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Oviductal and endometrial mRNA expression of implantation candidate biomarkers during early pregnancy in rabbit

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Summary

Prenatal losses are a complex problem. Pregnancy requires orchestrated communication between the embryo and the uterus that includes secretions from the embryo to signal pregnancy recognition and secretion and remodelling from the uterine epithelium. Most of these losses are characterized by asynchronization between embryo and uterus. To better understand possible causes, an analysis was conducted of gene expression of a set of transcripts related to maternal recognition and establishment of rabbit pregnancy (uteroglobin, *SCGB1A1*; integrin α_1, *ITGA1*; interferon-γ, *IFNG*; vascular endothelial growth factor, *VEGF*) in oviduct and uterine tissue at 16, 72 or 144 h post-ovulation and insemination. In the oviduct tissue, a significant decrease in the level of *SCGB1A1* mRNA expression was observed from 144 h post-ovulation. In the case of *ITGA1,* the transcript abundance was initially lower, but mRNA expression increased significantly at 72 and 144 h post-ovulation. For *IFNG,* a huge decrease was observed from 16 to 72 h post-ovulation. Finally, no significant differences were observed in the *VEGF* transcript. For the endometrium, the results showed a significant decline in the level of *SCGB1A1* mRNA expression from 16 to 144 h post-ovulation induction. The highest levels of *ITGA1* transcript were detected at 144 h, followed by the 16 h group and lower at 72 h post-ovulation. For *IFNG* there were no significant differences among post-ovulation induction times. Finally, it was possible to observe that *VEGF* mRNA abundance was present at low levels at 16 h post-ovulation and remained low at 72 h, but increased at 144 h. The functional significance of these observations may provide new insights into the maternal role in prenatal losses.

Keywords: Endometrium, Implantation biomarkers, Oviduct, Rabbit.

Introduction

Determination of the genetic basis of prenatal survival or the genetic or environmental causes of prenatal losses is a complex problem. Most losses are characterized by asynchronization between embryo and uterus that leads to problems in the process of implantation and/or placentation. The successful establishment and maintenance of pregnancy requires orchestrated communication between embryo and uterus that includes secretions from the embryo to signal pregnancy recognition and secretion and remodelling from the uterine epithelium to support attachment, development, and growth of the embryo.

Attachment of the embryo to the maternal endometrium is considered to be an active process facilitated by the attainment of a period of uterine receptivity. This interval, known as the implantation window, was first suggested by McLaren & Michie (1954). Subsequent comparative studies refined this concept and, in some instances, the differences between species have been very informative (Psychoyos, 1986; Enders, 1994; Weitlauf, 1994). Several key maternal factors that may contribute to maximal uterine receptivity have been identified: ultrastructural components such

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as pinopodes (Psychoyos & Nikas, 1994); steroids or cytokines and growth factors (Pollard *et al.*, 1991; Stewart *et al.*, 1992; Fukuda *et al.*, 1995, Zhu *et al.*, 1998; Hoffman *et al.*, 1998).

Rabbits are good experimental models in embryology and developmental biology because of their reproductive characteristics. The precise timing of ovulation (8–10 h after induction) is advantageous for documenting the moment of embryo development, apposition and attachment (Yang & Foote, 1987; Hoffman *et al.*, 1998); several biochemical markers have been described that define the period of receptivity in this species (Denker 1977; Winterhager *et al.*, 1994). In addition, the points of blastocyst attachment to the uterine epithelium are unique structures, known as trophoblastic knobs, and are readily identifiable during early pregnancy (Enders & Schlafke, 1971). This animal model has been studied to examine the expression of several endometrial biomarkers during implantation, such as uteroglobin (Krishnan & Daniel, 1967; Beier 1968), MUC-1 (Hoffman *et al.*, 1998), *VEGF* (Das *et al.*, 1997), integrin (Illera *et al.*, 2003) or cytokines (Muscettola *et al.*, 2003).

