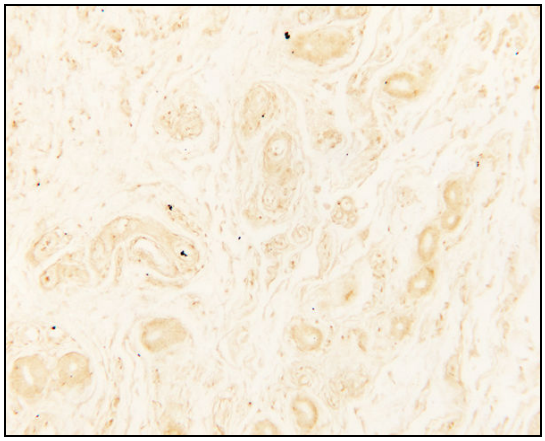
C1R



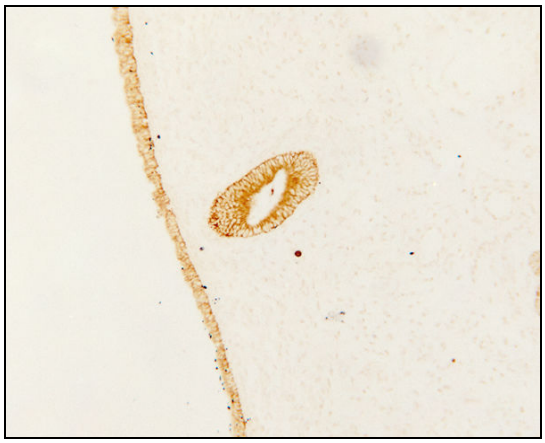
C1R



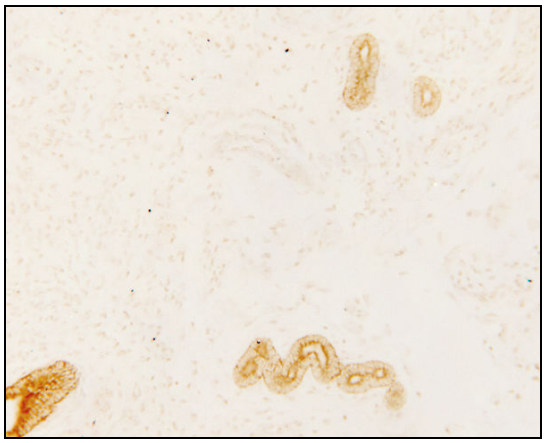
C1S



C1S

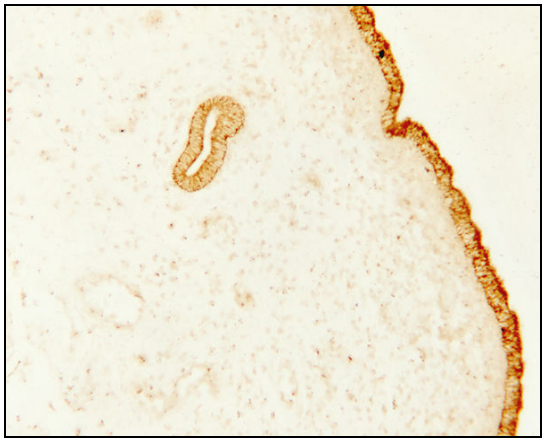


SERPING1

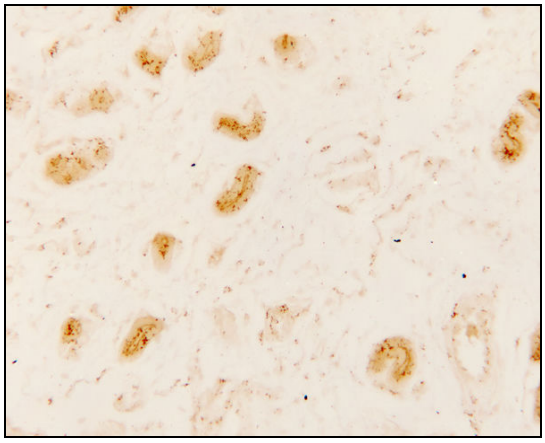


SERPING1

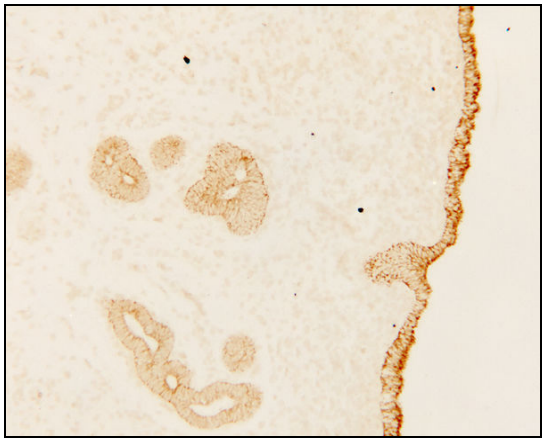
Fig. 21: *In situ* hybridization for the detection of C1S, C1R, and SERPING1 mRNA. On the left hand site endometrial sections near the epithelial surface are shown, on the right hand site endometrial sections near the deep uterine glands are shown. Magnification: 50-fold.



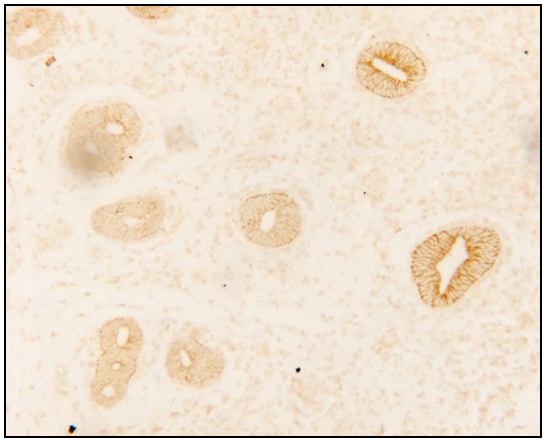
IFITM3



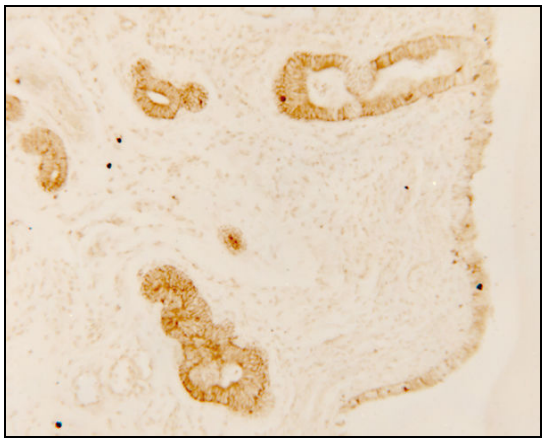
IFITM3



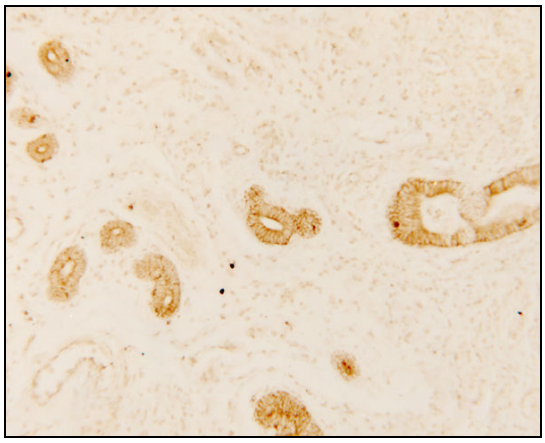
UBE1L



UBE1L

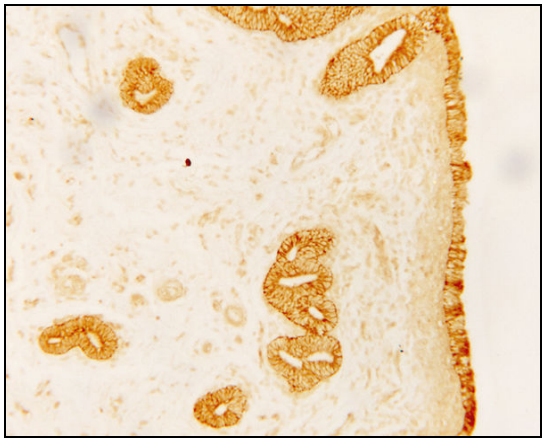


DTX3L

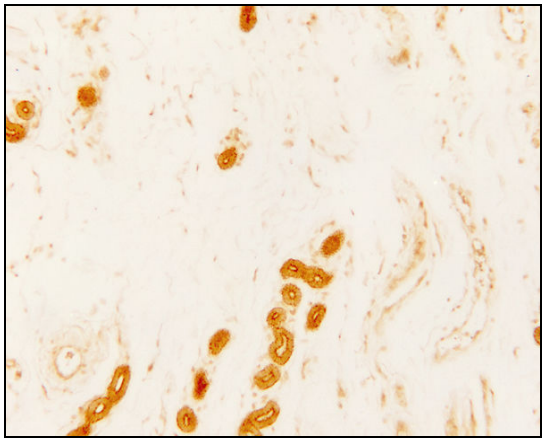


DTX3L

Fig. 22: ***In situ*** **hybridization for the detection of IFITM3, UBE1L, and DTX3L mRNA. On the left hand site endometrial sections near the epithelial surface are shown, on the right hand site endometrial sections near the deep uterine glands are shown. Magnification: 50-fold.**



HSXIAPAF1



HSXIAPAF1

Fig. 23: *In situ* hybridization for the detection of HSXIAPAF1 mRNA. On the left hand site an endometrial section near the epithelial surface is shown, on the right hand site an endometrial section near the deep uterine glands is shown. Magnification: 50-fold.

5. Discussion

The objective of the present study was to elucidate changes in gene expression of the bovine endometrium due to the presence of a conceptus during the pre-implantation stage, i.e. day 18 of gestation. Only few other studies have been conducted so far to investigate genes involved in the process of embryonic implantation in the rhesus monkey (Sun et al., 2004) and in mice (Yoshioka et al., 2000; Reese et al., 2001), and this is to our knowledge the first study of this kind in the bovine species. A combination of subtracted cDNA libraries and cDNA array hybridization was applied to detect cDNAs of genes differentially expressed in the bovine endometrium of pregnant versus control animals at day 18 of gestation. One subtracted cDNA library was constructed according to the SSH method and a second one was constructed of subtracted cDNA purchased from the vertis Biotechnologie AG. A total of 4068 individual cDNA clones were randomly picked from the subtracted libraries and analyzed by array hybridization using ^{33}P-dCTP labeled cDNA probes derived from endometrial tissue samples obtained from five monozygotic twin pairs. The usage of monozygotic twin pairs as biological model is a unique possibility to eliminate genetic variability as a factor potentially affecting the results of gene expression analyses as depicted in chapter 2.6. Eighty-seven different genes were detected as upregulated in the bovine endometrium of pregnant versus control animals at day 18 of gestation. Upregulation was assumed for all genes with a difference in signal intensity of two-fold or more in at least four out of five twin pairs. Significance of changes in gene expression levels was tested using a paired Student's t-test and considered to be significant when displaying a p-value of less than 0.05. Although four genes exhibited a p-value slightly higher than 0.05, they were assumed to be of interest, either because of their more than two-fold upregulation in four out of five twin pairs (*IFIT2*, *HLA-C*, *SAMD9*) or because of the high level of the average upregulation (*LY6G6C*). In addition, the coefficient of variation of the mean fold change was calculated. The moderate variation of expression ratios between pregnant and non-pregnant animals (mean coefficient of variation 41%) indicates consistent mechanisms of gene regulation. In view of the fact that the microarray data were adjusted for effects arising from variation in the technology (a process referred to as normalization, see chapter 3.2.7), the coefficient of variation is an indicator for the biological difference existing between the RNA samples obtained from the five twin pairs.

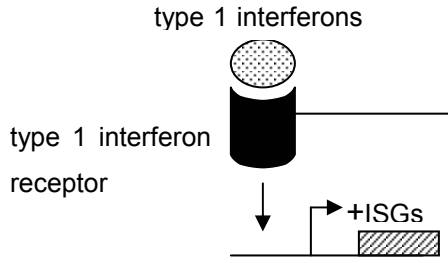
Real-time RT-PCR was used to confirm the expression data obtained by array hybridization for selcected genes. The ratios found by qPCR were in most cases slightly higher and in some cases, e.g. *UTMP* and *ISG15* clearly higher compared to those obtained by array hybridization. This phenomen can easily be explained: for array hybridization, a double stranded hybridization probe was used. Although hybridization to the cDNA fragments on the array should be preferred, re-association to double stranded cDNA in the hybridization solution can occur. The rate of re-association depends on the concentration of each cDNA, which is higher in the probe where the particular cDNA is more abundant. Additionally, background subtraction using the function “weighted image regions” contributes to the small variation in expression ratios found by array hybridization and qPCR. Overall, the results of qPCR and array hybridization correlated very well.

In situ hybridization was applied to detect the site of mRNA expression of certain genes within the endometrium. Interestingly, strong expression of interferon stimulated genes was detected in the luminal and superficial glandular epithelium (e.g. *IFITM3* and *UBE1L*). This contradicts the common view, that the expression of interferon stimulated genes is downregulated in the luminal and superficial glandular epithelium by IRF-2, a potent transcriptional repressor (view 2.2). One might hypothesize that certain interferon stimulated genes like *IFITM3* and *UBE1L* are missing an IRF-2 response element and thereby are not influenced by the transcriptional repressor activity of IRF-2. Yet, this remains to be proven.

Genes known to be stimulated by type I interferons

The expression of 38 of the identified genes is known to be stimulated by type I interferons (overview see fig. 24). The large number of genes inducible by type I interferons reflects the response to IFNτ, which is the embryonic pregnancy recognition signal in ruminants. It is through the interferon stimulated genes that interferons generate diverse cellular and physiological states involving antiviral, apoptotic, antitumor, and immune modulatory activities. Some of these genes have already been described in the context of early pregnancy in ruminants, e.g. *ISG15* (Austin et al., 1996; Johnson et al., 1998; Austin et al., 2004) *OAS1* (Schmitt et al., 1993), *IRF1* (Spencer et al., 1998), *IFITM1* and *IFITM3* (Pru et al., 2001), MX protein (Ott et al., 1998; Hicks et al., 2003), *IFIT1* (Kim et al., 2003), *ISG12* (Kim et al., 2003), MHC class I genes and *B2M* (Choi et al., 2003), but only little information exists concerning the putative roles of these ISGs in the context of early pregnancy.

The genes listed in Fig. 24 are now discussed in detail, either in this section, or, those marked with an asterisk, in one of the following sections below.



1. B2M*
2. BST2*
3. C1R*
4. C1S*
5. DDX58
6. DKFZP564J0863
7. G1P3
8. GBP1
9. GBP3
10. GBP4
11. GBP5
12. GRIM19*
13. HLA-A*
14. HLA-C*
15. HSXIAPAF1
16. IFI16*
17. IFIH1
18. IFIT1
19. IFIT2
20. IFIT3
21. IFIT5
22. IFITM1
23. IFITM3
24. IFRG28
25. IRF1
26. IRG6
27. ISG12
28. ISG15
29. LY6G6C
30. MX1
31. MX2
32. OAS1
33. OAS2
34. SERPING1*
35. SP110
36. SP140
37. STAT1
38. TPA 6-16

Fig. 24: Overview of genes known to be stimulated by type I IFNs

The genes are discussed in detail, either in this section, or, those marked with an asterisk, in one of the following sections below.

The gene product of ***DDX58*** belongs to the DEAD box proteins, which are characterized by the conserved motif Asp-Glu-Ala-Asp (DEAD) and are putative RNA helicases. DEAD box proteins are implicated in a number of cellular processes involving alteration of RNA secondary structure, such as translation initiation, nuclear and mitochondrial splicing, and ribosome and spliceosome assembly. Based on their distribution patterns, some members of this family are believed to be involved in embryogenesis, spermatogenesis, and cellular growth (Entrez Gene), further, DDX58 is believed to play some role in inflammatory reactions in the urinary tract epithelium (Imaizumi et al., 2004).

Interferon alpha and beta are known to induce transcription of Homo sapiens interferon alpha-inducible protein (clone IFI-6-16), ***G1P3***, while interferon gamma is a poor transcription inducer for this gene. In HeLa cells, *G1P3* mRNA can constitute as much as 0.1 % of the total mRNA after interferon stimulation. The encoded product was shown to be a 34 kDa glycosylated protein, which is localized at mitochondria

and is highly expressed in gastric cancer cell lines and tissues. The protein exhibits anti-apoptotic functions through inhibiting caspase-3 and might therefore function as cell survival protein by inhibiting mitochondrial-mediated apoptosis (Tahara E Jr et al., 2005). Genomic and cDNA sequence indicate that the mRNA encodes a hydrophobic polypeptide of 130 amino acids with a putative N-terminal signal sequence (Kelly et al., 1986). Despite a gene disruption study, cellular and biochemical roles for the G1P3 gene product have not been identified (Porter and Itzhaki, 1993).

The guanylate-binding proteins (GBPs) form a group of interferon inducible GTP-binding proteins, which belong to the family of dynamin-related proteins. A unique feature of the GBPs is their ability to hydrolyze GTP to GDP and GMP (Praefcke et al., 2004), and four members of this gene family, ***GBP1***, ***GBP3***, ***GBP4*** and ***GBP5*****,** were found to be upregulated. *GBP1* mRNA has been shown to be specifically induced during the midsecretory phase of the menstrual cycle in humans, which coincidences with the putative window of implantation. *In situ* hybridization revealed that *GBP1* mRNA expression is localized to glandular epithelial cells as well as to stromal cells. Treatment of human endometrial adenocarcinoma cells with IFN-gamma or IFN-alpha markedly induced the expression of *GBP1* mRNA; IFN-gamma was, however, a more potent inducer of *GBP1* than IFN-alpha (Kumar et al., 2001). In consideration of the important role of GTPases in cell proliferation, differentiation, signal transduction, and intracellular protein transport (Cheng et al., 1985; Cheng et al., 1991; Schwemmle and Staeheli, 1994), the unique expression of *GBP1* overlapping the putative window of implantatin in humans and the increased expression in endometrium of day 18 pregnant animals shown for *GBP1* and *GBP3-5* in the present study suggests, that this gene family could play an important role in the process of implantation. Moreover *GBP1* and *GBP4* mRNA were identified to be upregulated in intercaruncular endometrium of pregnant heifers after artificial insemination (Bauersachs, personal communication).

The ***IFIH1*** gene encodes an early type I interferon-responsive gene (Kang et al., 2004), consisting of a caspase recruitment domain (CARD) and a RNA helicase domain. The gene product belongs, like DDX58, to the DEAD box proteins, which are implicated in a number of cellular processes, as already mentioned above (Entrez Gene).

The mRNAs of four interferon induced proteins with tetratricopeptide repeats were identified: *IFIT1*, *IFIT2*, *IFIT3*, and *IFIT5*. The gene product of ***IFIT1***, the interferon induced protein with tetratricopeptide repeats 1, is a protein with a molecular mass of 56 kDa and unknown function. Microinjection of labeled protein into Xenopus laevis oocytes indicates that the protein is cytoplasmatic (Wathelet et al., 1986) and, furthermore, it has been shown that IFNs alpha, beta or gamma are able to activate the expression of this gene in human fibroblasts as well as in lymphoblastoid cells (Chebath et al., 1983). An IFIT1 protein-protein interaction study has shown that IFIT1 may interact with Rho/Rac guanine nucleotide exchange factor and regulate the activation of Rho/Rac proteins. In addition, IFIT1 is the first gene described as candidate gene for systemic lupus erythematosus (Ye et al., 2003). ***IFIT2*** encodes a polypeptide of 54 kDa, whose function remains to be determined (Entrez Gene). IFIT1 and IFIT2 are very closely related at the mRNA and protein level, additionally they possess similar promoters, which suggests that *IFIT1* and *IFIT2* are members of a gene family, which probably arose from duplication of an ancestor gene (Wathelet et al., 1988). Additionally it has been shown, that four members of the human gene family, IFIT1-4, are clustered together at chromosome 10q23.3 and the upstream regulatory region of these genes shows a conservation in structure, each containing two interferon-stimulated response elements upstream of a conserved TATA box. It is proposed that the four members of the family evolved by a series of duplication events from a common gene of origin (de Veer et al., 1998).

The cDNA of ***ISG12***, encoding a putative interferon stimulated protein of 12 kDa, was originally cloned as an estrogen-induced gene in human breast epithelial cell line, MCF7, and designated as p27. Even though it was demonstrated that the level of expression of *ISG12* mRNA is induced by estrogen in MCF7 cells, and high expression levels of *ISG12* mRNA are found in many breast carcinomas, ISG12 expression does not correlate with the presence of the estrogen receptor, neither in the cell lines tested nor in breast carcinomas (Rasmussen et al., 1993). It has then been shown by quantitative RT-PCR that *ISG12* is an interferon induced gene, whereat IFN-alpha is a strong, and IFN-gamma a slight inducer of *ISG12* mRNA expression (Gjermandsen et al., 2000). The promoter region of the *ISG12* gene contains putative interferon inducible elements, i.e. ISRE, IRF1/IRF2 and STAT and the subcellular localization of ISG12 was localized to the nuclear membrane (Martensen et al., 2001). Li et al. (2001) showed that ISG12 expression is strongly

enhanced in human endometrium during the midsecretory phase of the menstrual cycle in humans, overlapping with the putative window of implantation. The authors also observed a synergistic interaction between IFN-alpha and estrogen receptor signaling pathways that led to maximal induction of *ISG12* mRNA in Ishikawa cells (a human endometrial adenocarcinoma cell line). However, further experiments are needed to shed light on its function especially with regard to embryonic implantation.