In rabbit, uteroglobin (*SCGB1A1*) comprises 40–60% of the total protein from the histotroph uterine secretion on day 5 of pregnancy. These high levels observed close to early events of implantation (Krishnan & Daniel, 1967; Beier 1968) are induced by the progressive increase of progesterone levels together with the decreasing levels of oestrogens in this period (Kopu *et al.*, 1979; Chandra *et al.*, 1980; Muller & Beato, 1980; Snead *et al.*, 1981, Shen *et al.*, 1983).

Vascular endothelial growth factor (*VEGF*) is considered to be a potent promoter of vascular endothelial cell proliferation, microvascular endothelial cell proliferation and migration associated with neovascularization in implantation, embryogenesis, corpus luteus development, ovarian follicle development and tumorigenesis (Chakraborty *et al.*, 1995; Ferrara & Davis-Smyth, 1997; Artini *et al.*, 2008). The expression of *VEGF* in uterine tissue has been detected in many species, including in rabbit at the sixth day of gestation (Llobat *et al.*, 2012a).

Integrins are a major class of cell adhesion molecules. Both constitutive and cyclical expression of integrins has been observed in the uterus, and they are now considered to be the most decisive criteria for determining uterine receptivity (Lessey *et al.*, 1996). Apical localization of $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins in the mouse, human, baboon, rabbit, pig and sheep luminal epithelium makes these specific integrin pairs appropriate candidates for mediating trophoblast/epithelial interactions (Bowen *et al.*, 1996; Lessey *et al.*, 1996; Fazleabas *et al.*, 1997; Burghardt *et al.*, 2002; Illera *et al.*, 2003). Moreover, the $\alpha_V\beta_3$ integrin has also been shown on the surface of the blastocyst (Sutherland *et al.*, 1993), so a reciprocal and cooperative role in attachment is suggested.

Maternal-embryonic recognition is mainly related to the expression of different cytokines in various species (Sharkey, 1998). Embryos synthesize factors that stimulate the production of cytokines and prevent local activation of cytotoxic cells. Some of these cytokines are interferons (IFN) that have been linked to pregnancy recognition (IFN-α in pigs, IFN-α and IFN-β in humans, ω48 and IFN-γ in rabbits or IFN-τ in ruminants) (Cross & Roberts, 1989; Aboagye-Mathiesen *et al.*, 1995; Muscettola *et al.*, 2003; Spencer *et al.*, 2004). Moreover, IFNs are involved in the angiogenesis process and the activation of natural killer cells (IFN-α in mouse or IFN-γ in rabbits) (Krusche *et al.*, 2002; Murata *et al.*, 2005; Godornes *et al.*, 2007).

The aim of the present study was to evaluate the mRNA expression of a set of transcripts related to maternal recognition and the establishment of early rabbit pregnancy (uteroglobin, *SCGB1A1*; integrin α_1, *ITGA1*; interferon-γ, *IFNG*; vascular endothelial growth factor, VEGF) in oviduct and uterine tissue at 16, 72 or 144 h post-ovulation.

Materials and methods

Animals

Twenty four nulliparous does belonging to the New Zealand White line from the ICTA at the Polytechnic University of Valencia (UPV, Spain) were used to obtain preimplantation oviduct and uterus tissues. All experimental procedures involving animals were approved by the Research Ethics Committee of the UPV and licensed by the *European Community Directive 86/609/EC*.

Donor females were inseminated with 0.5 ml of fresh heterospermic pool semen at a rate of 40×10^6 spermatozoa/ml in Tris–citric–glucose extender (Viudes-De-Castro & Vicente, 1997). Motility was examined at room temperature under a microscope with phase-contrast optics at ×40 magnitude. Only those ejaculates with >70% motile sperm (minimum requirements commonly used in artificial insemination) were pooled (Marco-Jiménez *et al.*, 2010). Immediately after insemination, ovulation was induced by an intramuscular injection of 1 μg buserelin acetate

Oviduct and uterus tissue recovery

Eight samples from both tissues (oviduct and uterus) were recovered for each experimental group. Donor does were slaughtered at 16, 72 or 144 h after

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insemination and induction of ovulation. Oviduct and uterine samples were obtained by gently scraping from the ampulla section and endometrium and plunged into Trizol reagent (Invitrogen S.A, Barcelona, Spain).