The gene ***LY6G6C***, lymphocyte antigen 6 complex locus G6C, belongs to the lymphocyte antigen-6 (LY-6) superfamily and its expression in the bovine endometrium is known to be enhanced during the secretory phase of the estrous cycle (Bauersachs et al., 2005). Members of the LY-6 superfamily are cysteine-rich, generally GPI-anchored cell surface proteins, which have definite or putative immune-related roles and are important in leukocyte maturation. The gene *LYG6C* is located in the human major histocompatibility complex class III region (Ribas et al., 1999), and elevated expression has been shown in patients suffering from systemic lupus erythematosus (Tang et al., 2004). Secretion of human leukocyte antigen-G by human embryos is associated with a higher *in vitro* fertilization pregnancy rate as shown by Yie et al. (2005), even though a precise function of LY6G6C is not known.

Two members of the MX proteins were identified: MX1 and MX2. MX proteins are large GTPases, belonging to the dynamin superfamily (van der Bliek, 1999), which are specifically induced by IFN-alpha/beta (Simon et al., 1991), and in mammals two or more genes have been identified (Horisberger and Gunst, 1991). As with other dynamin-like proteins, the MX family contains a N-terminal GTPase domain and a variable C terminus, which modifies the function and location of the different MX proteins (Pavlovic et al., 1993). The protein encoded by the *Bos taurus* myxovirus (influenza) resistance 1 (***MX1***) gene is similar to the mouse MX1 protein, as determined by its antigenic relatedness, induction conditions, physicochemical properties, and amino acid analysis. MX1 is located in the cytoplasm and known to exert an antiviral effect by targeting specific, yet poorly defined steps of the viral replication cycle (Pavlovic et al., 1992). The **MX2** protein has similar, yet distinct properties compared to MX1: it has a nuclear and cytoplasmatic form and is also upregulated by interferon alpha but does not exhibit the antiviral activity of MX1. *MX1* and *MX2* belong to the same gene family as the guanylate binding proteins, suggesting an important, yet undefined role for large GTPases in the process of implantation. The MX protein is furthermore assumed to function as a conceptus-

induced component of the anti-luteolytic mechanism and/or regulator of endometrial secretion or uterine remodeling (Hicks et al., 2003).

The expression of ***OAS1*** and ***OAS2*** mRNA was shown to be enhanced. The 2´,5´-oligoadenylate synthetases (OAS) represent a family of interferon induced proteins, and in humans three forms of OAS, corresponding to proteins of 40/46 (OAS1), 69/71 (OAS2), and 100 kDa (OAS3), have been described. These OAS share a homologous region of about 350 amino acids, which could represent the functional domain. OAS1 contains one single domain, whereas OAS2 and OAS3 contain two and three adjacent domains, respectively. In humans, it has been shown that the three OAS genes are clustered, which might reflect their evolutionary relationship, possibly through the duplication of the conserved functional domain (Hovnanian et al., 1998). Oligoadenylate synthetases catalyze the synthesis of 2´-5´-linked oligoadenylates from ATP (Justesen et al., 2000), which in turn can bind to the latent RNase L, which, in the presence of double stranded RNA, subsequently dimerizes into the active form. Thereby this enzyme family plays a role in viral infection resistance by degrading viral RNAs (Dong and Silverman, 1995). Concerning reproductive processes, the 2', 5'-oligoadenylate synthetase is hypothesized to affect $PGF_{2\alpha}$ secretion by the endometrial epithelium, possibly by altering arachidonic acid metabolism (Schmitt et al., 1993).

Of particular interest among the interferon stimulated genes is ***ISG15*****,** and recently several studies were dedicated to clarify the role of ISG15 at the embryo-maternal interface (Johnson et al., 1998; Austin et al., 2004; Joyce et al., 2005). *ISG15,* one of the most markedly upregulated genes in the present study, encodes an ubiquitin-like protein that is conjugated to intracellular proteins (Narasimhan et al., 1996). The function of ubiquitin is well characterized and includes targeting cellular proteins for degradation by the 26S proteasome complex (Alberts et al., 2004). Proteins become ubiquitinated through a multienzyme ATP-dependent conjugation process, which requires the sequential action of three enzymes: E1, the ubiquitin activating enzyme, E2, the ubiquitin conjugating enzyme, and E3, the ubiquitin ligase (Haas and Siepmann, 1997). Recently identified proteins that become conjugated to ISG15 include several serine proteinase inhibitors (Hamerman et al., 2002) and signal transduction proteins like phospholipase C gamma1, JAK1, ERK1, and STAT1 (Malakhova et al., 2003; Malakhov et al., 2003). Because conjugated ISG15 remains in the uterus as late as day 45 of pregnancy, Austin and coworkers (2004) postulated

that one function of ISG15 is to stabilize proteins rather than target them to degradation as described for polyubiquitination. In the present study, the gene for bovine ubiquitin activating enzyme-1 like protein (*UBE1L*), the initiating enzyme for ISG15ylation (Rempel et al., 2004), was also identified as upregulated gene. Furthermore, the mRNAs for *IFITM1* and *IFITM3,* encoding proteins hypothesized to possess E2 enzyme activity based on a conserved E2 motif in the protein sequence were found to be upregulated in endometrium from pregnant animals (Pru et al., 2001). Until now, E3 enzymes specific for ISG15 have not been identified. In this study we found upregulated mRNA levels for *DTX3L*, coding for a protein with E3 ubiquitin ligase activity (Takeyama et al., 2003). In addition, *HSXIAPAF1* (*XAF1*) mRNA levels were increased in the pregnant endometrium. The product of this gene was described as antagonist of XIAP (Liston et al., 2001), an anti-apoptotic protein that possesses E3 ubiquitin ligase activity (MacFarlane et al., 2002). If XIAP plays a role in ISG15ylation due to its E3 ligase activity, it would be possible that HSXIAPAF1 as inhibitor of XIAP plays a role in regulation of the ISG15ylation system. The mRNA expression patterns of *IFITM3*, *DTX3L*, and *HSXIAPAF1* revealed by *in situ* hybridization in the bovine endometrium were very similar to that of *UBE1L*. Based on this result and the described functions in the literature, these genes can be seen as potential components of the ISG15 system and are probably involved in the regulation of the response of the endometrium to the signaling of the embryo.

With the signal transducer and activator of transcription 1 (***STAT1***), a central member of the interferonτ signaling cascade was identified. In response to IFNτ STAT proteins become tyrosine-phosphorylated, translocate into the nucleus and form hetero- and homodimers. These hetero- and homodimers bind to specific response elements present in the regulatory region of genes stimulated by interferons (Stewart et al., 2001). STAT1 homodimers increase the expression of genes such as the interferon regulatory factor 1 (*IRF1*) (Pine et al., 1994), which mRNA exhibited a 3-fold upregulation in the present study. The interferon regulatory 1 itself stimulates the expression of certain type I interferon stimultated genes through binding to cis-elements in the promotor regions (Harada et al., 1990).

Regulation of the maternal immune system in the endometrium

The intense cross-talk of the maternal endometrium with the fetal semi-allograft is reflected in the upregulation of various genes with immune-related functions, such as

MHC class I molecules (HLA-A, HLA-C, beta-2 microglobulin), factors of the complement system (C1S, C1R, SERPING1), and the lipopolysacharide binding protein, LBP. Major histocompatibility complex (MHC) class I molecules consist of a MHC encoded heavy chain and a non MHC encoded light chain (beta-2 microglobulin, β2MG), whereat the major histocompatibility complex is a region of high polymorphic genes. The principal function of these molecules is to bind fragments of foreign proteins, thereby forming complexes that can be recognized by T lymphocytes. Due to this ability of discriminating self and nonself, they are the principal determinants of graft rejection. It is well known, that both type I and type II interferons induce the expression of MHC class I molecules (Wan et al., 1987; Garofalo et al., 1996), and a few studies have been conducted so far to elucidate the spatial and temporal pattern of MHC class I expression in the ruminant uterus. Choi and coworkers (2003) studied the effects of the estrous cycle, pregnancy, and intrauterine administration of recombinant ovine interferon-tau on expression of MHC class I α chain and β2MG genes in the ovine endometrium using slot-blot hybridization, *in situ* hybridization and immunofluorescence analyses. The study revealed that both, MHC class I α chain and β2MG mRNA and protein, are expressed in the luminal epithelium, glandular epithelium and stroma of cyclic and early pregnant ewes on days 10 and 12. However, expression of these genes was undetectable in the luminal and superficial glandular epithelium after day 14 of pregnancy, but was increased in stroma and deep glandular epithelium. This pattern of expression is similar to that of other ISGs, and IRF-2, a potent transcriptional repressor in the IFN signaling system, is made responsible for this phenomenon (see chapter 2.2). Choi et al. hypothesize, that the absence of MHC class I α chain and β2MG genes in the luminal epithelium is advantageous because the luminal epithelium directly interfaces with the conceptus trophectoderm. The silencing of these genes may therefore be a critical mechanism to prevent immune rejection of the conceptus allograft. Todd and coworkers (1998) also found that IFNτ enhances the expression of MHC class I molecules on ovine endometrial cells. They hypothesize that the upregulation of MHC class I molecules may have an effect on endometrial natural killer (NK) cells. NK cells, present in the endometrium, increase in number during pregnancy, partially due to the action of IFNτ and are potentially harmful for the conceptus. These cells bear specific receptors for MCH class I molecules, also called “killer cells inhibitory receptors” because interaction with MHC

class I inhibits cytotoxic activity. Thus Todd et al. propose that enhancement of endometrial MHC class I expression helps to prevent potential damage by the NK cells.

Three factors of the complement system, C1s, C1r and C4A, are upregulated. The complement system is a group of serum proteins which are functionally linked and interact with one another in a highly regulated manner. The complement system is not antigen specific and is activated immediately in the presence of pathogens and is considered to be part of innate immunity. Several complement proteins are pro-enzymes and when activated they become proteases and activate other complement proteins in turn (a cascade). There are three different ways the complement cascade can be activated but the effector functions are the same, the formation of a membrane attack complex (MAC). C1S, C1R, and C4A are part of the classical pathway of complement activation, which is initiated by antigen-antibody complexes (Abbas et al., 2005) (see fig. 25).

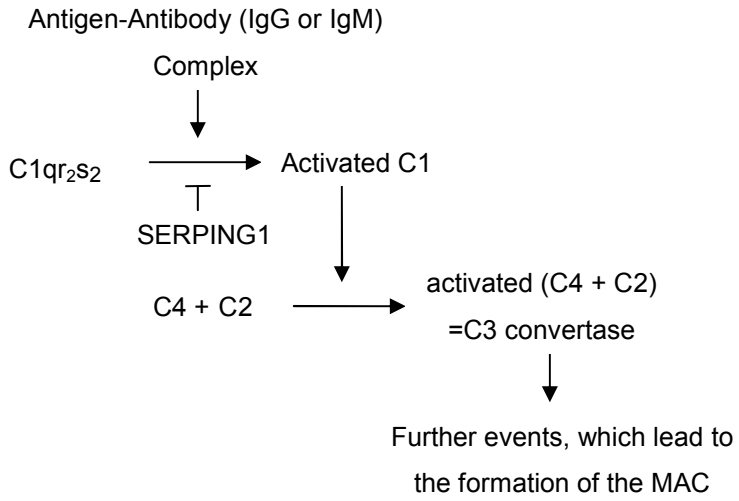


Fig. 25: Classical pathway of complement activation

Simultaneously, the gene expression of *SERPING1* is enhanced. The protein encoded by this gene is a member of the serpin (serin protease inhibitor) family and is also called C1 inhibitor (Ratnoff et al., 1969). It inhibits the ability of C1R and C1S to cleave their normal substrates by presenting a “bait” sequence that mimics the normal substrates of C1R and C1S. When it is then cleaved by C1R or C1S, it forms stable ester linkages with these serine proteases. The *in situ* hybridization experiments revealed specific expression of *C1S* and *C1R* mRNA mainly in the luminal and glandular epithelial cells but also weakly in the stromal cells. The

simultaneous upregulation of *SERPING1* in the luminal and glandular epithelial cells could be a mechanism to protect the embryo against an attack of the complement system.

UTMP is another gene that may play a role in the modulation of the maternal immune system. For the ovine UTMP protein inhibition of NK-like activity was shown and a role in protecting the conceptus from maternal cytotoxic lymphocytes was suggested (Tekin and Hansen, 2002). As in our study of pregnant cows, UTMP mRNA was found to be upregulated in endometrium of pregnant sheep (Stewart et al., 2000).

IFITM1 has been shown to be highly expressed in gastric tumor tissues and in gastric cell lines, where it plays a role in malignant progression by suppressing natural killer cells and by increasing the invasive potential of gastric cancer cells (Yang et al., 2005). For that reason, *IFITM1* might be another gene playing a role in preventing maternal rejection of the fetal semi-allograft.

Remodeling of the endometrium

The categorization of the identified genes according their function revealed the orchestrated interaction of various processes and mechanisms with regard to preparation of the maternal endometrium for embryonic implantation. Besides *CTGF*, *EPSTI1* and components of the MMP system, *MMP 19* and *TIMP2*, genes involved in cell growth, proliferation and differentiation as well as regulation of gene expression and regulation of apoptosis underline the extensive molecular and structural changes taking place during the pre-implantation stage. For an overview of genes involved in “cell growth”, “cell differentiation”, “cell proliferation” and “cell cycle” see fig. 26.

The enhanced expression of genes involved in these biological processes is in accordance with several physiological events occurring in the endometrium during the pre-implantation period. One event is the synthesis and secretion of histotroph by the endometrial glandular epithelium, which supports conceptus development and nourishment (Spencer and Bazer, 2004). A further event is reorganization of the endometrial luminal epithelium to allow its intimate association with the conceptus trophectoderm, e.g. receptors in the luminal epithelium become exposed or induced during the receptive period. Wathes and Wooding (1980) describe the changes occurring in the uterine epithelium of the cow between days 18 and 28 of gestation using electron microscopy. Throughout the estrous cycle the uterine epithelium was irregular columnar with a height of 15 - 35 µm. None of these cells contained more than one nucleus. At day 18 of gestation the uterine epithelium was more regular

than in non-pregnant animals and generally columnar in form. The cell height decreased to 20 - 25 µm and it was found that about 3 % of the cells contained two nuclei. All these physiological events require the endometrial cells to proliferate, to grow and to differentiate. The growth and differentiation of the endometrium and in particular of the endometrial glands during the period of early pregnancy is possible despite the antiproliferative effects of interferons because the endometrium exhibits a reduced responsiveness to the antiproliferative effects of type I interferons (Davidson et al., 1994). The genes involved in the processes mentioned above are discussed regarding their function either in this section or, those marked with an asterisk, in the next section, which deals with genes involved in the regulation of transcription, or elsewhere.

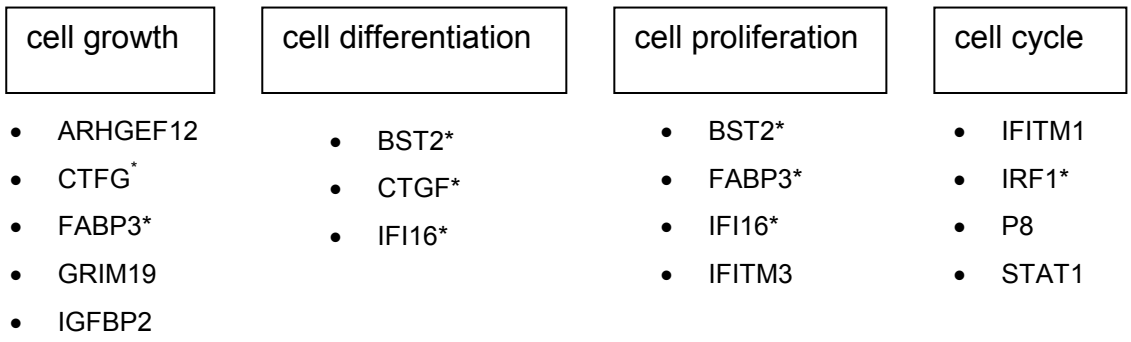


Fig. 26: Genes involved in cell growth, cell proliferation, and cell cycle

The genes are discussed regarding their function either in this section or, those marked with an asterix, in the next section, which deals with genes involved in the regulation of transcription, or elsewhere.

Rho guanine-nucleotide exchange factor 12 (**ARHGEF12**) activates Ras proteins and thereby regulates cell growth (Kristelly et al., 2004). Ras proteins belong to the large Ras superfamily of monomeric GTPases, which help to broadcast signals from the cell surface to other parts of the cell. They are often required, for example, when receptor tyrosine kinases signal to the nucleus to stimulate cell proliferation or differentiation by altering gene expression. Like other GTP-binding proteins, Ras functions as a switch, cycling between two distinct conformational states – active when GTP is bound and inactive when GDP is bound. ARHGEF12 promotes the exchange of bound nucleotide by stimulating the dissociation of GDP and the

subsequent uptake of GTP from the cytosol, thereby activating Ras (Alberts et al., 2004).

GRIM19, the cell death regulatory protein is involved in the negative regulation of cell growth. Its function is described in detail in the next section.

The insulin-like growth factor binding protein 2 (**IGFBP2**) is also a regulator of cell growth. Insulin-like growth factors (IGFs) are growth-promoting peptides that show significant homology with insulin and their bioavailability is controlled through several binding proteins. IGFBP2 is an inhibitor of IGF action by binding IGFs and subsequent prevention of their binding to the IGF receptors. IGFBP2 therefore negatively influences IGF-induced cell proliferation (Rajaram et al., 1997).

With ***MMP19*** and ***TIMP2*** two genes coding for components of the matrix metalloproteinase system, which is classically considered to be involved in tissue remodeling, were identified. Until now, neither *MMP19* nor *TIMP2* mRNA has been described to be upregulated in the bovine endometrium around the time of embryonic implantation. A study in normal breast tissue and mammary gland tumors revealed strong expression of MMP19 protein in all tumor cells of benign lesions, whereas the progression towards an invasive phenotype and neoplastic dedifferentiation led to the disappearance of MMP19 and a concomitant rise in the levels of MMP2 protein was observed (Djonov et al., 2001). These findings suggest an important role of MMP19 and TIMP2, the inhibitor of MMP2 (Stetler-Stevenson et al., 1989), for the regulation of the conceptus attachment.

Connective tissue growth factor (**CTGF**), which is assumed to be involved in stromal remodeling and uterine cell growth, has been studied at the utero-placental interface in pigs (Moussad et al., 2002), mice (Surveyor et al., 1998) and humans (Uzumcu et al., 2000). This is the first description of *CTGF* mRNA expression in the bovine endometrium at day 18 of gestation, and CTGF seems to be a universal factor involved in endometrial remodeling with regard to the variety of species exhibiting increased expression of *CTGF* mRNA in the maternal environment during the implantation period. As another gene important for endometrium remodeling ***EPSTI1*** was found, and mRNA expression in bovine endometrium was shown for the first time. The expression of *EPSTI1* mRNA has been shown in tissues characterized by extensive epithelial-stromal interaction and there is evidence that *EPSTI1* expression reflects an important event associated with organ development and tissue remodeling. Using a tissue mRNA panel the most prominent expression has been

detected in human placenta (Nielsen et al., 2002), underlining the potential importance of this gene for endometrial remodeling prior to attachment of the embryo.

Genes involved in regulation of transcription

Regulation of transcription, the synthesis of RNA from DNA, is one of the most important steps in control of cell growth and differentiation and the Gene Ontology classification has shown, that several gene products are involved in the regulation of gene expression or exhibit transcription regulator activity, respectively (see fig. 27).

In the following, the different genes are discussed regarding the known or potential function of their products.

BST2 is involved in the regulation of gene expression through positive regulation of the NF-kappaB cascade. NF-kappaB proteins are latent gene regulatory proteins that lie at the heart of most inflammatory responses, and, once activated, NF-kappaB turns on the transcription of more than 60 known genes (Alberts et al., 2004).

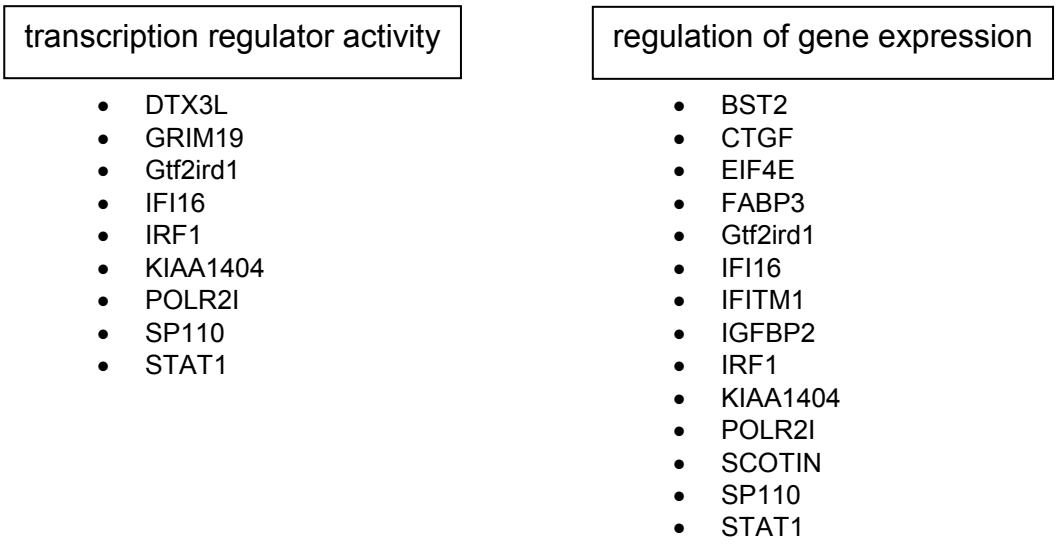


Fig. 27: Genes exhibiting transcription regulator activity or beeing involved in the regulation of gene expression, respectively

CTGF has been shown to enhance growth factor-induced DNA synthesis in NIH 3T3 fibroblasts and human vascular endothelial cells (Kireeva et al., 1997).