RNA extraction and reverse transcription

Total RNA was extracted using the traditional phenol/chloroform extraction method by sonication of samples in Trizol reagent (Invitrogen S.A, Barcelona, Spain). To prevent DNA contamination, one deoxyribonuclease treatment step (gDNA Wipeout Buffer, Qiagen Iberia S.L., Madrid, Spain) was performed from total RNA (1000 ng). Afterwards, reverse transcription was carried out using a Reverse Transcriptase (RT) Quantitect kit (Qiagen Iberia S.L.) according to the manufacturer's instructions.

SYBR® Green assay (quantitative real-time polymerase chain reactions)

Real-time PCR were conducted in an Applied Biosystems 7500 PCR system (Applied Biosystems, Foster City, CA, USA). Every PCR was performed from 5 μl diluted 1:40 cDNA template, 250 nM of forward and reverse specific primers (Table 1) and 10 μl of PowerSYBR Green PCR Master Mix (Fermentas GMBH, Madrid, Spain) in a final volume of 20 μl. The PCR protocol included an initial step of 50 °C (2 min), followed by 95 °C (10 min) and 42 cycles of 95 °C (15 s) and 60 °C (60 s). After real-time PCR, a melting curve analysis was performed by slowly increasing the temperature from 65–95 °C, with continuous recording of changes in fluorescent emission intensity. The amplification products were confirmed by SYBR Green-stained 2% agarose gel electrophoresis in 1× bionic buffer. Serial dilutions of cDNA pool made from several samples were done to assess PCR efficiency. A $^{\Delta\Delta}C_t$ method adjusted for PCR efficiency was used (Weltzien *et al.* 2005), employing the geometric average of *H2AFZ* (H2A histone family member Z) and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) as housekeeping normalization factor. Target and reference genes in unknown samples were run in duplicate. The expression of a cDNA pool from various samples was used as a calibrator to normalize all samples within one PCR run or between several runs.

Statistical analysis

After data normalization by logarithm transformation, the differences in mRNA expression among different post-ovulation induction times in both tissues (oviduct or uterus) were analysed by one-way analysis of variance (ANOVA), using the General Linear Models

Table 1 Information on primers used for qRT-PCR

Gene symbol	Accession number	Forward primer	Reverse primer	Fragment (bp)	Efficiency (%)	R^2
H2AFZ	AF030235	AGAGCCGGCTGCCAGTTCC	CAGTCGCGCCCACACGTCC	85	98.4	0.99
GAPDH	L23961	GCCGCTTCTTCTCGTGCAG	ATGGATCATTGATGGCGACAACAT	144	94.8	0.99
VEGF	AY196796	CTACCTCCACCATGCCAAGT	CACACTCCAGGCTTTCATCA	236	96.5	0.99
SCGB1A1	ENSOCUT00000014246	CCAGTTACGAGACATCCCTGA	CATACACAGTGGGCTCTTCACT	155	96.5	0.99
ITGA1	ENSOCUT00000011375	GCCTGTTCTTGATGATTCTCTACC	GCATCTTTCCTTGTTTCCACAG	81	97.5	0.99
IFNG	NM_001081991	GTCTGCATTCTGAGCCACTG	ATTCAGGGGCAGTCACAGTT	151	96.9	0.99

GAPDH: glyceraldehyde-3-phosphate dehydrogenase (Navarrete-Santos *et al.*, 2008); *H2AFZ*: H2A histone family member Z (Mamo *et al.*, 2008); *IFNG*: interferon-γ (Llobat *et al.*, 2012*a*); *ITGA1*: integrin α_1 (Saenz-de-Juano *et al.*, 2012); *SCGB1A1*: uteroglobin (Saenz-de-Juano *et al.*, 2012); *VEGF*: vascular endothelial growth factor (Saenz-de-Juano *et al.*, 2011).