DTX3L has already been mentioned above in the context of ISG15ylation because it possesses ubiquitin-protein isopeptide ligase activity. DTX3L is a member of the DTX (Deltex) family, which act as Notch signaling modifiers and may also regulate

transcription through interactions with specific transcription factors (Takeyama et al., 2003).

EIF4E, the eukaryotic translation initiation factor 4E, is the mRNA cap-binding protein required for the translation of cellular mRNAs utilizing the 5´cap structure (Li et al., 2004). Since EIF4E is the rate-limiting factor for mRNA recruitment to ribosomes, it is a major target for regulation of translation by growth factors, hormones, and other extracellular stimuli, e.g. IFNτ.

The fatty acid binding protein (heart) like **FABP3** is also believed to be responsible for modulation of cell growth and proliferation through the regulation of gene expression. It belongs to a multigene family and the fatty acid binding proteins are divided into at least three distinct types, the hepatic-, intestinal- and cardiac-type. They form 14 - 15 kDa proteins and also participate in the uptake, intracellular metabolism and/or transport of long chain fatty acids (Entrez Gene).

The cell death regulatory protein **GRIM19** is a negative regulator of transcription. “GRIM” is the abbreviation of Gene associated with Retinoid-IFN-induced Mortality. It has been shown, that GRIM-19 interacts with STAT3 (Lufei et al., 2003), which is a latent cytoplasmatic transcription factor that can be activated by cytokines, such as IFNτ. The cell death regulatory protein GRIM19 interacts specifically with STAT3 to repress its transcriptional activity and its target gene expression. GRIM 19 does not interact with STAT1 or STAT5a. Huang and coworkers (2004) showed that GRIM19 is a functional component of the mitochondrial complex I and that it is essential for early embryonic development. They generated mice deficient in GRIM19 by gene targeting; homologous deletion of GRIM19 causes embryonic lethality at embryonic day 9.5.

The protein encoded by ***Gtf2ird1***, general transcription factor II I repeat domain-containing 1, contains five GTF2I-like repeats and each repeat possess a potential helix-loop-helix (HLH) motif. The protein functions as a positive transcriptional regulator under the control of Retinoblastoma protein.

IFI16, interferon gamma inducible protein 16, is a member of the family of interferon-inducible proteins. The gene product contains a domain at the amino terminus capable of binding double-stranded DNA and Johnstone and coworkers (1998) showed that IFI16 is a transcriptional repressor with a modular structure typical of many known transcription regulators.

The gene ***POLR2I*** encodes a subunit of RNA polymerase II, the polymerase responsible for synthesizing messenger RNA in eukaryotes. This subunit forms, in combination with two other polymerase subunits, the DNA binding domain of the polymerase, a groove in which the DNA template is transcribed into RNA.

SCOTIN was identified as a novel p53-inducible protein localized to the endoplasmatic reticulum and the nuclear membrane. This protein can induce apoptosis in a caspase-dependent manner (Bourdon et al., 2002). Scotin enhances gene expression, like BST2, through the positive regulation of the NF-kappaB cascade.

SP110 belongs to the nuclear body, which is a multiprotein complex that can function as an activator of gene transcription and may also serve as a nuclear hormone receptor coactivator. The N-terminal domain of SP110 is homologous to two other components of the nuclear body protein complex (SP100 and SP140, which was identified also), the C-terminal portion is homologous to the transcription intermediary factor 1 (TIF1) family of proteins (Bloch et al., 2000).

The protein encoded by ***STAT1*** is a member of the STAT (signal transducer and activator of transcription) protein family. As already mentioned in chapter 2.3, STAT family members are phosphorylated by receptor associated kinases in response to cytokines and growth factors. After phosphorylation they form homo- or heterodimers that translocate to the nucleus where they act as transcription activators.

The interferon regulatory factor 1 (**IRF1**) is an interferon responsive gene and its expression is enhanced in response to binding of IFNτ to its receptor (see 2.3). IRF1 exhibits transcription factor activity through binding to cis-elements within the promoters of type I interferon inducible genes (Harada et al., 1990).

Genes involved in cell communication

A large number of the genes identified to display higher mRNA levels in the pregnant animals than in the control group participate in cell communication (see fig. 28), which indicates once more the importance of reciprocal communication between the embryo and the maternal environment during the pre-implantation period. Since the majority of these genes has already been mentioned in one of the previous sections, only some of them are shortly discussed regarding their contribution to cell communication.

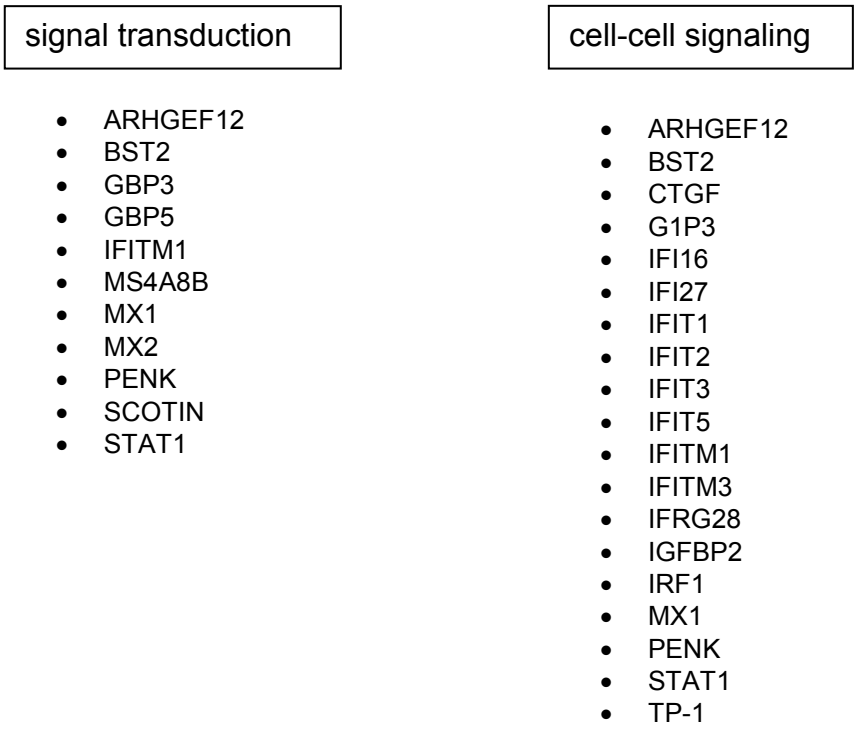


Fig. 28: Genes involved in cell communication

ARHGEF12, the Rho guanine-nucleotide exchange factor 12, helps to transmit signals from the cell surface to other parts of the cell by activating Ras proteins, as already described above (see: Remodeling of the endometrium)

BST2, as a transmembrane protein, is involved in cell-cell signaling via the exhibition of signal transducer activity, but the precise mechanism is not known yet (Entrez Gene).

Homo sapiens membrane-spanning 4-domains, subfamily A, member 8B (**MS4A8B**) is a member of the membrane-spanning 4A (MS4A) gene family. This gene family was designated by Liang and Tedder (2001) and, currently, contains at least 12 subgroups (MS4A1 through MS4A12) that encode at least 21 proteins expressed by hematopoetic cells and diverse cell types in non-hematopoetic tissues. MS4A family proteins share structural similarity, amino acid sequence homology and chromosomal location. They contain 4 highly conserved membrane-spanning regions, flanked by N- and C-terminal cytoplasmatic regions and are likely to be components of oligomeric cell surface complexes involved in signal transduction (Liang et al., 2001). This gene was also found to be upregulated in the bovine cycling endometrium at day 12 of the estrous cycle as compared to the day of standing heat (Bauersachs et al., 2005).

Genes previously described in the context of reproductive processes

Several genes identified in this study, such as *PENK*, *CTGF*, *MMP*s, *TIMP*s and *UTMP*, have already been examined regarding their relevance regarding reproductive processes.

Proenkephalin, **PENK** belongs to the mammalian opioid system which is composed of opioid receptors (at least three different types) and endogenous ligands of these receptors. The endogenous ligands include more than 20 opioid peptides derived from three precursors: proenkephalin (PENK), prodynorphin (PDYN) and proopiomelanocortin (POMC) (Brownstein, 1993). In several studies components of the opioid system from human placental tissue have been isolated (Sastry et al., 1980; Liotta et al., 1982; Ahmed et al., 1986; Belisle et al., 1988), but the physiological role of the opioid receptors and peptides in the placenta, an organ lacking innervation, is not fully understood yet. It has been shown, that opioids regulate the release of human chorionic gonadotropin from trophoblast tissue (Cemerikic et al., 1991). The presence of mRNA and peptide products derived from POMC, PENK (Jin et al., 1988) and DYN (Douglass et al., 1987), as well as opioid receptor binding sites (Baraldi et al., 1985), has been reported from the rat uterus. The study conducted by Zhu and Pintar (1998) revealed the presence of mRNA of the opioid receptors and their endogenous ligand precursors POMC, PENK and PDYN in the mouse uterus and placenta in sequential pregnancy states, using *in situ* hybridization. The results clearly indicate that the expression of the opioid system in these tissues begins soon after implantation and continues until late gestation. For PENK, Zhu and Pintar (1998) suggested a role of the enkephalin derived peptides in decidualization. They further suggest that enkephalins may be involved in the regulation of hypertrophy of the uterine smooth muscle and in modulating myometrial activity during pregnancy. Taken together, PENK seems to play a role during early gestation in several species, even though the precise role of PENK in the context of embryo-maternal communication is not clearly understood, and this study is the first one to describe the expression of *PENK* mRNA in the ruminant uterus during the pre-implantation period.

Numerous studies have been conducted so far in several species to elucidate the role of the connective tissue growth factor, **CTGF**, in reproductive processes. CTGF is a heparin-binding mitogen for fibroblasts and smooth muscle cells. Moussad and coworkers (2002) examined the temporal and spatial expression of CTGF at the

utero-placental interface in the pig using *in situ* hybridization and immunohistochemistry. Up to day 10 of pregnancy, CTGF mRNA and protein were abundant in luminal epithelial cells and glandular epithelial cells with lesser amount in stromal fibroblast. On days 12-27 there was a striking decrease in the amount of CTGF in luminal epithelial cells which was associated with maternal matrix reorganization and the onset of subepithelial neovascularization. This decrease was transient in nature and high amounts of CTGF appeared again on days 17-21 when endometrial neovascularization and matrix remodeling was completed. Moussad et al. (2002) concluded, that CTGF plays a role in stromal remodeling and neovascularization. Uzumcu and coworkers (2000) investigated the production of CTGF by human uterine tissues using immunhistochemical and Northern blotting analyses. During pregnancy, the decidual, epithelial and endothelial cells were all immunoreactive to CTGF and the authors suggested a role for this molecule in regulating aspects of uterine cell growth, migration and/or matrix production during pregnancy. On days 1.5 - 3.5 of pregnancy CTGF was mainly localized to uterine epithelial cells in the mouse uterus. On day 4.5 reduced levels of CTGF were determined, whereas on days 5.5 and 6.5 high levels of CTGF were present in the decidual cells (Surveyor et al., 1998). The aim of the study conducted by Rageh and coworkers (2001) was to determine mechanisms regulating the production of CTGF and TGF-beta1 in the mouse uterus. *In situ* hybridization and immunhistochemistry were used to localize CTGF in pseudopregant or ovariectomized mice. The ovariectomized animals received estradiol-17beta or progesterone, either alone or in combination. The results indicated that maternal factors are principal cues for CTGF production in the uterus, because expression of CTGF during pseudopregnancy is comparable to that seen in pregnancy and is regulated by ovarian steroids. Even though CTGF is a TGF-beta1 inducible gene, TGF-beta1 dependent and independent mechanisms of CTGF exist in the uterus.

As already pointed out in “Remodeling of the endometrium”, two components of the matrix metalloproteinase system were found to be upregulated: matrix metalloproteinase 19, **MMP19**, and tissue inhibitor of metalloproteinase 2, **TIMP2**. Proteins of the matrix metalloproteinase (MMP) family are enzymes capable of degrading both the collagenous and noncollagenous components of the extracellular matrix (ECM) (Okada et al., 1986). Common features of the MMP family include the presence of zinc in the active site of the catalytic domain, synthesis of the MMPs as

preproenzymes that are secreted in an inactive form, activation of the latent zymogen in the extracellular space, recognition and cleavage of the ECM by the catalytic domain of the enzyme, and inhibition of enzyme activity by metalloproteinase inhibitors (Curry, Jr. and Osteen, 2003). TIMP2 belongs to the tissue inhibitor of metalloproteinase (TIMP) gene family. The proteins encoded by this gene family are locally produced and are natural inhibitors of the matrix metalloproteinases (Curry, Jr. and Osteen, 2003). The ability of TIMPs to inhibit the MMP action occurs through the interaction of the N-terminal domain of TIMP with the active site of the catalytic domain and the substrate binding groove of the MMP. The MMP system is involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction and tissue remodeling, as well as in disease processes, such as arthritis and metastasis. Numerous studies have been conducted to elucidate the role of the MMP system in several reproductive processes, such as the menstrual cycle in humans and the process of implantation in different species. Goffin and coworkers (2003) used reverse polymerase chain reaction to evaluate the mRNA expression of a large array of MMPs and TIMPs in the cycling human endometrium. The expression of MMP9 and TIMP3 was studied in the implantation window phase of the human endometrium using immunhistochemical analyses (Li et al., 2000). Both MMP9 and TIMP3 expression was highest during the window of implantation. Gao and coworkers (2001) examined the expression of MMP2, TIMP1, and TIMP3 mRNA at the implantation site in the uterus of rhesus monkeys during early stages of pregnancy using in situ hybridization and Northern Blot analyses. Hurst and Palmay (1999) studied developing embryonic and uterine tissues during the implantation period in the rat to determine if MMPs and TIMPS are detectable. MMP2, MMP9, and TIMP2 were detectable on each day studied. A similar yet distinct study was done by Zhao et al. (2002). They determined the expression of MMP2 and MMP9 and TIMPs1-3 mRNAs in the rat uterus during early pregnancy.

The uterine milk protein (**UTMP**) is a member of the serpin superfamily of serine protease inhibitors even though no inhibitory activity towards any serine protease has been demonstrated so far (Ing and Roberts, 1989). The uterine milk protein is an important regulator of uterine immune function since it inhibits lymphocyte proliferation (Skopets et al., 1992) and reduces cytotoxic activity of NK cells (Liu and Hansen, 1993). NK cells are an important component of the natural immune system involved in immune surveillance against infectious diseases and autologous and

allogenic tumors (Trinchieri, 1989). NK like cells are also present in the endometrium of humans (King and Loke, 1990), mice (Croy et al., 2003) and sheep (Liu and Hansen, 1993), but the precise function is not elucidated yet. As described in chapter 2.1.4, the expression of UTMP is presumably driven by progesterone and therefore UTMP is assumed to mediate the immunosuppressive effects of progesterone.

Placenta expressed transcript protein (**PLET8**), also called C11orf34, has been previously reported to be highly expressed in pig and mouse placenta (Zhao et al., 2004). Hitherto, nothing is known about its function.

Surprisingly, expression of *IFNτ* mRNA itself **(bovine trophoblast protein-1, TP-1)** was found in the endometrial tissue samples by array hybridization. On the first glance, this might be due to residual trophoblast tissue adhering to the endometrium, but on day 18 of gestation no adhesion of the conceptus to the endometrial epithelium has occurred yet, as shown by an electron microscopy study conducted by Wathes und Wooding (1980). Moreover, *IFNτ* mRNA was identified to be upregulated in intercaruncular endometrium of pregnant heifers after artificial insemination as shown by array hybridization and qPCR (Bauersachs, personal communication), implicating, that the expression of IFNτ is a specific reaction of the ruminant endometrium around the time of embryonic implantation. Furthermore, it has been shown that human ISG15 induces IFN-gamma release by cultured T cells and Pru and coworkers (2000) hypothesized that bovine ISG15 would also induce release of IFN-gamma from peripheral blood mononuclear cells (PBMCs) in a manner similar to that described for human ISG15. Indeed, bovine ISG15 upregulated IFN-gamma mRNA in PBMCs. They further detected IFN-gamma mRNA in higher amounts in endometrium of pregnant compared to non-pregnant cows, and suggested that this increase could be mediated directly by ISG15. But ISG15 failed to induce IFN-gamma mRNA in cultured BEND cells, which might be explained by means of the nature of these cells: BEND cells are a highly enriched epithelial cell line primarily derived from uterine luminal epithelium, so ISG15 could have failed to induce IFN-gamma mRNA, because the IFN-gamma mRNA found in uterine endometrium from pregnant cows may have originated from cells other than the luminal epithelium, e.g. cells of the stroma or deep glandular epithelium or leukocytes. So it might be possible that ISG15 induces the expression of *IFNτ* in endometrial cells, but this hypothesis remains to be proven, e.g. by *in situ* hybridization.

Genes involved in cell adhesion

In domestic ruminants endometrial invasion, as it takes place in humans and rodents, does not occur; thus definite implantation is achieved by tight adhesion of the mononuclear trophoblast cells to the endometrial luminal epithelium. The present study revealed four genes, whose products are involved in cell adhesion; *CLEC4F CTGF*, *GPLD1*, and *MFGE8*. None of them has been mentioned to play a role in bovine implantation yet. **CLEC4F** is a member of the C-type lectins, which represent a large family of Ca^{2+}-dependent lectins that share primary structural homology in their carbohydrate-recognition domains. Members of the large family include many endocytic receptors, many proteoglycans, and all known collectins and selectins, which have diverse functions, such as cell-adhesion and cell-cell signaling amongst others (Alberts et al., 2004). The connective tissues growth factor (**CTGF**) is described in "Genes previously described in the context of reproductive processes". It has been shown that CTGF promotes cell adhesion by directly binding to two integrins, namely alpha(v)beta(3) and alpha(2b)beta(3) (Babic et al., 1999; Jedsadayanmata et al., 1999). The analysis of the deduced amino acid sequence of **GPLD1** revealed four regions of internal homology, which show a significant similarity with the metal ion binding domains of the alpha subunits of integrins. These sequences share an aspartate-rich core, flanked by short, conserved segments that occur only in the integrins (Scallon et al., 1991). The protein encoded by ***MFGE8*** is an abundant peripheral protein associated with the milk fat globule membrane but is also found in a wide range of tissues and is present in several body fluids (Butler et al., 1980; Andersen et al., 1997). MFGE8 consists of two N-terminal EGF-like domains, followed by two repeated C domains with homology to the C1 and C2 domains of blood clotting factor V and VIII. The second EGF motif domain contains an Arg-Gly-Asp (RGD) cell adhesion sequence motif recognized by integrin receptors (Hvarregaard et al., 1996; Couto et al., 1996). The RGD motif is located at the turn of the B-loop in the EGF domain and is thereby likely to be exposed to the surroundings. Moreover, the sequence motif is conserved in all species and MFGE8 proved to be an integrin receptor ligand, as affinity chromatography against bovine MFGE8 purified the $\alpha_v\beta_5$ integrin receptor (Andersen et al., 1997). Likewise, human and murine orthologous proteins have been demonstrated to interact with endothelial cells expressing the $\alpha_v\beta_5$ integrin (Taylor et al., 1997; Hidai et al., 1998). Furthermore, functional analyses carried out by Andersen and colleagues (2000) demonstrated,

that MFGE8 is a specific ligand for the $\alpha_v\beta_5$ and $\alpha_5\beta_v$ integrin receptors through its N-terminus, and that it can link to surfaces by binding to phospholipids through its C-terminus.

Genes, whose function remains to be elucidated in the context of early embryo-maternal communication

Neighbor of BRCA1 gene 2 (***NBR2***) was identified by its close proximity on human chromosome 17 to tumor suppressor gene BRCA1. The physical distance between the transcription start sites of the NBR2 and BRC1 genes is 218 base pairs, suggesting that regulation of the expression of both genes may be coordinated through a bi-directional prometer. The NBR2 cDNA contains an open reading frame of 112 amino acids and is predicted to encode a protein of approximately 12 kDa (Xu et al., 1997). The specific function of the gene product has not been determined yet.

BST2, bone marrow stromal cell antigen-2, is a type-2 transmembrane protein which is expressed not only on certain bone marrow stromal cell lines, but also on various tissues. Bone marrow stromal cells regulate B-cell growth and development through their surface molecules and cytokines, and there are hints that BST2 facilitates pre-B-cell growth (Ishikawa et al., 1995). On the first glance, the enhanced expression of this gene seems bewildering in the context of this study, but interestingly, Lysiak and Lala (1992) showed that certain mouse decidual stromal cells (DSC) and endometrial stromal cells (ESC) are actually of bone marrow origin. Decidual tissue is the maternal component of the maternal-fetal interface in humans and rodents and is composed predominantly of typical stromal-type cells as well as glandular cells and leukocytes. Decidual stromal cells constitute a distinctive cell class that appears in the endometrium during pregnancy. Initially DSC have classically been considered as fibroblastic cells (Riddick and Kusmik, 1977), but more recent studies demonstrated that DSC express antigens associated with hematopoietic cells (Imai et al., 1992; Montes et al., 1996; Olivares et al., 1997) and that they are, as already mentioned, of bone marrow orgigin (Lysiak and Lala, 1992). Furthermore, Simmons and Torok-Storb (1991) isolated DSC with a stable antigen phenotype similar to that of bone marrow stromal precursors. Studies in mice have suggested that the precursor cells of DSC can migrate from their origin (yolk sac and/or bone marrow) to the uterus at any time between embryonic life and the onset of reproductive life (Lysiak and Lala, 1992). Evaluation of the endometrium from four HLA-mismatched bone marrow transplant recipients demonstrated that endometrial cells can originate from donor-

derived bone marrow cells, suggesting that nonuterine stem cells contribute to the regeneration of human endometrial tissue (Taylor, 2004). The results of a study conducted by Johnson and coworkers (2003b) indicate that the uterine stroma of sheep undergoes a program of differentiation similar to decidualization in invasive implanting species. One might hypothesize that a similar process of differentiation takes place in the bovine endometrium, thereby explaining the enhanced expression of BST2 around the time of embryonic implantation.