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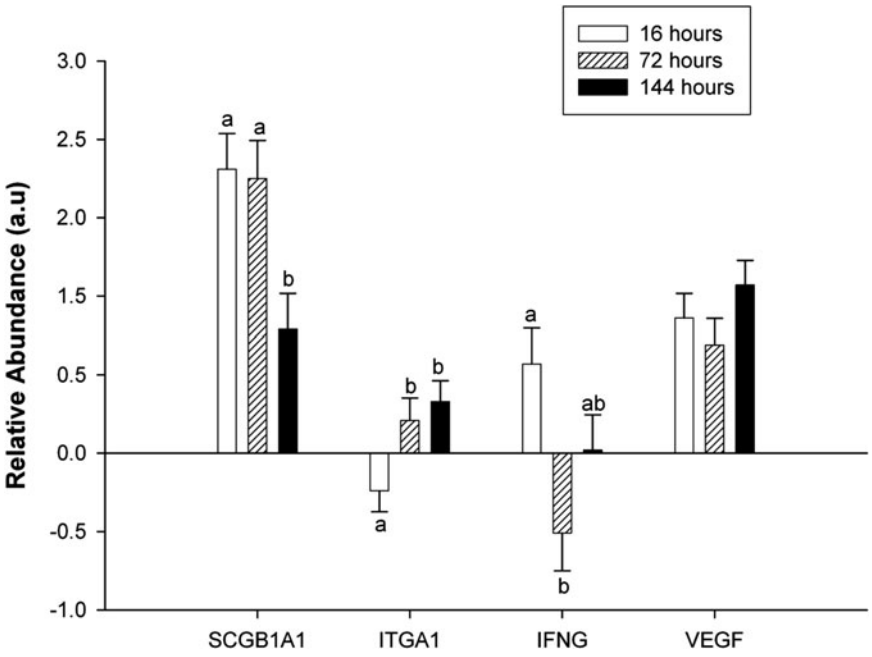

Figure 1 Relative abundance of *SCGB1A1* (uteroglobin), *ITGA1* (integrin α_1), *IFNG* (interferon-γ) and *VEGF* (vascular endothelial growth factor) mRNA expression for oviduct tissue in 16 h, 72 h and 144 h post-ovulation induction. Relative abundance values are expressed in arbitrary units (a.u.), showing the mean value ± standard error of the mean (SEM). [a,b]Different letters represent significant differences.

(GLM) procedure of Statgraphics Plus 5.1. Significance was taken as a *P*-value <0.05.

Results

The relative transcript abundance of *SCGB1A1*, *VEGF*, *ITGA1*, and *IFNG* for oviduct and uterine tissues among different post-ovulation induction times are shown in Figs 1 and 2, respectively.

In the oviduct tissue, a significant decrease in the level of *SCGB1A1* mRNA expression was observed from 72 to 144 h post-ovulation. In the case of *ITGA1,* the transcript abundance was lowest at 16 h post-ovulation, but the mRNA expression increased significantly at 72 and 144 h. For *IFNG*, a huge decrease was observed from 16 to 72 h post-ovulation, but this mRNA expression did not remain low and increased at 144 h. Finally, no significant differences were observed in *VEGF* transcript abundance between experimental days (Fig. 1).

For uterine tissue, the current results showed a significant decline in the level of *SCGB1A1* mRNA expression from 16 to 72 h post-ovulation induction. The highest levels of *ITGA1* transcript were detected at 144 h, followed by 72 h. In the case of *IFNG*, the mRNA expression pattern was similar to oviduct tissue, and a decrease was observed from 16 to 72 h post-ovulation followed by an increase at 144 h. Finally, it was possible to observe that *VEGF* mRNA abundance was present at low levels at 16 h post-ovulation and remained low at 72 h, but the level increased at 144 h (Fig. 2).

Discussion

In rabbits, losses from ovulation to days 6 to 7 post-insemination have been estimated at 8–14% (Adams, 1960; Mocé *et al.*, 2002; Llobat *et al.*, 2012b). From fertilization to implantation, embryonic development is influenced during its migration by the maternal environment (Fleming *et al.*, 2004). As the current results show, the oviduct exhibits a spatial-temporal pattern of transcripts involved in peri-implantation events. In rabbits, the embryo remains in the oviduct from fertilization until days 3 to 4 of development. During these days, the zygote should be converted into a competent embryo for implantation, requiring several changes such as cell cleavage divisions, activation of the embryonic genome, segmentation and compaction of the morula and blastocyst formation (Lonergan *et al.*, 2003). Carney *et al.* (1990) found that co-culture of rabbit zygotes with rabbit oviduct epithelial cells increased blastocyst formation. Ovarian steroids, growth factors, glucose, lactate, pyruvate, proteins, cholesterol, phospholipids and ions as sodium, potassium, chloride and calcium have been found in oviduct fluid (Leese, 1988; Henault & Killian,

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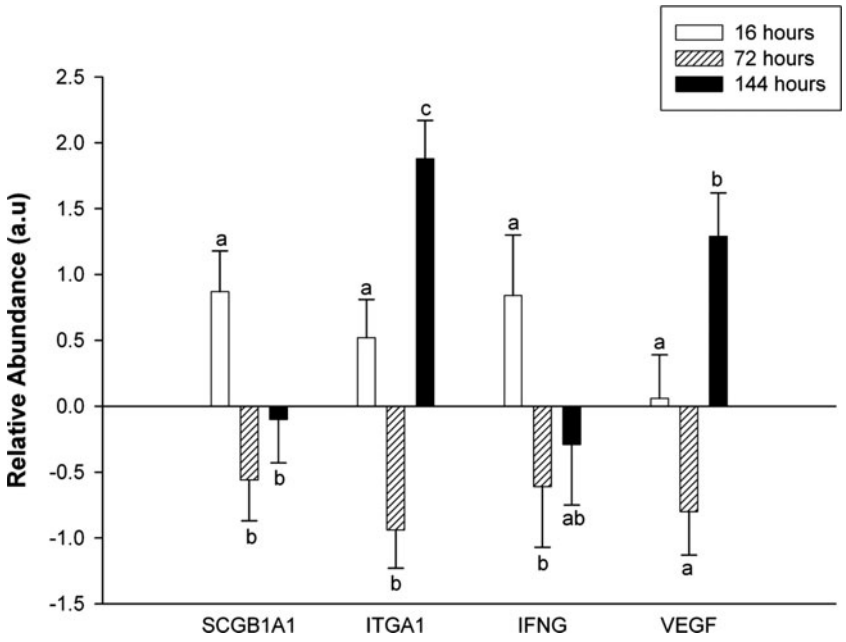

Figure 2 Relative abundance of *SCGB1A1* (uteroglobin), *ITGA1* (integrin α_1), *IFNG* (interferon-γ) and *VEGF* (vascular endothelial growth factor) mRNA expression for endometrium tissue in 16 h, 72 h or 144 h post-ovulation induction. Relative abundance values are expressed in arbitrary units (a.u.), showing the mean value ± standard error of the mean (SEM). [a,b,c]Different letters represent significant differences.

1993; Grippo *et al.*, 1994; Killian, 2004; Aviles *et al.*, 2010; Vecchio *et al.*, 2010) and several reports have confirmed that this support of oviduct secretions to embryo development are not species specific (Minami *et al.*, 1994; Lai *et al.*, 1996; Yadav *et al.*, 1998; Lloyd *et al.*, 2009). In the current experiment, the mRNA expression of a set of genes (*SCGB1A1*, *ITGA1*, *IFNG* and *VEGF*) associated with maternal recognition and establishment of rabbit pregnancy was examined. The specific hours (16, 72 and 144 h post-induction of ovulation) were selected because at 16 h the zygotes are in the oviduct, at 72 h the morulae or early blastocysts are exiting the oviduct and entering the uterus, and finally at 144 h the late blastocyst are in the uterus before the onset of gastrulation and adhesion to endometrium. As expected, the gene expression pattern of the oviduct changed from 16 to 144 h post-ovulation induction. It seemed that after ovulation the oviduct started to prepare the best case scenario to carry out the first steps of preimplantation development, by maintaining or increasing the quantity of crucial molecules such as uteroglobin, integrins or growth factors. Although the uteroglobin gene (*SCGB1A1*) was first identified in rabbit as a specific uterus protein, previous studies have detected mRNA expression in the oviduct (Kay & Feigelson, 1972). As stated previously, studies that focussed on regulation of uteroglobin in the uterus have identified that progesterone had the ability to induce it and oestrogen to repress it. However, in the case of the oviduct, it has been detected that *SCGB1A1* expression was induced by oestrogen, not progesterone (Mukherjee *et al.*, 2007), a finding that could explain why its expression is higher at 16 h than at 144 h post-ovulation induction. Integrins comprise a large family of heterodimeric transmembrane receptors linked with a great variety of extracellular matrix ligands. Regulation of the transport and stability of gametes and early embryos in the oviduct requires the support of cell adhesion molecules and, for this reason, it was possible to observe an increase in mRNA expression of *ITGA1* from 72 h. It is well known that interferons have a multipotential role in the immune response throughout pregnancy. In particular, successful pregnancy requires a protective immunomodulatory mechanism, including a reduction in inflammatory and cytotoxic reactions mainly carried out via *IFNG*, *IL-2* and *TNF* (Druckmann & Druckmann, 2005). As the expression of *IFNG* is considered an immunoreaction related to pregnancy failure, it could be posited that transcript abundance was reduced significantly at 72 h in order to avoid embryo abortion. Moreover, it has been suggested that IFN-γ is also enhanced by oestrogens (Platt & Hunt, 1998), a suggestion that would correlate with the high transcript abundance observed after ovulation. Regarding *VEGF* mRNA expression, no differences were found in the oviduct tissue between post-ovulation and preimplantation stage. The current results complemented the observations