CNDP dipetidase 2 (**CNDP2**) is a ubiquitous protein of 52.7 kDa exhibiting peptidase activity and belongs to the M20 metalloprotease family. CNDP2 requires Mn^{2+} for full activity, and is sensitive to inhibition by betastatin (Teufel et al., 2003).

The protein encoded by Homo sapiens chromosome 5 open reading frame 18 (**C5orf18**) is integral to membrane, but nothing about its function is known yet. The expression of this gene has previously been identified to be enhanced during the secretory phase of the estrous cycle in the bovine endometrium (Bauersachs et al., 2005).

The C-terminal modulator protein (**CTMP**) has been identified as a negative regulatory component of the pathway controlling protein kinase B activity (Maira et al., 2001). Protein kinase B (PKB) (also called Akt) is a major downstream target of receptor tyrosine kinases that signal via phosphatidylinositol 3-kinase and once activated through phosphorylation by a phospaditylinositol-dependent protein kinase called PDK1, protein kinase B phosphorylates a variety of cytosolic target proteins (Alberts et al., 2004). Epigenetic downregulation of CTMP transcription is a common aberration in glioblastomas (Knobbe et al., 2004).

Homo sapiens likely ortholog of mouse D11lgp2 (**LGP2**) is a cytoplasmatic protein of 678 amino acids and was identified in a study which aimed to identify genes in the STAT3/5 locus that may participate in normal and neoplastic development of the mammary gland (Cui et al., 2001).

Only little information is available about ***LOC401623***, *Homo sapiens* similar to LINE-1 reverse transcriptase homolog. Long interspersed elements (LINE-1) are abundant retrotransposons in mammalian genomes that probably retrotranspose by target site-primed reverse transcription (TPRT). During TPRT, the LINE-1 endonuclease cleaves genomic DNA, freeing a 3'hydroxyl that serves as primer for reverse transcription of LINE-1 RNA by LINE-1 reverse transcriptase.

Abundance levels of the cytosolic NADP-isocitrate dehydrogenase (**IDH1**) protein were shown to be dependent on the stage of the sexual cycle in human endometrium, with a maximum in the secretory phase (Byrjalsen et al., 1995). Additionally, in a holistic differential analysis of embryo-induced alterations in the proteome of bovine endometrium in the preattachment period, IDH1 protein showed a significantly higher abundance in samples derived from pregnant animals (Berendt et al., 2005).

Myotubularin and related proteins, like **MTMR6**, constitute a large and highly conserved family possessing phosphoinositide 3-phosphatase activity, although not all members show this activity. Members of this family contain a conserved region called the GRAM domain that is found in a variety of proteins associated with membrane-coupled processes and signal transduction (Tsujita et al., 2004). As compared to the day of standing heat, *MTMR3* is upregulated in the cycling bovine endometrium at day 12 of the estrous cycle (Bauersachs et al., 2005).

Placental ribonuclease/angiogenin inhibitor (**RNH1**) is a member of a family of proteinaceous cytoplasmatic RNase inhibitors that occur in many tissues and bind to both intracellular and extracellular RNases. In addition to control of intracellular RNases, the inhibitor may have a role in the regulation of angiogenin. RNH1 may be essential for control of mRNA turnover, since neutral and alkaline ribonucleases play a critical role in the turnover of RNA in eukaryotic cells. RNH1 further displays a unusual combination of high affinity and relaxed stringency (Iyer et al., 2005).

The sterile alpha motif domain present in **SAMD9** is a protein interaction module that is present in diverse signal-transducing proteins such as the Eph family of receptor tyrosine kinases, diaglycerol kinases, serine-threonine kinases, Src homology 2 domain-containing adapter proteins, and others (Thanos et al., 1999).

SLC15A3 is a member of the solute carrier family 15 of electrogenic transporters that utilize the proton-motive force for uphill transport of short chain peptides and peptide-mimetics into a variety of cells. The prototype tranporters of this family are SLC15A1 and SLC15A2, which mediate the uptake of peptides into intestinal and renal epithelial cells. SLC15A3 was identified by Daniel and Kottra (2004), and shown to transport free histidine and certain di- and tripeptides, but it is not clear yet whether it is located in the plasma membrane or represents a lysosomal transporter for proton-dependent export of histidine and dipeptides from lysosomal protein degradation into the cytosol. In humans, SLC15A1 is upregulated during the late secretory phase of

the cycling endometrium and its expression is restricted to glandular and luminal epithelial cells (Riesewijk et al., 2003).
The HBxAG transactivated protein 2 (***XTP2***) gene is amplified and overexpressed in bladder cancer (Huang et al., 2002).

Insulin-like growth factor system
The insulin growth factor system is assumed to play a vital role in endometrial tissue remodeling by regulating uterine growth (Wolf et al., 2003). The changes in abundance of the mRNAs of *IGFBP2* and *CTGF*, encoding a low-affinity IGF-binding protein, indicate the potential importance of IGFBP regulation of uterine IGFs during this time period. Furthermore, upregulation of MMP19 might lead to proteolysis of IGFBP3, which was shown to result in increased IGF-signaling in human keratinocytes (Sadowski et al., 2003).

Expression levels of some genes are rather maintained at a higher level than induced by the embryo
The expression of six genes, namely *ATP1B2*, *IDH1*, *LY6G6C*, *MS4A8B*, *PENK*, and *TIMP2* is likely to be not specifically upregulated by the presence of a conceptus since they were identified as upregulated at diestrus in a study of transcriptome changes in bovine intercaruncular endometrium comparing late estrus and diestrus (Bauersachs et al., 2005). The regulation of these genes can be explained in the way that the presence of the embryo maintains a high level of expression by preventing the progression of the estrous cycle.

Comparison to other studies respectively the literature
Three previous studies dealing with global gene expression analysis were conducted in the past. Sun et al. (2004) performed suppression subtractive hybridization to find genes that are differentially expressed in the implantation site of the pregnant rhesus monkey uterus, Reese et al. (2001) compared RNAs from implantation and interimplantation sites to identify genes that are specifically up- or downregulated at the implantation site in mice, and Yoshioka et al. (2000) examined the expression of uterine genes before and after conceptus implantation in mice. There are very few genes mutually identified in one of the three studies mentioned above and in the current study, which deals with the identification of genes induced by the conceptus in the bovine endometrium during the pre-implantation period. There are two main

reasons for the missing concurrence regarding the set of identified genes. On the one hand, the process of embryonic implantation was investigated in different species, i.e. rhesus monkeys, mice and cows, which all exhibit fundamental differences concerning the mode of embryonic implantation. On the other hand, only Sun and coworkers virtually compared the mRNA profiles of pregnant and non-pregnant uteri, as it was performed in the current study. The two remaining studies compared the mRNA profiles of implantation and interimplantation sites, both tissue samples derived from pregnant uteri (Reese et al., 2001) or the mRNA profiles of whole uterine tissues from two different days of gestation (Yoshioka et al., 2000), respectively, thus, the studies substantially differ from the current study concerning their experimental design.

In chapter 2.1 various cytokines and growth factors are reviewed regarding their known or potential function in the context of embryonic implantation. Some of these factors, e.g. LIF, M-CSF, and HOXA10, have been demonstrated to be essential for embryonic implantation in species such as mice and humans. In the current study none of the cytokines or growth factors reviewed in chapter 2.1 was identified. This might be attributable to the fact that, instead of the total subtracted cDNA library, only a representational number of clones was analyzed by cDNA array hybridization. Nevertheless, several studies conducted in our laboratory have proven that the analysis of 1536 clones per subtracted cDNA library is sufficient to identify the most relevant set of differentially expressed genes (Bauersachs et al., 2003; Bauersachs et al., 2004). It is more likely, that some of the factors, which have been shown to be indispensable for embryonic implantation in the murine and human species, was identified in the present study because the mode of implantation differs substantially between these species and the cow, as already mentioned above.

The efficiency of SSH

During the course of this research project, the efficiency of two subtraction methods for generating subtracted cDNA was compared: on the one hand, subtracted cDNA was generated using the SSH method according to Diatchenko and coworkers (1996) (in the following referred to as “SSH library”), and, on the other hand, subtracted cDNA was purchased from the vertis Biotechnologie AG (in the following referred to as “vertis library”). Both subtracted cDNA populations were used to construct a cDNA library, which was subsequently analyzed by cDNA array hybridization.

Two major differences concerning the technique of subtraction exist between both approaches. The first step in the process of subtraction involves hybridization of cDNA from one population (tester) to excess of cDNA from another population (driver). The SSH method utilizes double stranded (ds) cDNA for hybridization, whereas the vertis Biotechnologie AG made use of single stranded (ss) cDNA. Due to the fact that complementary cDNA strands cannot form between tester molecules when using ss cDNA for hybridization, the equalization of the concentration of high- and low abundance sequences (a process referred to as normalization, view chapter 2.4) only occurs when employing the SSH method. An additional difference consists in the second step of subtraction, which involves separation of the unhybridized fraction (target) from the hybridized common sequences. The SSH methods utilizes the so-called suppressor PCR, which selectively amplifies target cDNA fragments and simultaneously suppresses non-target cDNA amplification. Thereby any intermediate step for physical separation of ss and ds cDNAs is circumvented. The vertis Biotechnolgie AG, however, uses hydroxylapatite chromatography to separate ss and ds cDNA in a physical manner.

From the SSH library 3072 individual cDNA clones were analyzed, whereas only 1536 individual cDNA clones were analyzed from the vertis library, because ongoing sequence analysis of differentially expressed cDNAs revealed high redundancy of the identified genes belonging to the “vertis library”. This predominance of certain genes in the “vertis library” is due to the fact that, as already mentioned above, only when performing subtraction according the SSH method the process of normalization occurs. Collectively, it can be stated, that, even though the percentage of differentially expressed genes is almost the same in both libraries, the library constructed using the SSH method is more suitable for analyzing a large number of individual cDNA clones. Analysis of a larger number of cDNA clones from the vertis library is much more effort by reason of the lacking equalization of the concentration of high- and low abundance sequences.

In general, cDNAs that were represented by three or more cDNA clones, either on the “SSH array” or “vertis array”, were present in both arrays, indicating that both methods are suitable for identifying the relevant subset of differentially regulated genes . In contrast, cDNAs that were represented by one or two cDNA clones, either on the “SSH array” or “vertis array”, were only present on one array in the majority of cases. However, some cDNAs (i.e. *KIAA1404*, *ISG12*, *G1P3*, and *PENK*) were

identified to be present only on one array, even though they were represented by five or more cDNA fragments. There is no reasonable explanation for this observation. Probably, for some reason, certain cDNA fragments can only be amplified and identified with one of the two methods.

Final Conclusions

In conclusion this study provides a holistic view of the quantitative changes of endometrial transcript levels associated with the complex embryo-maternal cross-talk in the bovine species. Even though the precise function is not known for each gene identified, the study revealed the orchestrated upregulation of genes important for embryonic implantation during the pre-implantation period in the bovine endometrium. The findings underline the importance of IFNτ as an embryo-derived pregnancy recognition signal. The categorization of the identified genes according their function revealed the orchestrated upregulation of genes essential for remodeling of the endometrium, and for modulation of the maternal immunsystem, and of several putative components of the ISG15ylation system. Moreover, many increased transcript levels were found for novel candidate genes which might be important for conditioning the endometrium for conceptus attachment. These findings represent interesting starting points for more detailed investigations and are a solid basis for building a systems biology of embryo-maternal communication and implantation.

6. Summary

Identification of genes induced by the conceptus in the bovine endometrium during the pre-implantation period

An intact embryo-maternal communication in the pre-implantation period is particularly critical for establishment of pregnancy and early embryonic losses have been identified as the major cause of reproductive failure in cattle. Thus, to gain deeper insight into this complex embryo-maternal crosstalk, a combination of subtracted cDNA libraries and cDNA array hybridization was applied to identify mRNAs differentially regulated genes in the bovine endometrium by the presence of a conceptus. One cDNA library was constructed according the suppression subtractive hybridization method (Diatchenko et al., 1996) with minor modifications; a second cDNA library was constructed of subtracted cDNA purchased from the vertis Biotechnologie AG. As biological model endometrial tissue samples of monozygotic twins (generated by embryo splitting) collected at day 18 of gestation were used, which is a unique possibility to eliminate genetic variability as a factor potentially affecting the results of gene expression analyses. Array hybridization was carried out using ^{33}P-labeled cDNA probes obtained from five monozygotic twin pairs. Sequence analysis revealed 87 different genes or mRNAs, respectively, which displayed a difference in signal intensity of 2.0 fold or more in at least four out of five twin pairs. Eighty genes corresponded to genes with known or inferred function, either the bovine gene or the human orthologue. For 7 mRNAs a match with bovine ESTs was obtained only. For nine selected genes the expression in the bovine endometrium was quantified by the use of quantitative real-time RT-PCR to verify the results obtained by array hybridization and to perform more precise quantitative measurements for these genes. Overall, the results of array hybridization and real-time RT-PCR correlated very well.

Almost half of the identified genes are known to be stimulated by type I interferons reflecting the response to IFNτ, which is the pregnancy recognition signal in ruminants. Of particular interest among the interferon stimulated genes is *ISG15*, one of the most markedly upregulated genes in the present study, which is hypothesized to stabilize intracellular endometrial proteins through conjugation processes. For the ISG15ylation system mRNAs of four potential components (*IFITM1*, *IFITM3*, *HSXIAPAF1*, and *DTX3L*) were found in addition to *ISG15* and *UBE1L*, and *in situ*

hybridization revealed similar mRNA expression patterns of these genes. It is therefore suggested, that modification of endometrial proteins through ISG15ylation plays a fundamental role in the IFNτ signaling.

A classification of the identified genes according to their assignment to Gene Ontologies revealed the orchestrated interaction of various processes and mechanisms with regard to the preparation of the maternal endometrium for embryonic implantation. As particular interesting, genes were identified involved in modulation of the maternal immune system at the humoral and cellular level, cell adhesion, cell communication, regulation of transcription, cell differentiation, cell growth, and cell proliferation. These findings underline that an intense embryo-maternal dialogue takes place during the pre-implantation period, which culminates in a receptive endometrium prepared for implantation of the conceptus.

To conclude, this is the first study of its kind for cattle in the pre-implantation stage of embryonic development and revealed the orchestrated upregulation of genes important for embryonic implantation during the pre-implantation period in the bovine endometrium. The presented results provide new starting points for detailed investigations of the embryo-maternal dialogue by which the endometrium is prepared for conceptus attachment.

7. Zusammenfassung

Identifizierung von Genen im bovinen Endometrium, welche durch den Konzeptus im prä-implantatorischen Zeitraum induziert werden

Eine intakte embryo-maternale Kommunikation in der prä-Implantationsphase ist im Besonderen wichtig für die Etablierung einer Gravidität und frühe embryonale Verluste wurden als eine der Hauptursachen für Fruchtbarkeitsstörungen beim Rind identifiziert. Deshalb wurde, um einen tieferen Einblick in diesen komplexen embryo-maternalen Dialog zu bekommen, eine Kombination aus subtraktiven cDNA Banken und cDNA Array Hybridisierung angewendet, mit dem Ziel, Gene zu identifizieren, die aufgrund der Gegenwart des Konzeptus differentiell reguliert werden. Eine subtraktive cDNA Bank wurde, mit kleinen Modifikationen, nach der suppression subtractive hybridization Methode (Diatchenko et al., 1996) hergestellt; eine zweite subtraktive cDNA Bank wurde aus subtraktiver cDNA hergestellt, welch von der vertis Biotechnologie AG erworben wurde. Als biologisches Modell dienten endometriale Gewebeproben von monozygoten Zwillingen (generiert durch Embryosplitting), welche an Tag 18 der Gravidität gewonnen wurden. Diese Vorgehensweise ist eine einmalige Gelegenheit um den Einfluss genetischer Variabilität auf die Ergebnisse von Genexpressionanalysen zu eliminieren. Die Array Hybridisierung wurde mit ^{33}P-markierten Proben von fünf monozygoten Zwillingspaaren durchgeführt. Die Sequenzanalyse ergab 87 verschiedene Gene beziehungsweise mRNAs, die einen Unterschied in der Signalintensität von 2,0 oder mehr in mindesten vier von fünf Zwillingspaaren zeigten. Achtzig mRNAs konnten Genen mit bekannter oder mutmaßlicher Funktion zugeordnet werden, entweder dem bovinen Gen oder dem humanen Ortholog. Für 7 mRNAs konnte nur ein Treffer mit bovinen ESTs gefunden werden. Um die Ergebnisse der Array Hybridisierung zu verifizieren und um eine genauere quantitative Messung durchzuführen, wurde die Expression neun ausgesuchter Gene durch quantitative real-time RT-PCR quantifiziert. Die Ergebnisse der Array Hybridisierung und der real-time RT-PCR korrelierten sehr gut.

Von fast der Hälfte aller identifizierten Gene ist bekannt, dass die Expression durch Typ I Interferone stimuliert, was die Antwort auf IFNτ, dem Trächtigkeitserkennungsignal bei Ruminanten, widerspiegelt. Unter den Interferon stimulierten Genen ist das *ISG15*, eines der am deutlichsten hochregulierten Gene in

der vorliegenden Studie, von besonderem Interesse, von welchem angenommen wird, dass es intrazelluläre endometriale Proteine durch Konjugationsprozesse stabilisiert. Neben *ISG15* und *UBE1L* wurden vier potentielle Komponenten des ISG15 Systems gefunden (*IFITM1*, *IFITM3*, *HSXIAPAF1* und *DTX3L*) und die *in situ* Hybridisierung zeigte ähnliche mRNA Expressionsmuster dieser Gene. Es wird deswegen angenommen, dass die Modifikation endometrialer Proteine durch ISG15ylierung eine fundamentale Rolle im IFNτ Signaltransduktionsweg spielt.

Eine Klassifizierung der identifizierten Gene nach ihrer Zuordnung zu Gene Ontologies deckte die koordinierte Interaktion verschiedenster Prozesse und Mechanismen im Hinblick auf die Vorbereitung des maternalen Endometriums auf die Implantation des Embryos auf. Als besonders interessant wurden Gene erachtet, die eine Rolle spielen in der Regulation des maternalen Immunsystems auf zellulärer und humoraler Ebene, der Zelladhäsion, der Zellkommunikation, der Regulation der Transkription, der Zelldifferenzierung und der Zellproliferation. Diese Ergebnisse unterstrichen, dass ein intensiver embryo-maternaler Dialog in der prä-implantatorischen Phase stattfindet, welcher in einem rezeptiven Endometrium, vorbereitet auf die Implantation des Konzeptus, gipfelt.

Abschließend ist dies die erste Studie ihrer Art in der prä-Implantationsphase der embryonalen Entwicklung beim Rind, wobei die koordinierte Hochregulierung von Genen, die im Kontext der embryonalen Implantation eine wichtige Rolle spielen, gezeigt werden konnte. Die Ergebnisse liefern somit neue Ausgangspunkte für detailliertere Untersuchungen des embryo-maternalen Dialogs, welcher das Endometrium auf die Adhäsion des Konzeptus vorbereitet.

8. References

Abbas,A.K., Lichtman,A.H., and Pober,J.S. (2005). Cellular and Molecular Immonology. W.B. Saunders Company.

Abbondanzo,S.J., Cullinan,E.B., McIntyre,K., Labow,M.A., and Stewart,C.L. (1996). Reproduction in mice lacking a functional type 1 IL-1 receptor. Endocrinology *137*, 3598-3601.

Ace,C.I. and Okulicz,W.C. (2004). Microarray profiling of progesterone-regulated endometrial genes during the rhesus monkey secretory phase. Reprod. Biol. Endocrinol. *2*, 54.

Acosta,A.A., Elberger,L., Borghi,M., Calamera,J.C., Chemes,H., Doncel,G.F., Kliman,H., Lema,B., Lustig,L., and Papier,S. (2000). Endometrial dating and determination of the window of implantation in healthy fertile women. Fertil. Steril. *73*, 788-798.

Aghajanova,L. (2004). Leukemia inhibitory factor and human embryo implantation. Ann. N. Y. Acad. Sci. *1034*, 176-183.

Ahmed,M.S., Randall,L.W., Cavinato,A.G., Desiderio,D.M., Fridland,G., and Sibai,B. (1986). Human placental opioid peptides: correlation to the route of delivery. Am. J. Obstet. Gynecol. *155*, 703-706.

Alberts,B., Johnson,A., Lewis,J., Raff,M., Roberts,K., and Walter,P. (2004). Molekularbiologie der Zelle, 4. Auflage. WILEY-VCH Verlag, Weinheim.

Alexander,C.M., Hansell,E.J., Behrendtsen,O., Flannery,M.L., Kishnani,N.S., Hawkes,S.P., and Werb,Z. (1996). Expression and function of matrix metalloproteinases and their inhibitors at the maternal-embryonic boundary during mouse embryo implantation. Development *122*, 1723-1736.