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by Wijayagunawardane *et al.* (2005), which showed that, after ovulation, the elevated *VEGF* mRNA expression is immediately downregulated by negative feedback regulation; the current results suggest that this expression remains constant in the days before implantation.

To establish embryo–uterine cross-talk and begin the implantation process, the uterus must differentiate into a receptive state (Paria *et al.*, 2001). This change means that the endometrial epithelium is functionally and structurally ready to accept the embryo for implantation (Salilew-Wondim *et al.*, 2012). The importance of progesterone in pregnancy recognition and uterine receptivity has been studied widely in many species; inadequate progesterone levels could reduce the ability of the uterus to support embryo development (Rizos *et al.*, 2010; Salilew-Wondim *et al.*, 2012). Apart from ovarian hormones, there are other components such as growth factors, cytokines, chemokines and adhesion molecules, among others, that participate in this dialogue between endometrium and embryo (van Mourik *et al.*, 2009); any modification or absence of these molecules may hinder the implantation process. The results of the present research agree with previous studies that reported the presence of uteroglobin in the uterus during early pregnancy (Peri *et al.*, 1995). In particular, this uteroglobin has been associated with cell proliferation and stimulation of blastocyst growth (Beier, 2000; Riffo *et al.*, 2007; Mukherjee *et al.*, 2007). Previous studies have detected mRNA expression in rabbit blastocysts embryos (Saenz-de-Juano *et al.*, 2012; Naturil-Alfonso *et al.*, 2013), so the synthesis of this protein by the embryo itself could explain the decrease in mRNA expression observed from 72 to 144 h in the uterine tissue. Integrins are considered to be immunohistochemical markers of uterine receptivity (Lessey, 1998), and it has been observed that they could be expressed in the endometrium either constitutively or in a cycle-dependent manner. Recently, Tesfaye *et al.* (2011) analysed the endometrial gene expression of heifers that eventually resulted in calf delivery and those that resulted in no pregnancy, and observed that expression of integrins was up-regulated in successfully pregnant heifers. Interferons have a crucial role in the uterine immune system and make both implantation and maintenance of pregnancy possible (Szekeres-Bartho, 2002). In the current experiment, as occurs in the oviduct tissue, a significant decrease was observed in *IFNG* gene expression from 16 to 72 h or 144 h post-ovulation induction; this decrease was also correlated with high progesterone levels at these stages. Finally, in the case of *VEGF*, up-regulation in expression of this transcript was found in uterus tissue at 144 h. *VEGF* has been associated with the process of *de novo* angiogenesis (Lee & DeMayo, 2004); its expression and function has been regarded as ensuring a suitable vasculogenesis during implantation and early placentation (Torry *et al.*, 2007). So, as occurs for the *ITGA1* gene, its importance grows as the implantation window approaches.