Allison,G.C., Bartol,F.F., Taylor,K.M., Wiley,A.A., Ramsey,W.S., Ott,T.L., Bazer,F.W., and Spencer,T.E. (2000). Ovine uterine gland knock-out model: effects of gland ablation on the estrous cycle. Biol. Reprod. *62*, 448-456.

Andersen,M.H., Berglund,L., Rasmussen,J.T., and Petersen,T.E. (1997). Bovine PAS-6/7 binds alpha v beta 5 integrins and anionic phospholipids through two domains. Biochemistry *36*, 5441-5446.

Andersen,M.H., Graversen,H., Fedosov,S.N., Petersen,T.E., and Rasmussen,J.T. (2000). Functional analyses of two cellular binding domains of bovine lactadherin. Biochemistry *39*, 6200-6206.

Andrade,P.M., Silva,I.D., Borra,R.C., de Lima,G.R., and Baracat,E.C. (2002). Estrogen regulation of uterine genes in vivo detected by complementary DNA array. Horm. Metab Res. *34*, 238-244.

Aplin,J.D. (1999). MUC-1 glycosylation in endometrium: possible roles of the apical glycocalyx at implantation. Hum. Reprod. *14 Suppl 2*, 17-25.

Apparao,K.B., Illera,M.J., Beyler,S.A., Olson,G.E., Osteen,K.G., Corjay,M.H., Boggess,K., and Lessey,B.A. (2003). Regulated expression of osteopontin in the peri-implantation rabbit uterus. Biol. Reprod. *68*, 1484-1490.

Austin,K.J., Bany,B.M., Belden,E.L., Rempel,L.A., Cross,J.C., and Hansen,T.R. (2003). Interferon-stimulated gene-15 (Isg15) expression is up-regulated in the mouse uterus in response to the implanting conceptus. Endocrinology *144*, 3107-3113.

Austin,K.J., Carr,A.L., Pru,J.K., Hearne,C.E., George,E.L., Belden,E.L., and Hansen,T.R. (2004). Localization of ISG15 and conjugated proteins in bovine endometrium using immunohistochemistry and electron microscopy. Endocrinology *145*, 967-975.

Austin,K.J., Ward,S.K., Teixeira,M.G., Dean,V.C., Moore,D.W., and Hansen,T.R. (1996). Ubiquitin cross-reactive protein is released by the bovine uterus in response to interferon during early pregnancy. Biol. Reprod. *54*, 600-606.

Babic,A.M., Chen,C.C., and Lau,L.F. (1999). Fisp12/mouse connective tissue growth factor mediates endothelial cell adhesion and migration through integrin alphavbeta3, promotes endothelial cell survival, and induces angiogenesis in vivo. Mol. Cell Biol. *19*, 2958-2966.

Baraldi,M., Giarre,G., Santi,M., Facchinetti,F., Petraglia,F., and Genazzani,A.R. (1985). Pregnancy related changes of opiate receptors identified in rat uterine membranes by 3H-naloxone binding. Peptides *6*, 971-974.

Bartocci,A., Pollard,J.W., and Stanley,E.R. (1986). Regulation of colony-stimulating factor 1 during pregnancy. J. Exp. Med. *164*, 956-961.

Basak,S., Dhar,R., and Das,C. (2002). Steroids modulate the expression of alpha4 integrin in mouse blastocysts and uterus during implantation. Biol. Reprod. *66*, 1784-1789.

Bauersachs,S., Blum,H., Mallok,S., Wenigerkind,H., Rief,S., Prelle,K., and Wolf,E. (2003). Regulation of ipsilateral and contralateral bovine oviduct epithelial cell function in the postovulation period: a transcriptomics approach. Biol. Reprod. *68*, 1170-1177.

Bauersachs,S., Rehfeld,S., Ulbrich,S.E., Mallok,S., Prelle,K., Wenigerkind,H., Einspanier,R., Blum,H., and Wolf,E. (2004). Monitoring gene expression changes in bovine oviduct epithelial cells during the oestrous cycle. J. Mol. Endocrinol. *32*, 449-466.

Bauersachs,S., Ulbrich,S.E., Gross,K., Schmidt,S.E., Meyer,H.H., Einspanier,R., Wenigerkind,H., Vermehren,M., Blum,H., Sinowatz,F., and Wolf,E. (2005). Gene expression profiling of bovine endometrium during the oestrous cycle: detection of molecular pathways involved in functional changes. J. Mol. Endocrinol. *34*, 889-908.

Bayless,K.J., Meininger,G.A., Scholtz,J.M., and Davis,G.E. (1998). Osteopontin is a ligand for the alpha4beta1 integrin. J. Cell Sci. *111 (Pt 9)*, 1165-1174.

Bazer,F.W. and Roberts,R.M. (1983). Biochemical aspects of conceptus--endometrial interactions. J. Exp. Zool. *228*, 373-383.

Bazer,F.W., Spencer,T.E., and Ott,T.L. (1997). Interferon tau: a novel pregnancy recognition signal. Am. J. Reprod. Immunol. *37*, 412-420.

Bebington,C., Doherty,F.J., and Fleming,S.D. (1999). Ubiquitin cross-reactive protein gene expression is increased in decidualized endometrial stromal cells at the initiation of pregnancy. Mol. Hum. Reprod. *5*, 966-972.

Belisle,S., Petit,A., Gallo-Payet,N., Bellabarba,D., Lehoux,J.G., and Lemaire,S. (1988). Functional opioid receptor sites in human placentas. J. Clin. Endocrinol. Metab *66*, 283-289.

Benson,G.V., Lim,H., Paria,B.C., Satokata,I., Dey,S.K., and Maas,R.L. (1996). Mechanisms of reduced fertility in Hoxa-10 mutant mice: uterine homeosis and loss of maternal Hoxa-10 expression. Development *122*, 2687-2696.

Berendt,F.J., Frohlich,T., Schmidt,S.E., Reichenbach,H.D., Wolf,E., and Arnold,G.J. (2005). Holistic differential analysis of embryo-induced alterations in the proteome of bovine endometrium in the preattachment period. Proteomics. *5*, 2551-2560.

Bhatt,H., Brunet,L.J., and Stewart,C.L. (1991). Uterine expression of leukemia inhibitory factor coincides with the onset of blastocyst implantation. Proc. Natl. Acad. Sci. U. S. A *88*, 11408-11412.

Bilalis,D.A., Klentzeris,L.D., and Fleming,S. (1996). Immunohistochemical localization of extracellular matrix proteins in luteal phase endometrium of fertile and infertile patients. Hum. Reprod. *11*, 2713-2718.

Bloch,D.B., Nakajima,A., Gulick,T., Chiche,J.D., Orth,D., de La Monte,S.M., and Bloch,K.D. (2000). Sp110 localizes to the PML-Sp100 nuclear body and may function as a nuclear hormone receptor transcriptional coactivator. Mol. Cell Biol. *20*, 6138-6146.

Boomsma,R.A., Mavrogianis,P.A., Fazleabas,A.T., Jaffe,R.C., and Verhage,H.G. (1994). Detection of insulin-like growth factor binding protein-1 in cat implantation sites. Biol. Reprod. *51*, 392-399.

Borthwick,J.M., Charnock-Jones,D.S., Tom,B.D., Hull,M.L., Teirney,R., Phillips,S.C., and Smith,S.K. (2003). Determination of the transcript profile of human endometrium. Mol. Hum. Reprod. *9*, 19-33.

Bowen,J.A., Bazer,F.W., and Burghardt,R.C. (1996). Spatial and temporal analyses of integrin and Muc-1 expression in porcine uterine epithelium and trophectoderm in vivo. Biol. Reprod. *55*, 1098-1106.

Braga,V.M. and Gendler,S.J. (1993). Modulation of Muc-1 mucin expression in the mouse uterus during the estrus cycle, early pregnancy and placentation. J. Cell Sci. *105 (Pt 2)*, 397-405.

Brownstein,M.J. (1993). A brief history of opiates, opioid peptides, and opioid receptors. Proc. Natl. Acad. Sci. U. S. A *90*, 5391-5393.

Butler,J.E., Pringnitz,D.J., Martens,C.L., and Crouch,N. (1980). Bovine-associated mucoprotein: I. Distribution among adult and fetal bovine tissues and body fluids. Differentiation *17*, 31-40.

Byrjalsen,I., Larsen,P.M., Fey,S.J., and Christiansen,C. (1995). Human endometrial proteins with cyclic changes in the expression during the normal menstrual cycle: characterization by protein sequence analysis. Hum. Reprod. *10*, 2760-2766.

Cann,C.H., Fairclough,R.J., Sutton,R., and Gow,C.B. (1997). Endometrial expression of mRNA encoding insulin-like growth factors I and II and IGF-binding proteins 1 and 2 in early pregnant ewes. J. Reprod. Fertil. *111*, 7-13.

Carson,D.D., DeSouza,M.M., Kardon,R., Zhou,X., Lagow,E., and Julian,J. (1998). Mucin expression and function in the female reproductive tract. Hum. Reprod. Update. *4*, 459-464.

Carson,D.D., Lagow,E., Thathiah,A., Al Shami,R., Farach-Carson,M.C., Vernon,M., Yuan,L., Fritz,M.A., and Lessey,B. (2002). Changes in gene expression during the early to mid-luteal (receptive phase) transition in human endometrium detected by high-density microarray screening. Mol. Hum. Reprod. *8*, 871-879.

Cemerikic,B., Cheng,J., Agbas,A., and Ahmed,M.S. (1991). Opioids regulate the release of human chorionic gonadotropin hormone from trophoblast tissue. Life Sci. *49*, 813-824.

Cerro,J.A. and Pintar,J.E. (1997). Insulin-like growth factor binding protein gene expression in the pregnant rat uterus and placenta. Dev. Biol. *184*, 278-295.

Charlier,M., Hue,D., Martal,J., and Gaye,P. (1989). Cloning and expression of cDNA encoding ovine trophoblastin: its identity with a class-II alpha interferon. Gene *77*, 341-348.

Charpigny,G., Reinaud,P., Huet,J.C., Guillomot,M., Charlier,M., Pernollet,J.C., and Martal,J. (1988). High homology between a trophoblastic protein (trophoblastin) isolated from ovine embryo and alpha-interferons. FEBS Lett. *228*, 12-16.

Chebath,J., Merlin,G., Metz,R., Benech,P., and Revel,M. (1983). Interferon-induced 56,000 Mr protein and its mRNA in human cells: molecular cloning and partial sequence of the cDNA. Nucleic Acids Res. *11*, 1213-1226.

Chee,M., Yang,R., Hubbell,E., Berno,A., Huang,X.C., Stern,D., Winkler,J., Lockhart,D.J., Morris,M.S., and Fodor,S.P. (1996). Accessing genetic information with high-density DNA arrays. Science *274*, 610-614.

Chen,D., Jin,Z., and Xing,F. (1998). [The expression of integrin beta 3 in cycling and early pregnant endometrium and its relationship with primary unexplained infertility]. Zhonghua Fu Chan Ke. Za Zhi. *33*, 487-489.

Chen,J.R., Cheng,J.G., Shatzer,T., Sewell,L., Hernandez,L., and Stewart,C.L. (2000). Leukemia inhibitory factor can substitute for nidatory estrogen and is essential to inducing a receptive uterus for implantation but is not essential for subsequent embryogenesis. Endocrinology *141*, 4365-4372.

Cheng,Y.S., Becker-Manley,M.F., Chow,T.P., and Horan,D.C. (1985). Affinity purification of an interferon-induced human guanylate-binding protein and its characterization. J. Biol. Chem. *260*, 15834-15839.

Cheng,Y.S., Patterson,C.E., and Staeheli,P. (1991). Interferon-induced guanylate-binding proteins lack an N(T)KXD consensus motif and bind GMP in addition to GDP and GTP. Mol. Cell Biol. *11*, 4717-4725.

Cheon,Y.P., Li,Q., Xu,X., DeMayo,F.J., Bagchi,I.C., and Bagchi,M.K. (2002). A genomic approach to identify novel progesterone receptor regulated pathways in the uterus during implantation. Mol. Endocrinol. *16*, 2853-2871.

Choi,Y., Johnson,G.A., Burghardt,R.C., Berghman,L.R., Joyce,M.M., Taylor,K.M., Stewart,M.D., Bazer,F.W., and Spencer,T.E. (2001). Interferon regulatory factor-two restricts expression of interferon-stimulated genes to the endometrial stroma and glandular epithelium of the ovine uterus. Biol. Reprod. *65*, 1038-1049.

Choi,Y., Johnson,G.A., Spencer,T.E., and Bazer,F.W. (2003). Pregnancy and interferon tau regulate major histocompatibility complex class I and beta2-microglobulin expression in the ovine uterus. Biol. Reprod. *68*, 1703-1710.

Chwetzoff,S. and d'Andrea,S. (1997). Ubiquitin is physiologically induced by interferons in luminal epithelium of porcine uterine endometrium in early pregnancy: global RT-PCR cDNA in place of RNA for differential display screening. FEBS Lett. *405*, 148-152.

Clark,D.E., Hurst,P.R., McLennan,I.S., and Myers,D.B. (1993). Immunolocalization of collagen type I and laminin in the uterus on days 5 to 8 of embryo implantation in the rat. Anat. Rec. *237*, 8-20.

Couto,J.R., Taylor,M.R., Godwin,S.G., Ceriani,R.L., and Peterson,J.A. (1996). Cloning and sequence analysis of human breast epithelial antigen BA46 reveals an RGD cell adhesion sequence presented on an epidermal growth factor-like domain. DNA Cell Biol. *15*, 281-286.

Cui,Y., Li,M., Walton,K.D., Sun,K., Hanover,J.A., Furth,P.A., and Hennighausen,L. (2001). The Stat3/5 locus encodes novel endoplasmic reticulum and helicase-like proteins that are preferentially expressed in normal and neoplastic mammary tissue. Genomics *78*, 129-134.

Curry,T.E., Jr. and Osteen,K.G. (2003). The matrix metalloproteinase system: changes, regulation, and impact throughout the ovarian and uterine reproductive cycle. Endocr. Rev. *24*, 428-465.

Daftary,G.S., Troy,P.J., Bagot,C.N., Young,S.L., and Taylor,H.S. (2002). Direct regulation of beta3-integrin subunit gene expression by HOXA10 in endometrial cells. Mol. Endocrinol. *16*, 571-579.

Daniel,H. and Kottra,G. (2004). The proton oligopeptide cotransporter family SLC15 in physiology and pharmacology. Pflugers Arch. *447*, 610-618.

Danilovich,N., Wernsing,D., Coschigano,K.T., Kopchick,J.J., and Bartke,A. (1999). Deficits in female reproductive function in GH-R-KO mice; role of IGF-I. Endocrinology *140*, 2637-2640.

Das,S.K., Wang,X.N., Paria,B.C., Damm,D., Abraham,J.A., Klagsbrun,M., Andrews,G.K., and Dey,S.K. (1994). Heparin-binding EGF-like growth factor gene is induced in the mouse uterus temporally by the blastocyst solely at the site of its apposition: a possible ligand for interaction with blastocyst EGF-receptor in implantation. Development *120*, 1071-1083.

Das,S.K., Yano,S., Wang,J., Edwards,D.R., Nagase,H., and Dey,S.K. (1997). Expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in the mouse uterus during the peri-implantation period. Dev. Genet. *21*, 44-54.

Davidson,J.A., Betts,J.G., Tiemann,U., Kamwanja,L.A., Monterroso,V.H., and Hansen,P.J. (1994). Effects of interferon-tau and interferon-alpha on proliferation of bovine endometrial cells. Biol. Reprod. *51*, 700-705.

de Veer,M.J., Sim,H., Whisstock,J.C., Devenish,R.J., and Ralph,S.J. (1998). IFI60/ISG60/IFIT4, a new member of the human IFI54/IFIT2 family of interferon-stimulated genes. Genomics *54*, 267-277.

De,M., Sanford,T.R., and Wood,G.W. (1993). Expression of interleukin 1, interleukin 6 and tumour necrosis factor alpha in mouse uterus during the peri-implantation period of pregnancy. J. Reprod. Fertil. *97*, 83-89.

Dhara,S., Lalitkumar,P.G., Sengupta,J., and Ghosh,D. (2001). Immunohistochemical localization of insulin-like growth factors I and II at the primary implantation site in the Rhesus monkey. Mol. Hum. Reprod. *7*, 365-371.

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

Djonov,V., Hogger,K., Sedlacek,R., Laissue,J., and Draeger,A. (2001). MMP-19: cellular localization of a novel metalloproteinase within normal breast tissue and mammary gland tumours. J. Pathol. *195*, 147-155.

Döcke,F. (1994). Veterinärmedizinische Endokrinologie, 3. Auflage. Gustav Fischer Verlag, Jena.

Dong,B. and Silverman,R.H. (1995). 2-5A-dependent RNase molecules dimerize during activation by 2-5A. J. Biol. Chem. *270*, 4133-4137.

Douglass,J., Cox,B., Quinn,B., Civelli,O., and Herbert,E. (1987). Expression of the prodynorphin gene in male and female mammalian reproductive tissues. Endocrinology *120*, 707-713.

Drakakis,P., Loutradis,D., Milingos,S., Michalas,S., Kallianidis,K., Bletsa,R., Aravantinos,D., and Kiessling,A.A. (1995). A preliminary study of the effect of growth

hormone on mouse preimplantation embryo development in vitro. Gynecol. Obstet. Invest *40*, 222-226.

Emond,V., Asselin,E., Fortier,M.A., Murphy,B.D., and Lambert,R.D. (2000). Interferon-tau stimulates granulocyte-macrophage colony-stimulating factor gene expression in bovine lymphocytes and endometrial stromal cells. Biol. Reprod. *62*, 1728-1737.

Emond,V., MacLaren,L.A., Kimmins,S., Arosh,J.A., Fortier,M.A., and Lambert,R.D. (2004). Expression of cyclooxygenase-2 and granulocyte-macrophage colony-stimulating factor in the endometrial epithelium of the cow is up-regulated during early pregnancy and in response to intrauterine infusions of interferon-tau. Biol. Reprod. *70*, 54-64.

Evans,M.J., Gurer,C., Loike,J.D., Wilmut,I., Schnieke,A.E., and Schon,E.A. (1999). Mitochondrial DNA genotypes in nuclear transfer-derived cloned sheep. Nat. Genet. *23*, 90-93.

Farrar,J.D. and Carson,D.D. (1992). Differential temporal and spatial expression of mRNA encoding extracellular matrix components in decidua during the peri-implantation period. Biol. Reprod. *46*, 1095-1108.

Fazleabas,A.T., Jaffe,R.C., Verhage,H.G., Waites,G., and Bell,S.C. (1989). An insulin-like growth factor-binding protein in the baboon (Papio anubis) endometrium: synthesis, immunocytochemical localization, and hormonal regulation. Endocrinology *124*, 2321-2329.

Feinberg,A.P. and Vogelstein,B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. *132*, 6-13.

Feinberg,A.P. and Vogelstein,B. (1984). "A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity". Addendum. Anal. Biochem. *137*, 266-267.

Flanagan,A.M. and Lader,C.S. (1998). Update on the biologic effects of macrophage colony-stimulating factor. Curr. Opin. Hematol. *5*, 181-185.

Flint,A.P. and Sheldrick,E.L. (1983). Evidence for a systemic role for ovarian oxytocin in luteal regression in sheep. J. Reprod. Fertil. *67*, 215-225.

Flores,J.M., Sanchez,M.A., Garcia,P., Sanchez,B., and Nieto,A. (1998). Immunohistochemical localization of epidermal growth factor, transforming growth factor-alpha and growth factor-beta s in the caprine peri-implantation period. Theriogenology *50*, 931-944.

Floyd-Smith,G., Wang,Q., and Sen,G.C. (1999). Transcriptional induction of the p69 isoform of 2',5'-oligoadenylate synthetase by interferon-beta and interferon-gamma involves three regulatory elements and interferon-stimulated gene factor 3. Exp. Cell Res. *246*, 138-147.

Fu,X.Y., Kessler,D.S., Veals,S.A., Levy,D.E., and Darnell,J.E., Jr. (1990). ISGF3, the transcriptional activator induced by interferon alpha, consists of multiple interacting polypeptide chains. Proc. Natl. Acad. Sci. U. S. A *87*, 8555-8559.

Gao,F., Chen,X.L., Wei,P., Gao,H.J., and Liu,Y.X. (2001). Expression of matrix metalloproteinase-2, tissue inhibitors of metalloproteinase-1, -3 at the implantation site of rhesus monkey during the early stage of pregnancy. Endocrine. *16*, 47-54.

Garcia,P., Nieto,A., Sanchez,M.A., Pizarro,M., and Flores,J.M. (2004). Expression of alphav, alpha4, alpha5 and beta3 integrin subunits, fibronectin and vitronectin in goat peri-implantation. Anim Reprod. Sci. *80*, 91-100.

Garofalo,R., Mei,F., Espejo,R., Ye,G., Haeberle,H., Baron,S., Ogra,P.L., and Reyes,V.E. (1996). Respiratory syncytial virus infection of human respiratory epithelial cells up-regulates class I MHC expression through the induction of IFN-beta and IL-1 alpha. J. Immunol. *157*, 2506-2513.

Geisert,R.D., Lee,C.Y., Simmen,F.A., Zavy,M.T., Fliss,A.E., Bazer,F.W., and Simmen,R.C. (1991). Expression of messenger RNAs encoding insulin-like growth factor-I, -II, and insulin-like growth factor binding protein-2 in bovine endometrium during the estrous cycle and early pregnancy. Biol. Reprod. *45*, 975-983.

Genbacev,O.D., Prakobphol,A., Foulk,R.A., Krtolica,A.R., Ilic,D., Singer,M.S., Yang,Z.Q., Kiessling,L.L., Rosen,S.D., and Fisher,S.J. (2003). Trophoblast L-selectin-mediated adhesion at the maternal-fetal interface. Science *299*, 405-408.

Gharib-Hamrouche,N., Chene,N., and Martal,J. (1995). Comparative expression of TGF-alpha and EGF genes in the ovine conceptus and uterine endometrium in the peri-implantation period. Reprod. Nutr. Dev. *35*, 291-303.

Ghosh,D., Sharkey,A.M., Charnock-Jones,D.S., Dhawan,L., Dhara,S., Smith,S.K., and Sengupta,J. (2000). Expression of vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) in conceptus and endometrium during implantation in the rhesus monkey. Mol. Hum. Reprod. *6*, 935-941.

Gjermandsen,I.M., Justesen,J., and Martensen,P.M. (2000). The interferon-induced gene ISG12 is regulated by various cytokines as the gene 6-16 in human cell lines. Cytokine *12*, 233-238.

Gluckman,P.D., Grumbach,M.M., and Kaplan,S.L. (1981). The neuroendocrine regulation and function of growth hormone and prolactin in the mammalian fetus. Endocr. Rev. *2*, 363-395.

Goffin,F., Munaut,C., Frankenne,F., Perrier,D.S., Beliard,A., Fridman,V., Nervo,P., Colige,A., and Foidart,J.M. (2003). Expression pattern of metalloproteinases and tissue inhibitors of matrix-metalloproteinases in cycling human endometrium. Biol. Reprod. *69*, 976-984.

Gray,C.A., Burghardt,R.C., Johnson,G.A., Bazer,F.W., and Spencer,T.E. (2002). Evidence that absence of endometrial gland secretions in uterine gland knockout ewes compromises conceptus survival and elongation. Reproduction. *124*, 289-300.

Gray,C.A., Taylor,K.M., Ramsey,W.S., Hill,J.R., Bazer,F.W., Bartol,F.F., and Spencer,T.E. (2001). Endometrial glands are required for preimplantation conceptus elongation and survival. Biol. Reprod. *64*, 1608-1613.

Guillomot,M. (1999). Changes in extracellular matrix components and cytokeratins in the endometrium during goat implantation. Placenta *20*, 339-345.

Guillomot,M., Flechon,J.E., and Wintenberger-Torres,S. (1981). Conceptus attachment in the ewe: an ultrastructural study. Placenta *2*, 169-182.

Haas,A.L. and Siepmann,T.J. (1997). Pathways of ubiquitin conjugation. FASEB J. *11*, 1257-1268.

Hamerman,J.A., Hayashi,F., Schroeder,L.A., Gygi,S.P., Haas,A.L., Hampson,L., Coughlin,P., Aebersold,R., and Aderem,A. (2002). Serpin 2a is induced in activated macrophages and conjugates to a ubiquitin homolog. J. Immunol. *168*, 2415-2423.

Hey,N.A., Graham,R.A., Seif,M.W., and Aplin,J.D. (1994). The polymorphic epithelial mucin MUC1 in human endometrium is regulated with maximal expression in the implantation phase. J. Clin. Endocrinol. Metab *78*, 337-342.

Hicks,B.A., Etter,S.J., Carnahan,K.G., Joyce,M.M., Assiri,A.A., Carling,S.J., Kodali,K., Johnson,G.A., Hansen,T.R., Mirando,M.A., Woods,G.L., Vanderwall,D.K., and Ott,T.L. (2003). Expression of the uterine Mx protein in cyclic and pregnant cows, gilts, and mares. J. Anim Sci. *81*, 1552-1561.

Hidai,C., Zupancic,T., Penta,K., Mikhail,A., Kawana,M., Quertermous,E.E., Aoka,Y., Fukagawa,M., Matsui,Y., Platika,D., Auerbach,R., Hogan,B.L., Snodgrass,R., and Quertermous,T. (1998). Cloning and characterization of developmental endothelial locus-1: an embryonic endothelial cell protein that binds the alphavbeta3 integrin receptor. Genes Dev. *12*, 21-33.

Hild-Petito,S., Verhage,H.G., and Fazleabas,A.T. (1994). Characterization, localization, and regulation of receptors for insulin-like growth factor I in the baboon uterus during the cycle and pregnancy. Biol. Reprod. *50*, 791-801.

Hillis,G.S. and MacLeod,A.M. (1996). Integrins and disease. Clin. Sci. (Lond) *91*, 639-650.

Hoffman,L.H., Olson,G.E., Carson,D.D., and Chilton,B.S. (1998). Progesterone and implanting blastocysts regulate Muc1 expression in rabbit uterine epithelium. Endocrinology *139*, 266-271.

Hofig,A., Michel,F.J., Simmen,F.A., and Simmen,R.C. (1991). Constitutive expression of uterine receptors for insulin-like growth factor-I during the peri-implantation period in the pig. Biol. Reprod. *45*, 533-539.

Horisberger,M.A. and Gunst,M.C. (1991). Interferon-induced proteins: identification of Mx proteins in various mammalian species. Virology *180*, 185-190.

Horne,A.W., White,J.O., Margara,R.A., Williams,R., Winston,R.M., and Lalani,E. (2001). MUC 1: a genetic susceptibility to infertility? Lancet *357*, 1336-1337.

Horvath,C.M., Stark,G.R., Kerr,I.M., and Darnell,J.E., Jr. (1996). Interactions between STAT and non-STAT proteins in the interferon-stimulated gene factor 3 transcription complex. Mol. Cell Biol. *16*, 6957-6964.

Hovnanian,A., Rebouillat,D., Mattei,M.G., Levy,E.R., Marie,I., Monaco,A.P., and Hovanessian,A.G. (1998). The human 2',5'-oligoadenylate synthetase locus is composed of three distinct genes clustered on chromosome 12q24.2 encoding the 100-, 69-, and 40-kDa forms. Genomics *52*, 267-277.

Hu,D.D., Lin,E.C., Kovach,N.L., Hoyer,J.R., and Smith,J.W. (1995). A biochemical characterization of the binding of osteopontin to integrins alpha v beta 1 and alpha v beta 5. J. Biol. Chem. *270*, 26232-26238.

Huang,G., Lu,H., Hao,A., Ng,D.C., Ponniah,S., Guo,K., Lufei,C., Zeng,Q., and Cao,X. (2004). GRIM-19, a cell death regulatory protein, is essential for assembly and function of mitochondrial complex I. Mol. Cell Biol. *24*, 8447-8456.

Huang,W.C., Taylor,S., Nguyen,T.B., Tomaszewski,J.E., Libertino,J.A., Malkowicz,S.B., and McGarvey,T.W. (2002). KIAA1096, a gene on chromosome 1q, is amplified and overexpressed in bladder cancer. DNA Cell Biol. *21*, 707-715.

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

Hurst,P.R. and Palmay,R.D. (1999). Matrix metalloproteinases and their endogenous inhibitors during the implantation period in the rat uterus. Reprod. Fertil. Dev. *11*, 395-402.

Hvarregaard,J., Andersen,M.H., Berglund,L., Rasmussen,J.T., and Petersen,T.E. (1996). Characterization of glycoprotein PAS-6/7 from membranes of bovine milk fat globules. Eur. J. Biochem. *240*, 628-636.

Illera,M.J., Cullinan,E., Gui,Y., Yuan,L., Beyler,S.A., and Lessey,B.A. (2000). Blockade of the alpha(v)beta(3) integrin adversely affects implantation in the mouse. Biol. Reprod. *62*, 1285-1290.

Illera,M.J., Lorenzo,P.L., Gui,Y.T., Beyler,S.A., Apparao,K.B., and Lessey,B.A. (2003). A role for alphavbeta3 integrin during implantation in the rabbit model. Biol. Reprod. *68*, 766-771.

Imai,K., Maeda,M., Fujiwara,H., Okamoto,N., Kariya,M., Emi,N., Takakura,K., Kanzaki,H., and Mori,T. (1992). Human endometrial stromal cells and decidual cells express cluster of differentiation (CD) 13 antigen/aminopeptidase N and CD10 antigen/neutral endopeptidase. Biol. Reprod. *46*, 328-334.

Imaizumi,T., Yagihashi,N., Hatakeyama,M., Yamashita,K., Ishikawa,A., Taima,K., Yoshida,H., Yagihashi,S., and Satoh,K. (2004). Upregulation of retinoic acid-inducible gene-I in T24 urinary bladder carcinoma cells stimulated with interferon-gamma. Tohoku J. Exp. Med. *203*, 313-318.

Imakawa,K., Anthony,R.V., Kazemi,M., Marotti,K.R., Polites,H.G., and Roberts,R.M. (1987). Interferon-like sequence of ovine trophoblast protein secreted by embryonic trophectoderm. Nature *330*, 377-379.

Ing,N.H., Francis,H., McDonnell,J.J., Amann,J.F., and Roberts,R.M. (1989). Progesterone induction of the uterine milk proteins: major secretory proteins of sheep endometrium. Biol. Reprod. *41*, 643-654.

Ing,N.H. and Roberts,R.M. (1989). The major progesterone-modulated proteins secreted into the sheep uterus are members of the serpin superfamily of serine protease inhibitors. J. Biol. Chem. *264*, 3372-3379.

Isaacs,J. and Murphy,C.R. (2003). Ultrastructural localisation of Muc-1 on the plasma membrane of uterine epithelial cells. Acta Histochem. *105*, 239-243.

Ishikawa,J., Kaisho,T., Tomizawa,H., Lee,B.O., Kobune,Y., Inazawa,J., Oritani,K., Itoh,M., Ochi,T., Ishihara,K., and . (1995). Molecular cloning and chromosomal mapping of a bone marrow stromal cell surface gene, BST2, that may be involved in pre-B-cell growth. Genomics *26*, 527-534.

Ishiwata,H., Katsuma,S., Kizaki,K., Patel,O.V., Nakano,H., Takahashi,T., Imai,K., Hirasawa,A., Shiojima,S., Ikawa,H., Suzuki,Y., Tsujimoto,G., Izaike,Y., Todoroki,J., and Hashizume,K. (2003). Characterization of gene expression profiles in early bovine pregnancy using a custom cDNA microarray. Mol. Reprod. Dev. *65*, 9-18.

Iyer,S., Holloway,D.E., Kumar,K., Shapiro,R., and Acharya,K.R. (2005). Molecular recognition of human eosinophil-derived neurotoxin (RNase 2) by placental ribonuclease inhibitor. J. Mol. Biol. *347*, 637-655.

Jedsadayanmata,A., Chen,C.C., Kireeva,M.L., Lau,L.F., and Lam,S.C. (1999). Activation-dependent adhesion of human platelets to Cyr61 and Fisp12/mouse connective tissue growth factor is mediated through integrin alpha(IIb)beta(3). J. Biol. Chem. *274*, 24321-24327.

Jin,D.F., Muffly,K.E., Okulicz,W.C., and Kilpatrick,D.L. (1988). Estrous cycle- and pregnancy-related differences in expression of the proenkephalin and proopiomelanocortin genes in the ovary and uterus. Endocrinology *122*, 1466-1471.

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

Johnson,G.A., Austin,K.J., Van Kirk,E.A., and Hansen,T.R. (1998). Pregnancy and interferon-tau induce conjugation of bovine ubiquitin cross-reactive protein to cytosolic uterine proteins. Biol. Reprod. *58*, 898-904.

Johnson,G.A., Bazer,F.W., Jaeger,L.A., Ka,H., Garlow,J.E., Pfarrer,C., Spencer,T.E., and Burghardt,R.C. (2001). Muc-1, integrin, and osteopontin expression during the implantation cascade in sheep. Biol. Reprod. *65*, 820-828.

Johnson,G.A., Burghardt,R.C., Bazer,F.W., and Spencer,T.E. (2003a). Osteopontin: roles in implantation and placentation. Biol. Reprod. *69*, 1458-1471.

Johnson,G.A., Burghardt,R.C., Joyce,M.M., Spencer,T.E., Bazer,F.W., Pfarrer,C., and Gray,C.A. (2003b). Osteopontin expression in uterine stroma indicates a

decidualization-like differentiation during ovine pregnancy. Biol. Reprod. *68*, 1951-1958.

Johnson,G.A., Burghardt,R.C., Spencer,T.E., Newton,G.R., Ott,T.L., and Bazer,F.W. (1999b). Ovine osteopontin: II. Osteopontin and alpha(v)beta(3) integrin expression in the uterus and conceptus during the periimplantation period. Biol. Reprod. *61*, 892-899.

Johnson,G.A., Spencer,T.E., Burghardt,R.C., and Bazer,F.W. (1999c). Ovine osteopontin: I. Cloning and expression of messenger ribonucleic acid in the uterus during the periimplantation period. Biol. Reprod. *61*, 884-891.

Johnstone,R.W., Kerry,J.A., and Trapani,J.A. (1998). The human interferon-inducible protein, IFI 16, is a repressor of transcription. J. Biol. Chem. *273*, 17172-17177.

Joyce,M.M., White,F.J., Burghardt,R.C., Muniz,J.J., Spencer,T.E., Bazer,F.W., and Johnson,G.A. (2005). Interferon stimulated gene 15 conjugates to endometrial cytosolic proteins and is expressed at the uterine-placental interface throughout pregnancy in sheep. Endocrinology *146*, 675-684.

Juliano,R.L. and Haskill,S. (1993). Signal transduction from the extracellular matrix. J. Cell Biol. *120*, 577-585.

Justesen,J., Hartmann,R., and Kjeldgaard,N.O. (2000). Gene structure and function of the 2'-5'-oligoadenylate synthetase family. Cell Mol. Life Sci. *57*, 1593-1612.

Kang,D.C., Gopalkrishnan,R.V., Lin,L., Randolph,A., Valerie,K., Pestka,S., and Fisher,P.B. (2004). Expression analysis and genomic characterization of human melanoma differentiation associated gene-5, mda-5: a novel type I interferon-responsive apoptosis-inducing gene. Oncogene *23*, 1789-1800.

Kao,L.C., Tulac,S., Lobo,S., Imani,B., Yang,J.P., Germeyer,A., Osteen,K., Taylor,R.N., Lessey,B.A., and Giudice,L.C. (2002). Global gene profiling in human endometrium during the window of implantation. Endocrinology *143*, 2119-2138.

Kauma,S., Matt,D., Strom,S., Eierman,D., and Turner,T. (1990). Interleukin-1 beta, human leukocyte antigen HLA-DR alpha, and transforming growth factor-beta expression in endometrium, placenta, and placental membranes. Am. J. Obstet. Gynecol. *163*, 1430-1437.

Keller,M.L., Roberts,A.J., and Seidel,G.E., Jr. (1998). Characterization of insulin-like growth factor-binding proteins in the uterus and conceptus during early conceptus elongation in cattle. Biol. Reprod. *59*, 632-642.

Kelly,J.M., Porter,A.C., Chernajovsky,Y., Gilbert,C.S., Stark,G.R., and Kerr,I.M. (1986). Characterization of a human gene inducible by alpha- and beta-interferons and its expression in mouse cells. EMBO J. *5*, 1601-1606.

Kessler,D.S., Levy,D.E., and Darnell,J.E., Jr. (1988). Two interferon-induced nuclear factors bind a single promoter element in interferon-stimulated genes. Proc. Natl. Acad. Sci. U. S. A *85*, 8521-8525.

Kim,S., Choi,Y., Bazer,F.W., and Spencer,T.E. (2003). Identification of genes in the ovine endometrium regulated by interferon tau independent of signal transducer and activator of transcription 1. Endocrinology *144*, 5203-5214.

Kimmins,S. and MacLaren,L.A. (1999). Cyclic modulation of integrin expression in bovine endometrium. Biol. Reprod. *61*, 1267-1274.

King,A. and Loke,Y.W. (1990). Uterine large granular lymphocytes: a possible role in embryonic implantation? Am. J. Obstet. Gynecol. *162*, 308-310.

Kireeva,M.L., Latinkic,B.V., Kolesnikova,T.V., Chen,C.C., Yang,G.P., Abler,A.S., and Lau,L.F. (1997). Cyr61 and Fisp12 are both ECM-associated signaling molecules: activities, metabolism, and localization during development. Exp. Cell Res. *233*, 63-77.

Kliem,A., Tetens,F., Klonisch,T., Grealy,M., and Fischer,B. (1998). Epidermal growth factor receptor and ligands in elongating bovine blastocysts. Mol. Reprod. Dev. *51*, 402-412.

Knickerbocker,J.J., Thatcher,W.W., Bazer,F.W., Drost,M., Barron,D.H., Fincher,K.B., and Roberts,R.M. (1986). Proteins secreted by day-16 to -18 bovine conceptuses extend corpus luteum function in cows. J. Reprod. Fertil. *77*, 381-391.

Knobbe,C.B., Reifenberger,J., Blaschke,B., and Reifenberger,G. (2004). Hypermethylation and transcriptional downregulation of the carboxyl-terminal modulator protein gene in glioblastomas. J. Natl. Cancer Inst. *96*, 483-486.

Kolle,S., Sinowatz,F., Boie,G., Lincoln,D., Palma,G., Stojkovic,M., and Wolf,E. (1998). Topography of growth hormone receptor expression in the bovine embryo. Histochem. Cell Biol. *109*, 417-419.

Kolle,S., Sinowatz,F., Boie,G., Lincoln,D., and Waters,M.J. (1997). Differential expression of the growth hormone receptor and its transcript in bovine uterus and placenta. Mol. Cell Endocrinol. *131*, 127-136.

Kolle,S., Stojkovic,M., Boie,G., Wolf,E., and Sinowatz,F. (2002). Growth hormone inhibits apoptosis in in vitro produced bovine embryos. Mol. Reprod. Dev. *61*, 180-186.

Kolle,S., Stojkovic,M., Prelle,K., Waters,M., Wolf,E., and Sinowatz,F. (2001). Growth hormone (GH)/GH receptor expression and GH-mediated effects during early bovine embryogenesis. Biol. Reprod. *64*, 1826-1834.

Kolle,S., Stojkovic,M., Reese,S., Reichenbach,H.D., Wolf,E., and Sinowatz,F. (2004). Effects of growth hormone on the ultrastructure of bovine preimplantation embryos. Cell Tissue Res. *317*, 101-108.

Komatsu,N., Maekawa,T., Takeuchi,S., and Takahashi,S. (2003). Epidermal growth factor and transforming growth factor-alpha stimulate the proliferation of mouse uterine stromal cells. Zoolog. Sci. *20*, 639-645.

Korgun,E.T., Dohr,G., Desoye,G., Demir,R., Kayisli,U.A., and Hahn,T. (2003). Expression of insulin, insulin-like growth factor I and glucocorticoid receptor in rat

uterus and embryo during decidualization, implantation and organogenesis. Reproduction. *125*, 75-84.

Kristelly,R., Gao,G., and Tesmer,J.J. (2004). Structural determinants of RhoA binding and nucleotide exchange in leukemia-associated Rho guanine-nucleotide exchange factor. J. Biol. Chem. *279*, 47352-47362.

Kumar,S., Li,Q., Dua,A., Ying,Y.K., Bagchi,M.K., and Bagchi,I.C. (2001). Messenger ribonucleic acid encoding interferon-inducible guanylate binding protein 1 is induced in human endometrium within the putative window of implantation. J. Clin. Endocrinol. Metab *86*, 2420-2427.

Lacroix,M.C., Devinoy,E., Cassy,S., Servely,J.L., Vidaud,M., and Kann,G. (1999). Expression of growth hormone and its receptor in the placental and feto-maternal environment during early pregnancy in sheep. Endocrinology *140*, 5587-5597.

Lasky,L.A., Singer,M.S., Dowbenko,D., Imai,Y., Henzel,W.J., Grimley,C., Fennie,C., Gillett,N., Watson,S.R., and Rosen,S.D. (1992). An endothelial ligand for L-selectin is a novel mucin-like molecule. Cell *69*, 927-938.

Leslie,M.V. and Hansen,P.J. (1991). Progesterone-regulated secretion of the serpin-like proteins of the ovine and bovine uterus. Steroids *56*, 589-597.

Lessey,B.A., Castelbaum,A.J., Buck,C.A., Lei,Y., Yowell,C.W., and Sun,J. (1994). Further characterization of endometrial integrins during the menstrual cycle and in pregnancy. Fertil. Steril. *62*, 497-506.

Lessey,B.A., Gui,Y., Apparao,K.B., Young,S.L., and Mulholland,J. (2002). Regulated expression of heparin-binding EGF-like growth factor (HB-EGF) in the human endometrium: a potential paracrine role during implantation. Mol. Reprod. Dev. *62*, 446-455.

Levy,D.E., Kessler,D.S., Pine,R., and Darnell,J.E., Jr. (1989). Cytoplasmic activation of ISGF3, the positive regulator of interferon-alpha-stimulated transcription, reconstituted in vitro. Genes Dev. *3*, 1362-1371.

Li,J. and Roberts,R.M. (1994). Interferon-tau and interferon-alpha interact with the same receptors in bovine endometrium. Use of a readily iodinatable form of recombinant interferon-tau for binding studies. J. Biol. Chem. *269*, 13544-13550.

Li,L., Chen,S., and Xing,F. (2000). [Expression of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-3 protein in the implantation window phase of endometrium]. Zhonghua Fu Chan Ke. Za Zhi. *35*, 544-546.

Li,Q., Zhang,M., Kumar,S., Zhu,L.J., Chen,D., Bagchi,M.K., and Bagchi,I.C. (2001). Identification and implantation stage-specific expression of an interferon-alpha-regulated gene in human and rat endometrium. Endocrinology *142*, 2390-2400.

Li,S., Perlman,D.M., Peterson,M.S., Burrichter,D., Avdulov,S., Polunovsky,V.A., and Bitterman,P.B. (2004). Translation initiation factor 4E blocks endoplasmic reticulum-mediated apoptosis. J. Biol. Chem. *279*, 21312-21317.

Liang,P. and Pardee,A.B. (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science *257*, 967-971.

Liang,Y., Buckley,T.R., Tu,L., Langdon,S.D., and Tedder,T.F. (2001). Structural organization of the human MS4A gene cluster on Chromosome 11q12. Immunogenetics *53*, 357-368.

Liang,Y. and Tedder,T.F. (2001). Identification of a CD20-, FcepsilonRIbeta-, and HTm4-related gene family: sixteen new MS4A family members expressed in human and mouse. Genomics *72*, 119-127.

Liaw,L., Skinner,M.P., Raines,E.W., Ross,R., Cheresh,D.A., Schwartz,S.M., and Giachelli,C.M. (1995). The adhesive and migratory effects of osteopontin are mediated via distinct cell surface integrins. Role of alpha v beta 3 in smooth muscle cell migration to osteopontin in vitro. J. Clin. Invest *95*, 713-724.

Liotta,A.S., Houghten,R., and Krieger,D.T. (1982). Identification of a beta-endorphin-like peptide in cultured human placental cells. Nature *295*, 593-595.

Liston,P., Fong,W.G., Kelly,N.L., Toji,S., Miyazaki,T., Conte,D., Tamai,K., Craig,C.G., McBurney,M.W., and Korneluk,R.G. (2001). Identification of XAF1 as an antagonist of XIAP anti-Caspase activity. Nat. Cell Biol. *3*, 128-133.

Liu,H.C., Mele,C., Catz,D., Noyes,N., and Rosenwaks,Z. (1995). Production of insulin-like growth factor binding proteins (IGFBPs) by human endometrial stromal cell is stimulated by the presence of embryos. J. Assist. Reprod. Genet. *12*, 78-87.

Liu,W.J. and Hansen,P.J. (1993). Effect of the progesterone-induced serpin-like proteins of the sheep endometrium on natural-killer cell activity in sheep and mice. Biol. Reprod. *49*, 1008-1014.

Luetteke,N.C., Qiu,T.H., Fenton,S.E., Troyer,K.L., Riedel,R.F., Chang,A., and Lee,D.C. (1999). Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development. Development *126*, 2739-2750.

Lufei,C., Ma,J., Huang,G., Zhang,T., Novotny-Diermayr,V., Ong,C.T., and Cao,X. (2003). GRIM-19, a death-regulatory gene product, suppresses Stat3 activity via functional interaction. EMBO J. *22*, 1325-1335.

Lukashev,M.E. and Werb,Z. (1998). ECM signalling: orchestrating cell behaviour and misbehaviour. Trends Cell Biol. *8*, 437-441.

Lysiak,J.J. and Lala,P.K. (1992). In situ localization and characterization of bone marrow-derived cells in the decidua of normal murine pregnancy. Biol. Reprod. *47*, 603-613.

Ma,L., Benson,G.V., Lim,H., Dey,S.K., and Maas,R.L. (1998). Abdominal B (AbdB) Hoxa genes: regulation in adult uterus by estrogen and progesterone and repression in mullerian duct by the synthetic estrogen diethylstilbestrol (DES). Dev. Biol. *197*, 141-154.

MacFarlane,M., Merrison,W., Bratton,S.B., and Cohen,G.M. (2002). Proteasome-mediated degradation of Smac during apoptosis: XIAP promotes Smac ubiquitination in vitro. J. Biol. Chem. *277*, 36611-36616.

Machida,T., Taga,M., and Minaguchi,H. (1995). Effects of epidermal growth factor and transforming growth factor alpha on the mouse trophoblast outgrowth in vitro. Eur. J. Endocrinol. *133*, 741-746.

MacIntyre,D.M., Lim,H.C., Ryan,K., Kimmins,S., Small,J.A., and MacLaren,L.A. (2002). Implantation-associated changes in bovine uterine expression of integrins and extracellular matrix. Biol. Reprod. *66*, 1430-1436.

Maira,S.M., Galetic,I., Brazil,D.P., Kaech,S., Ingley,E., Thelen,M., and Hemmings,B.A. (2001). Carboxyl-terminal modulator protein (CTMP), a negative regulator of PKB/Akt and v-Akt at the plasma membrane. Science *294*, 374-380.

Mak,Y.T., Hampson,G., Beresford,J.N., and Spector,T.D. (2004). Variations in genome-wide gene expression in identical twins - a study of primary osteoblast-like culture from female twins discordant for osteoporosis. BMC. Genet. *5*, 14.

Malakhov,M.P., Kim,K.I., Malakhova,O.A., Jacobs,B.S., Borden,E.C., and Zhang,D.E. (2003). High-throughput immunoblotting. Ubiquitiin-like protein ISG15 modifies key regulators of signal transduction. J. Biol. Chem. *278*, 16608-16613.

Malakhova,O.A., Yan,M., Malakhov,M.P., Yuan,Y., Ritchie,K.J., Kim,K.I., Peterson,L.F., Shuai,K., and Zhang,D.E. (2003). Protein ISGylation modulates the JAK-STAT signaling pathway. Genes Dev. *17*, 455-460.

Mann,G.B., Fowler,K.J., Gabriel,A., Nice,E.C., Williams,R.L., and Dunn,A.R. (1993). Mice with a null mutation of the TGF alpha gene have abnormal skin architecture, wavy hair, and curly whiskers and often develop corneal inflammation. Cell *73*, 249-261.

Markoff,E., Henemyre,C., Fellows,J., Pennington,E., Zeitler,P.S., and Cedars,M.I. (1995). Localization of insulin-like growth factor binding protein-4 expression in the mouse uterus during the peri-implantation period. Biol. Reprod. *53*, 1103-1109.

Martensen,P.M., Sogaard,T.M., Gjermandsen,I.M., Buttenschon,H.N., Rossing,A.B., Bonnevie-Nielsen,V., Rosada,C., Simonsen,J.L., and Justesen,J. (2001). The interferon alpha induced protein ISG12 is localized to the nuclear membrane. Eur. J. Biochem. *268*, 5947-5954.

Martin,J., Dominguez,F., Avila,S., Castrillo,J.L., Remohi,J., Pellicer,A., and Simon,C. (2002). Human endometrial receptivity: gene regulation. J. Reprod. Immunol. *55*, 131-139.

Martinez-Moczygemba,M., Gutch,M.J., French,D.L., and Reich,N.C. (1997). Distinct STAT structure promotes interaction of STAT2 with the p48 subunit of the interferon-alpha-stimulated transcription factor ISGF3. J. Biol. Chem. *272*, 20070-20076.

Meyer,M.D., Hansen,P.J., Thatcher,W.W., Drost,M., Badinga,L., Roberts,R.M., Li,J., Ott,T.L., and Bazer,F.W. (1995). Extension of corpus luteum lifespan and reduction

of uterine secretion of prostaglandin F2 alpha of cows in response to recombinant interferon-tau. J. Dairy Sci. *78*, 1921-1931.

Moffatt,R.J., Bazer,F.W., Roberts,R.M., and Thatcher,W.W. (1987). Secretory function of the ovine uterus: effects of gestation and steroid replacement therapy. J. Anim Sci. *65*, 1400-1410.

Montes,M.J., Aleman,P., Garcia-Tortosa,C., Borja,C., Ruiz,C., and Garcia-Olivares,E. (1996). Cultured human decidual stromal cells express antigens associated with hematopoietic cells. J. Reprod. Immunol. *30*, 53-66.

Moussad,E.E., Rageh,M.A., Wilson,A.K., Geisert,R.D., and Brigstock,D.R. (2002). Temporal and spatial expression of connective tissue growth factor (CCN2; CTGF) and transforming growth factor beta type 1 (TGF-beta1) at the utero-placental interface during early pregnancy in the pig. Mol. Pathol. *55*, 186-192.

Muller-Doblies,D., Ackermann,M., and Metzler,A. (2002). In vitro and in vivo detection of Mx gene products in bovine cells following stimulation with alpha/beta interferon and viruses. Clin. Diagn. Lab Immunol. *9*, 1192-1199.

Narasimhan,J., Potter,J.L., and Haas,A.L. (1996). Conjugation of the 15-kDa interferon-induced ubiquitin homolog is distinct from that of ubiquitin. J. Biol. Chem. *271*, 324-330.

Nardo,L.G., Bartoloni,G., Di Mercurio,S., and Nardo,F. (2002). Expression of alpha(v)beta3 and alpha4beta1 integrins throughout the putative window of implantation in a cohort of healthy fertile women. Acta Obstet. Gynecol. Scand. *81*, 753-758.

Neuvians,T.P., Pfaffl,M.W., Berisha,B., and Schams,D. (2003). The mRNA expression of insulin receptor isoforms (IR-A and IR-B) and IGFR-2 in the bovine corpus luteum during the estrous cycle, pregnancy, and induced luteolysis. Endocrine. *22*, 93-100.

Nielsen,H.L., Ronnov-Jessen,L., Villadsen,R., and Petersen,O.W. (2002). Identification of EPSTI1, a novel gene induced by epithelial-stromal interaction in human breast cancer. Genomics *79*, 703-710.

Okada,Y., Nagase,H., and Harris,E.D., Jr. (1986). A metalloproteinase from human rheumatoid synovial fibroblasts that digests connective tissue matrix components. Purification and characterization. J. Biol. Chem. *261*, 14245-14255.

Olivares,E.G., Montes,M.J., Oliver,C., Galindo,J.A., and Ruiz,C. (1997). Cultured human decidual stromal cells express B7-1 (CD80) and B7-2 (CD86) and stimulate allogeneic T cells. Biol. Reprod. *57*, 609-615.

Oshima,K., Watanabe,H., Yoshihara,K., Kojima,T., Dochi,O., Takenouchi,N., Fukushima,M., and Komatsu,M. (2003). Gene expression of leukemia inhibitory factor (LIF) and macrophage colony stimulating factor (M-CSF) in bovine endometrium during early pregnancy. Theriogenology *60*, 1217-1226.

Ott,T.L., Yin,J., Wiley,A.A., Kim,H.T., Gerami-Naini,B., Spencer,T.E., Bartol,F.F., Burghardt,R.C., and Bazer,F.W. (1998). Effects of the estrous cycle and early

pregnancy on uterine expression of Mx protein in sheep (Ovis aries). Biol. Reprod. *59*, 784-794.

Pavlovic,J., Haller,O., and Staeheli,P. (1992). Human and mouse Mx proteins inhibit different steps of the influenza virus multiplication cycle. J. Virol. *66*, 2564-2569.

Pavlovic,J., Schroder,A., Blank,A., Pitossi,F., and Staeheli,P. (1993). Mx proteins: GTPases involved in the interferon-induced antiviral state. Ciba Found. Symp. *176*, 233-243.

Perry,D.J., Austin,K.J., and Hansen,T.R. (1999). Cloning of interferon-stimulated gene 17: the promoter and nuclear proteins that regulate transcription. Mol. Endocrinol. *13*, 1197-1206.

Pine,R., Canova,A., and Schindler,C. (1994). Tyrosine phosphorylated p91 binds to a single element in the ISGF2/IRF-1 promoter to mediate induction by IFN alpha and IFN gamma, and is likely to autoregulate the p91 gene. EMBO J. *13*, 158-167.

Pollard,J.W., Bartocci,A., Arceci,R., Orlofsky,A., Ladner,M.B., and Stanley,E.R. (1987). Apparent role of the macrophage growth factor, CSF-1, in placental development. Nature *330*, 484-486.

Pollard,J.W., Hunt,J.S., Wiktor-Jedrzejczak,W., and Stanley,E.R. (1991). A pregnancy defect in the osteopetrotic (op/op) mouse demonstrates the requirement for CSF-1 in female fertility. Dev. Biol. *148*, 273-283.

Porter,A.C. and Itzhaki,J.E. (1993). Gene targeting in human somatic cells. Complete inactivation of an interferon-inducible gene. Eur. J. Biochem. *218*, 273-281.

Porter,J.C. and Hogg,N. (1998). Integrins take partners: cross-talk between integrins and other membrane receptors. Trends Cell Biol. *8*, 390-396.

Praefcke,G.J., Kloep,S., Benscheid,U., Lilie,H., Prakash,B., and Herrmann,C. (2004). Identification of residues in the human guanylate-binding protein 1 critical for nucleotide binding and cooperative GTP hydrolysis. J. Mol. Biol. *344*, 257-269.

Pru,J.K., Austin,K.J., Haas,A.L., and Hansen,T.R. (2001). Pregnancy and interferon-tau upregulate gene expression of members of the 1-8 family in the bovine uterus. Biol. Reprod. *65*, 1471-1480.

Pru,J.K., Austin,K.J., Perry,D.J., Nighswonger,A.M., and Hansen,T.R. (2000). Production, purification, and carboxy-terminal sequencing of bioactive recombinant bovine interferon-stimulated gene product 17. Biol. Reprod. *63*, 619-628.

Qin,L., Wang,Y.L., Bai,S.X., Ji,S.H., Qiu,W., Tang,S., and Piao,Y.S. (2003). Temporal and spatial expression of integrins and their extracellular matrix ligands at the maternal-fetal interface in the rhesus monkey during pregnancy. Biol. Reprod. *69*, 563-571.

Rageh,M.A., Moussad,E.E., Wilson,A.K., and Brigstock,D.R. (2001). Steroidal regulation of connective tissue growth factor (CCN2; CTGF) synthesis in the mouse uterus. Mol. Pathol. *54*, 338-346.

Rajaram,S., Baylink,D.J., and Mohan,S. (1997). Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions. Endocr. Rev. *18*, 801-831.

Rasmussen,U.B., Wolf,C., Mattei,M.G., Chenard,M.P., Bellocq,J.P., Chambon,P., Rio,M.C., and Basset,P. (1993). Identification of a new interferon-alpha-inducible gene (p27) on human chromosome 14q32 and its expression in breast carcinoma. Cancer Res. *53*, 4096-4101.

Ratnoff,O.D., Pensky,J., Ogston,D., and Naff,G.B. (1969). The inhibition of plasmin, plasma kallikrein, plasma permeability factor, and the C'1r subcomponent of the first component of complement by serum C'1 esterase inhibitor. J. Exp. Med. *129*, 315-331.

Reese,J., Das,S.K., Paria,B.C., Lim,H., Song,H., Matsumoto,H., Knudtson,K.L., DuBois,R.N., and Dey,S.K. (2001). Global gene expression analysis to identify molecular markers of uterine receptivity and embryo implantation. J. Biol. Chem. *276*, 44137-44145.

Rempel,L.A., Francis,B.R., Austin,K.J., and Hansen,T.R. (2004). Isolation and Sequence of an Interferon-Tau-Inducible, Pregnancy- and ISG15-Specific, Bovine Ubiquitin-Activating E1-Like (UBE1L) Enzyme. Biol. Reprod.

Ribas,G., Neville,M., Wixon,J.L., Cheng,J., and Campbell,R.D. (1999). Genes encoding three new members of the leukocyte antigen 6 superfamily and a novel member of Ig superfamily, together with genes encoding the regulatory nuclear chloride ion channel protein (hRNCC) and an N omega-N omega-dimethylarginine dimethylaminohydrolase homologue, are found in a 30-kb segment of the MHC class III region. J. Immunol. *163*, 278-287.

Riddick,D.H. and Kusmik,W.F. (1977). Decidua: a possible source of amniotic fluid prolactin. Am. J. Obstet. Gynecol. *127*, 187-190.

Riesewijk,A., Martin,J., van Os,R., Horcajadas,J.A., Polman,J., Pellicer,A., Mosselman,S., and Simon,C. (2003). Gene expression profiling of human endometrial receptivity on days LH+2 versus LH+7 by microarray technology. Mol. Hum. Reprod. *9*, 253-264.

Roberts,R.M. (1996). Interferon-tau and pregnancy. J. Interferon Cytokine Res. *16*, 271-273.

Robinson,R.S., Mann,G.E., Lamming,G.E., and Wathes,D.C. (1999). The effect of pregnancy on the expression of uterine oxytocin, oestrogen and progesterone receptors during early pregnancy in the cow. J. Endocrinol. *160*, 21-33.

Rosen,S.D. (1993). Robert Feulgen Lecture 1993. L-selectin and its biological ligands. Histochemistry *100*, 185-191.

Rosenfeld,C.S., Han,C.S., Alexenko,A.P., Spencer,T.E., and Roberts,R.M. (2002). Expression of interferon receptor subunits, IFNAR1 and IFNAR2, in the ovine uterus. Biol. Reprod. *67*, 847-853.

Rowe,A.J., Wulff,C., and Fraser,H.M. (2003). Localization of mRNA for vascular endothelial growth factor (VEGF), angiopoietins and their receptors during the peri-implantation period and early pregnancy in marmosets (Callithrix jacchus). Reproduction. *126*, 227-238.

Ruoslahti,E. and Pierschbacher,M.D. (1987). New perspectives in cell adhesion: RGD and integrins. Science *238*, 491-497.

Rüsse,I. and Sinowatz,F. (1998). Lehrbuch der Embryologie der Haustiere, 2. Auflage. Verlag Paul Parey, Berlin.

Sadek,S., Unterman,T.G., and Bell,S.C. (1994). Epithelial localization of insulin-like growth factor binding protein 1 in the uterus of the rat during pregnancy, deciduoma-bearing pseudopregnancy and hormone treatment. J. Reprod. Fertil. *101*, 67-75.

Sadowski,T., Dietrich,S., Koschinsky,F., and Sedlacek,R. (2003). Matrix metalloproteinase 19 regulates insulin-like growth factor-mediated proliferation, migration, and adhesion in human keratinocytes through proteolysis of insulin-like growth factor binding protein-3. Mol. Biol. Cell *14*, 4569-4580.

Salamonsen,L.A., Nagase,H., and Woolley,D.E. (1995). Matrix metalloproteinases and their tissue inhibitors at the ovine trophoblast-uterine interface. J. Reprod. Fertil. Suppl *49*, 29-37.

Sastry,B.V., Barnwell,S.L., Tayeb,O.S., Janson,V.E., and Owens,L.K. (1980). Occurrence of methionine enkephalin in human placental villus. Biochem. Pharmacol. *29*, 475-478.

Satokata,I., Benson,G., and Maas,R. (1995). Sexually dimorphic sterility phenotypes in Hoxa10-deficient mice. Nature *374*, 460-463.

Sbracia,M., Scarpellini,F., Poverini,R., Alo,P.L., Rossi,G., and Di Tondo,U. (2004). Immunohistochemical localization of the growth hormone in human endometrium and decidua. Am. J. Reprod. Immunol. *51*, 112-116.

Scallon,B.J., Fung,W.J., Tsang,T.C., Li,S., Kado-Fong,H., Huang,K.S., and Kochan,J.P. (1991). Primary structure and functional activity of a phosphatidylinositol-glycan-specific phospholipase D. Science *252*, 446-448.

Schindler,C., Fu,X.Y., Improta,T., Aebersold,R., and Darnell,J.E., Jr. (1992). Proteins of transcription factor ISGF-3: one gene encodes the 91-and 84-kDa ISGF-3 proteins that are activated by interferon alpha. Proc. Natl. Acad. Sci. U. S. A *89*, 7836-7839.

Schmitt,R.A., Geisert,R.D., Zavy,M.T., Short,E.C., and Blair,R.M. (1993). Uterine cellular changes in 2',5'-oligoadenylate synthetase during the bovine estrous cycle and early pregnancy. Biol. Reprod. *48*, 460-466.

Schwemmle,M. and Staeheli,P. (1994). The interferon-induced 67-kDa guanylate-binding protein (hGBP1) is a GTPase that converts GTP to GMP. J. Biol. Chem. *269*, 11299-11305.

Simmons,P.J. and Torok-Storb,B. (1991). Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. Blood *78*, 55-62.

Simon,A., Fah,J., Haller,O., and Staeheli,P. (1991). Interferon-regulated Mx genes are not responsive to interleukin-1, tumor necrosis factor, and other cytokines. J. Virol. *65*, 968-971.

Simon,C., Frances,A., Piquette,G.N., el,D., I, Zurawski,G., Dang,W., and Polan,M.L. (1994). Embryonic implantation in mice is blocked by interleukin-1 receptor antagonist. Endocrinology *134*, 521-528.

Simon,C., Piquette,G.N., Frances,A., Westphal,L.M., Heinrichs,W.L., and Polan,M.L. (1993). Interleukin-1 type I receptor messenger ribonucleic acid expression in human endometrium throughout the menstrual cycle. Fertil. Steril. *59*, 791-796.

Skopets,B., Li,J., Thatcher,W.W., Roberts,R.M., and Hansen,P.J. (1992). Inhibition of lymphocyte proliferation by bovine trophoblast protein-1 (type I trophoblast interferon) and bovine interferon-alpha I1. Vet. Immunol. Immunopathol. *34*, 81-96.

Skrzypczak,J., Mikolajczyk,M., and Szymanowski,K. (2001). Endometrial receptivity: expression of alpha3beta1, alpha4beta1 and alphaVbeta1 endometrial integrins in women with impaired fertility. Reprod. Biol. *1*, 85-94.

Song,J.H., Houde,A., and Murphy,B.D. (1998). Cloning of leukemia inhibitory factor (LIF) and its expression in the uterus during embryonic diapause and implantation in the mink (Mustela vison). Mol. Reprod. Dev. *51*, 13-21.

Spencer,T.E., Bartol,F.F., Bazer,F.W., Johnson,G.A., and Joyce,M.M. (1999). Identification and characterization of glycosylation-dependent cell adhesion molecule 1-like protein expression in the ovine uterus. Biol. Reprod. *60*, 241-250.

Spencer,T.E. and Bazer,F.W. (2004). Conceptus signals for establishment and maintenance of pregnancy. Reprod. Biol. Endocrinol. *2*, 49.

Spencer,T.E., Becker,W.C., George,P., Mirando,M.A., Ogle,T.F., and Bazer,F.W. (1995). Ovine interferon-tau regulates expression of endometrial receptors for estrogen and oxytocin but not progesterone. Biol. Reprod. *53*, 732-745.

Spencer,T.E., Mirando,M.A., Mayes,J.S., Watson,G.H., Ott,T.L., and Bazer,F.W. (1996). Effects of interferon-tau and progesterone on oestrogen-stimulated expression of receptors for oestrogen, progesterone and oxytocin in the endometrium of ovariectomized ewes. Reprod. Fertil. Dev. *8*, 843-853.

Spencer,T.E., Ott,T.L., and Bazer,F.W. (1998). Expression of interferon regulatory factors one and two in the ovine endometrium: effects of pregnancy and ovine interferon tau. Biol. Reprod. *58*, 1154-1162.

Staggs,K.L., Austin,K.J., Johnson,G.A., Teixeira,M.G., Talbott,C.T., Dooley,V.A., and Hansen,T.R. (1998). Complex induction of bovine uterine proteins by interferon-tau. Biol. Reprod. *59*, 293-297.

Stavreus-Evers,A., Aghajanova,L., Brismar,H., Eriksson,H., Landgren,B.M., and Hovatta,O. (2002). Co-existence of heparin-binding epidermal growth factor-like growth factor and pinopodes in human endometrium at the time of implantation. Mol. Hum. Reprod. *8*, 765-769.

Stetler-Stevenson,W.G., Krutzsch,H.C., and Liotta,L.A. (1989). Tissue inhibitor of metalloproteinase (TIMP-2). A new member of the metalloproteinase inhibitor family. J. Biol. Chem. *264*, 17374-17378.

Stewart,C.L., Kaspar,P., Brunet,L.J., Bhatt,H., Gadi,I., Kontgen,F., and Abbondanzo,S.J. (1992). Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. Nature *359*, 76-79.

Stewart,D.M., Johnson,G.A., Vyhlidal,C.A., Burghardt,R.C., Safe,S.H., Yu-Lee,L.Y., Bazer,F.W., and Spencer,T.E. (2001). Interferon-tau activates multiple signal transducer and activator of transcription proteins and has complex effects on interferon-responsive gene transcription in ovine endometrial epithelial cells. Endocrinology *142*, 98-107.

Stewart,H.J., McCann,S.H., Barker,P.J., Lee,K.E., Lamming,G.E., and Flint,A.P. (1987). Interferon sequence homology and receptor binding activity of ovine trophoblast antiluteolytic protein. J. Endocrinol. *115*, R13-R15.

Stewart,H.J., McCann,S.H., Northrop,A.J., Lamming,G.E., and Flint,A.P. (1989). Sheep antiluteolytic interferon: cDNA sequence and analysis of mRNA levels. J. Mol. Endocrinol. *2*, 65-70.

Stewart,M.D., Johnson,G.A., Gray,C.A., Burghardt,R.C., Schuler,L.A., Joyce,M.M., Bazer,F.W., and Spencer,T.E. (2000). Prolactin receptor and uterine milk protein expression in the ovine endometrium during the estrous cycle and pregnancy. Biol. Reprod. *62*, 1779-1789.

Stojkovic,M., Westesen,K., Zakhartchenko,V., Stojkovic,P., Boxhammer,K., and Wolf,E. (1999). Coenzyme Q(10) in submicron-sized dispersion improves development, hatching, cell proliferation, and adenosine triphosphate content of in vitro-produced bovine embryos. Biol. Reprod. *61*, 541-547.

Strowitzki,T., Singer,G.A., Rettig,I., and Capp,E. (1996). Characterization of receptors for insulin-like growth factor type I on cultured human endometrial stromal cells: downregulation by progesterone. Gynecol. Endocrinol. *10*, 229-240.

Sugino,N., Kashida,S., Karube-Harada,A., Takiguchi,S., and Kato,H. (2002). Expression of vascular endothelial growth factor (VEGF) and its receptors in human endometrium throughout the menstrual cycle and in early pregnancy. Reproduction. *123*, 379-387.

Sun,X.Y., Li,F.X., Li,J., Tan,Y.F., Piao,Y.S., Tang,S., and Wang,Y.L. (2004). Determination of genes involved in the early process of embryonic implantation in rhesus monkey (Macaca mulatta) by suppression subtractive hybridization. Biol. Reprod. *70*, 1365-1373.

Surveyor,G.A., Gendler,S.J., Pemberton,L., Das,S.K., Chakraborty,I., Julian,J., Pimental,R.A., Wegner,C.C., Dey,S.K., and Carson,D.D. (1995). Expression and steroid hormonal control of Muc-1 in the mouse uterus. Endocrinology *136*, 3639-3647.

Surveyor,G.A., Wilson,A.K., and Brigstock,D.R. (1998). Localization of connective tissue growth factor during the period of embryo implantation in the mouse. Biol. Reprod. *59*, 1207-1213.

Tahara E Jr, Tahara,H., Kanno,M., Naka,K., Takeda,Y., Matsuzaki,T., Yamazaki,R., Ishihara,H., Yasui,W., Barrett,J.C., Ide,T., and Tahara,E. (2005). G1P3, an interferon inducible gene 6-16, is expressed in gastric cancers and inhibits mitochondrial-mediated apoptosis in gastric cancer cell line TMK-1 cell. Cancer Immunol. Immunother.

Takeyama,K., Aguiar,R.C., Gu,L., He,C., Freeman,G.J., Kutok,J.L., Aster,J.C., and Shipp,M.A. (2003). The BAL-binding protein BBAP and related Deltex family members exhibit ubiquitin-protein isopeptide ligase activity. J. Biol. Chem. *278*, 21930-21937.

Tamada,H., Kai,Y., and Mori,J. (1994). Epidermal growth factor--induced implantation and decidualization in the rat. Prostaglandins *47*, 467-475.

Tamada,H., Sakamoto,M., Sakaguchi,H., Inaba,T., and Sawada,T. (1997). Evidence for the involvement of transforming growth factor-alpha in implantation in the rat. Life Sci. *60*, 1515-1522.

Tamada,H., Tsubutani,D., Kawate,N., Inaba,T., Matsuyama,S., Imakawa,K., Sakai,S., Christenson,R.K., and Sawada,T. (2002). Detection of transforming growth factor-alpha and epidermal growth factor receptor mRNA and immunohistochemical localization of their proteins in the ovine uterus during the early implantation period. Histochem. J. *34*, 383-390.

Tamura,K., Hara,T., Kutsukake,M., Iwatsuki,K., Yanagida,M., Yoshie,M., and Kogo,H. (2004). Expression and the biological activities of insulin-like growth factor-binding protein related protein 1 in rat uterus during the periimplantation period. Endocrinology *145*, 5243-5251.

Tan,Y.F., Li,F.X., Piao,Y.S., Sun,X.Y., and Wang,Y.L. (2003). Global gene profiling analysis of mouse uterus during the oestrous cycle. Reproduction. *126*, 171-182.

Tanaka,T.S., Jaradat,S.A., Lim,M.K., Kargul,G.J., Wang,X., Grahovac,M.J., Pantano,S., Sano,Y., Piao,Y., Nagaraja,R., Doi,H., Wood,W.H., III, Becker,K.G., and Ko,M.S. (2000). Genome-wide expression profiling of mid-gestation placenta and embryo using a 15,000 mouse developmental cDNA microarray. Proc. Natl. Acad. Sci. U. S. A *97*, 9127-9132.

Tang,J.P., Gu,Y.Y., Shen,N., Ye,S., Qian,J., Hua,J., Chen,X.G., Chen,S.L., Bao,C.D., Wang,Y., and Zhang,W. (2004). [Interferon-inducible genes lymphocyte antigen 6 complex E and tetratricopeptide repeats 1 are correlated with clinical features of patients with systemic lupus erythematosus]. Zhonghua Yi. Xue. Za Zhi. *84*, 1157-1160.

Tarantino,S., Verhage,H.G., and Fazleabas,A.T. (1992). Regulation of insulin-like growth factor-binding proteins in the baboon (Papio anubis) uterus during early pregnancy. Endocrinology *130*, 2354-2362.

Taylor,H.S. (2004). Endometrial cells derived from donor stem cells in bone marrow transplant recipients. JAMA *292*, 81-85.

Taylor,H.S., Arici,A., Olive,D., and Igarashi,P. (1998). HOXA10 is expressed in response to sex steroids at the time of implantation in the human endometrium. J. Clin. Invest *101*, 1379-1384.

Taylor,M.R., Couto,J.R., Scallan,C.D., Ceriani,R.L., and Peterson,J.A. (1997). Lactadherin (formerly BA46), a membrane-associated glycoprotein expressed in human milk and breast carcinomas, promotes Arg-Gly-Asp (RGD)-dependent cell adhesion. DNA Cell Biol. *16*, 861-869.

Teixeira,M.G., Austin,K.J., Perry,D.J., Dooley,V.D., Johnson,G.A., Francis,B.R., and Hansen,T.R. (1997). Bovine granulocyte chemotactic protein-2 is secreted by the endometrium in response to interferon-tau (IFN-tau). Endocrine. *6*, 31-37.

Tekin,S. and Hansen,P.J. (2002). Natural killer-like cells in the sheep: functional characterization and regulation by pregnancy-associated proteins. Exp. Biol. Med. (Maywood.) *227*, 803-811.

Teufel,M., Saudek,V., Ledig,J.P., Bernhardt,A., Boularand,S., Carreau,A., Cairns,N.J., Carter,C., Cowley,D.J., Duverger,D., Ganzhorn,A.J., Guenet,C., Heintzelmann,B., Laucher,V., Sauvage,C., and Smirnova,T. (2003). Sequence identification and characterization of human carnosinase and a closely related non-specific dipeptidase. J. Biol. Chem. *278*, 6521-6531.

Thanos,C.D., Goodwill,K.E., and Bowie,J.U. (1999). Oligomeric structure of the human EphB2 receptor SAM domain. Science *283*, 833-836.

Thatcher,W.W., Meyer,M.D., and Danet-Desnoyers,G. (1995). Maternal recognition of pregnancy. J. Reprod. Fertil. Suppl *49*, 15-28.

Todd,I., McElveen,J.E., and Lamming,G.E. (1998). Ovine trophoblast interferon enhances MHC class I expression by sheep endometrial cells. J. Reprod. Immunol. *37*, 117-123.

Trinchieri,G. (1989). Biology of natural killer cells. Adv. Immunol. *47*, 187-376.

Troyer,K.L., Luetteke,N.C., Saxon,M.L., Qiu,T.H., Xian,C.J., and Lee,D.C. (2001). Growth retardation, duodenal lesions, and aberrant ileum architecture in triple null mice lacking EGF, amphiregulin, and TGF-alpha. Gastroenterology *121*, 68-78.

Tsujita,K., Itoh,T., Ijuin,T., Yamamoto,A., Shisheva,A., Laporte,J., and Takenawa,T. (2004). Myotubularin regulates the function of the late endosome through the gram domain-phosphatidylinositol 3,5-bisphosphate interaction. J. Biol. Chem. *279*, 13817-13824.

Ulbrich,S.E., Schoenfelder,M., Thoene,S., and Einspanier,R. (2004). Hyaluronan in the bovine oviduct--modulation of synthases and receptors during the estrous cycle. Mol. Cell Endocrinol. *214*, 9-18.

Ullrich,A. and Schlessinger,J. (1990). Signal transduction by receptors with tyrosine kinase activity. Cell *61*, 203-212.

Uzumcu,M., Homsi,M.F., Ball,D.K., Coskun,S., Jaroudi,K., Hollanders,J.M., and Brigstock,D.R. (2000). Localization of connective tissue growth factor in human uterine tissues. Mol. Hum. Reprod. *6*, 1093-1098.

Vallet,J.L., Bazer,F.W., Fliss,M.F., and Thatcher,W.W. (1988). Effect of ovine conceptus secretory proteins and purified ovine trophoblast protein-1 on interoestrous interval and plasma concentrations of prostaglandins F-2 alpha and E and of 13,14-dihydro-15-keto prostaglandin F-2 alpha in cyclic ewes. J. Reprod. Fertil. *84*, 493-504.

van der Bliek,A.M. (1999). Functional diversity in the dynamin family. Trends Cell Biol. *9*, 96-102.

Veals,S.A., Santa,M.T., and Levy,D.E. (1993). Two domains of ISGF3 gamma that mediate protein-DNA and protein-protein interactions during transcription factor assembly contribute to DNA-binding specificity. Mol. Cell Biol. *13*, 196-206.

Veals,S.A., Schindler,C., Leonard,D., Fu,X.Y., Aebersold,R., Darnell,J.E., Jr., and Levy,D.E. (1992). Subunit of an alpha-interferon-responsive transcription factor is related to interferon regulatory factor and Myb families of DNA-binding proteins. Mol. Cell Biol. *12*, 3315-3324.

Velculescu,V.E., Zhang,L., Vogelstein,B., and Kinzler,K.W. (1995). Serial analysis of gene expression. Science *270*, 484-487.

Vogiagis,D., Fry,R.C., Sandeman,R.M., and Salamonsen,L.A. (1997a). Leukaemia inhibitory factor in endometrium during the oestrous cycle, early pregnancy and in ovariectomized steroid-treated ewes. J. Reprod. Fertil. *109*, 279-288.

Vogiagis,D., Salamonsen,L.A., Sandeman,R.M., Squires,T.J., Butt,A.R., and Fry,R.C. (1997b). Effect of immunisation against leukaemia inhibitory factor on the establishment of pregnancy in sheep. Reprod. Nutr. Dev. *37*, 459-468.

Wan,Y.J., Orrison,B.M., Lieberman,R., Lazarovici,P., and Ozato,K. (1987). Induction of major histocompatibility class I antigens by interferons in undifferentiated F9 cells. J. Cell Physiol *130*, 276-283.

Wang,H., Li,Q., Lin,H., Yu,X., Qian,D., Dai,J., Duan,E., and Zhu,C. (2003). Expression of vascular endothelial growth factor and its receptors in the rhesus monkey (Macaca mulatta) endometrium and placenta during early pregnancy. Mol. Reprod. Dev. *65*, 123-131.

Wathelet,M., Moutschen,S., Defilippi,P., Cravador,A., Collet,M., Huez,G., and Content,J. (1986). Molecular cloning, full-length sequence and preliminary characterization of a 56-kDa protein induced by human interferons. Eur. J. Biochem. *155*, 11-17.

Wathelet,M.G., Clauss,I.M., Content,J., and Huez,G.A. (1988). The IFI-56K and IFI-54K interferon-inducible human genes belong to the same gene family. FEBS Lett. *231*, 164-171.

Wathes,D.C. and Wooding,F.B. (1980). An electron microscopic study of implantation in the cow. Am. J. Anat. *159*, 285-306.

Welter,H., Wollenhaupt,K., Tiemann,U., and Einspanier,R. (2003). Regulation of the VEGF-system in the endometrium during steroid-replacement and early pregnancy of pigs. Exp. Clin. Endocrinol. Diabetes *111*, 33-40.

Wolf,E., Arnold,G.J., Bauersachs,S., Beier,H.M., Blum,H., Einspanier,R., Frohlich,T., Herrler,A., Hiendleder,S., Kolle,S., Prelle,K., Reichenbach,H.D., Stojkovic,M., Wenigerkind,H., and Sinowatz,F. (2003). Embryo-maternal communication in bovine - strategies for deciphering a complex cross-talk. Reprod. Domest. Anim *38*, 276-289.

Wu,R.J. and Zhou,F.Z. (2004). [Insulin-like growth factor II and its receptor gene expression in the endometrium of women with unexplained infertility]. Zhonghua Fu Chan Ke. Za Zhi. *39*, 242-245.

Xu,C.F., Brown,M.A., Nicolai,H., Chambers,J.A., Griffiths,B.L., and Solomon,E. (1997). Isolation and characterisation of the NBR2 gene which lies head to head with the human BRCA1 gene. Hum. Mol. Genet. *6*, 1057-1062.

Yamada,O., Todoroki,J., Takahashi,T., and Hashizume,K. (2002). The dynamic expression of extracellular matrix in the bovine endometrium at implantation. J. Vet. Med. Sci. *64*, 207-214.

Yang,Y., Lee,J.H., Kim,K.Y., Song,H.K., Kim,J.K., Yoon,S.R., Cho,D., Song,K.S., Lee,Y.H., and Choi,I. (2005). The interferon-inducible 9-27 gene modulates the susceptibility to natural killer cells and the invasiveness of gastric cancer cells. Cancer Lett. *221*, 191-200.

Yang,Z.M., Le,S.P., Chen,D.B., and Harper,M.J. (1994). Temporal and spatial expression of leukemia inhibitory factor in rabbit uterus during early pregnancy. Mol. Reprod. Dev. *38*, 148-152.

Yarden,Y. (2001). The EGFR family and its ligands in human cancer. signalling mechanisms and therapeutic opportunities. Eur. J. Cancer *37 Suppl 4*, S3-S8.

Ye,S., Pang,H., Gu,Y.Y., Hua,J., Chen,X.G., Bao,C.D., Wang,Y., Zhang,W., Qian,J., Tsao,B.P., Hahn,B.H., Chen,S.L., Rao,Z.H., and Shen,N. (2003). Protein interaction for an interferon-inducible systemic lupus associated gene, IFIT1. Rheumatology. (Oxford) *42*, 1155-1163.

Yie,S.M., Balakier,H., Motamedi,G., and Librach,C.L. (2005). Secretion of human leukocyte antigen-G by human embryos is associated with a higher in vitro fertilization pregnancy rate. Fertil. Steril. *83*, 30-36.

Yoo,H.J., Barlow,D.H., and Mardon,H.J. (1997). Temporal and spatial regulation of expression of heparin-binding epidermal growth factor-like growth factor in the human endometrium: a possible role in blastocyst implantation. Dev. Genet. *21*, 102-108.

Yoshioka,K., Matsuda,F., Takakura,K., Noda,Y., Imakawa,K., and Sakai,S. (2000). Determination of genes involved in the process of implantation: application of GeneChip to scan 6500 genes. Biochem. Biophys Res. Commun. *272*, 531-538.

Yue,Z.P., Yang,Z.M., Li,S.J., Wang,H.B., and Harper,M.J. (2000). Epidermal growth factor family in rhesus monkey uterus during the menstrual cycle and early pregnancy. Mol. Reprod. Dev. *55*, 164-174.

Zhao,S.H., Simmons,D.G., Cross,J.C., Scheetz,T.E., Casavant,T.L., Soares,M.B., and Tuggle,C.K. (2004). PLET1 (C11orf34), a highly expressed and processed novel gene in pig and mouse placenta, is transcribed but poorly spliced in human. Genomics *84*, 114-125.

Zhao,Y.G., Xiao,A.Z., Cao,X.M., and Zhu,C. (2002). Expression of matrix metalloproteinase -2, -9 and tissue inhibitors of metalloproteinase -1, -2, -3 mRNAs in rat uterus during early pregnancy. Mol. Reprod. Dev. *62*, 149-158.

Zhou,J. and Bondy,C. (1992). Insulin-like growth factor-II and its binding proteins in placental development. Endocrinology *131*, 1230-1240.

Zhu,Y. and Pintar,J.E. (1998). Expression of opioid receptors and ligands in pregnant mouse uterus and placenta. Biol. Reprod. *59*, 925-932.

Acknowledgments

I would like to thank Prof. Dr. Eckhard Wolf for allowing me to carry out this research work at the Gene Center of the Ludwig-Maximillians-University of Munich and for critically reviewing this manuscript.

I am very grateful to Dr. Stefan Bauersachs who mentored the execution of this work, including reviewing this manuscript.

Thanks to Dr. Helmut Blum for allowing me to work in his laboratory and also to all members of the lab, especially to Karin Gross.

I am indebted to Prof. Dr. Dr. Sinowatz and Margarete Vermehren for preparing the *in situ* hybridization experiments, as well as to Dr. Susanne Ulbrich for carrying out the real-time RT-PCR experiments.

I wish to express my sincere thanks to Dr. Horst Reichenbach for excellent animal management and to Dr. Susanne Schmidt for her decisive support in collection of endometrial tissue samples.

The financial support from the H. Wilhelm Schaumann Stiftung and the Deutsche Forschungsgemeinschaft (Research Unit “Mechanisms of embryo-maternal communication”; FOR 478/1) is deeply acknowledged.

I am particular grateful to my parents for their financial suport all the years.

Curriculum vitae

Personal data

name:	Claudia Klein
birthday:	september 04, 1978
birthplace:	Wiesbaden
nationality:	german
religion:	various denominations
maritial status:	single

Education

1985 – 1989	primary school (Rudolf-Dietz-Schule) in Naurod
1989 - 1998	high school (Gutenberg-Gymnasium) in Wiesbaden
1998 – 2004	Justus-Liebig-University, Faculty of Veterinary Medicine, Giessen
2001	honoring for the best exam (Physikum)
april 2004	approbation as veterinarian
april 2004 – june 2005	postgraduate at the Institute of Molecular Animal Breeding and Biotechnology working at “Identification of genes induced by the conceptus in the bovine endometrium during the pre-implantation period”
2005	honoring for the best exam (Staatsexamen)
since july 2005	intern at the clinic for obstetric, gynecology and andrology of small and large animals with veterinary outpatients care, Justus-Liebig-University, Giessen