To understand why prenatal mortality continues to occur, it is important to characterize the causes from a biological point of view. The examination of biochemical changes and gene expression patterns of the oviduct and uterus in the presence of gametes or embryos could help us understand the molecular mechanisms of oviduct–oocyte, oviduct–embryo and uterus–embryo interactions.

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Conflict of interest

There are no conflicts of interest.

References

Aboagye-Mathiesen, G., Tóth, F.D., Zdravkovic, M. & Ebbesen, P. (1995). Human trophoblast interferons: production and possible roles in early pregnancy. *Early Pregnancy* **1**, 41–53.

Adams, C.E. (1960). Prenatal mortality in the rabbit *Oryctolagus cuniculus*. *J. Reprod. Fertil.* **1**, 36–44.

Artini, P.G., Valentino, V., Montleone, P., Simi, G., Parisen-Toldin, M.R., Critello, F., Cela, V. & Genazzani, A.R. (2008). Vascular endothelial growth factor level changes during human embryo development in culture medium. *Gynecol. Endocrinol.* **24**, 184–7.

Aviles, M., Gutierrez-Adan, A. & Coy, P. (2010). Oviductal secretions: will they be key factors for the future ARTs? *Mol. Hum. Reprod.* **16**, 896–906.

Beier, H.M. (1968). Uteroglobin: a hormone-sensitive endometrial protein involved in blastocyst development. *Biochim. Biophys. Acta* **160**, 289–91.

Beier, H.M. (2000). The discovery of uteroglobin and its significance for reproductive biology and endocrinology. *Ann. N Y Acad. Sci.* **923**, 9–24.

Bowen, J.A., Bazer, F.W. & 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**, 1098–106.

Burghardt, R.C., Johnson, G.A., Jaeger, L.A., Ka, H., Garlow, J.E., Spencer, T.E. & Bazer, F.W. (2002). Integrins and extracellular matrix proteins at the maternal–fetal interface in domestic animals. *Cells Tissues Organs* **171**, 202–17.

Carney, E.W., Tobback, C. & Foote, R.H. (1990). Co-culture of rabbit one-cell embryos with rabbit oviduct epithelial cells. *In Vitro Cell Dev. Biol.* **26**, 629–35.

Chakraborty, I., Das, S.K. & Dey, S.K. (1995). Differential expression of vascular endothelial growth factor and its receptor mRNAs in the mouse uterus around the time of implantation. *J. Endocrinol.* **147**, 339–52.

Chandra, T., Woo, S.L. & Bullock, D.W. (1980). Cloning of thc rabbit uteroglobin structural gene. *Biochem. Biophys. Res. Commun.* **95**, 197–204.

Cross, J.C. & Roberts, R.M. (1989). Porcine conceptuses secrete an interferon during the preattachment period of early pregnancy. *Biol. Reprod.* **40**, 1109–18.

Das, S.K., Chakraborty, I., Wang, J., Dey, S.K. & Hoffman, L.H. (1997). Expression of vascular endothelial growth factor (VEGF) and VEGF-receptor messenger ribonucleic acids in the peri-implantation rabbit uterus. *Biol. Reprod.* **56**, 1390–9.

Denker, H.W. (1977). Implantation: the role of proteinases and blockage of implantation by proteinase inhibitors. *Adv. Anat. Embryol. Cell Biol.* **53**, 1–123.

Druckmann, R. & Druckmann, M.A. (2005). Progesterone and the immunology of pregnancy. *J. Steroid Biochem. Mol. Biol.* **97**, 389–96.

Enders, A.C. (1994). Contributions of comparative studies to understanding mechanisms of implantation. In *Endocrinology of Embryo-Endometrium Interactions* (eds S.R. Glasser, J. Mulholland & A. Psychoyos) pp. 11–16. New York and London: Plenum Press.

Enders, A.C. & Schlafke, S. (1971). Penetration of the uterus epithelium during implantation in the rabbit. *Am. J. Anat.* **132**, 219–30.

Fazleabas, A.T.F., Donnelly, K.M., Hild-Petito, S., Hausermann, H.M. & Verhage, H.G. (1997). Secretory proteins of the baboon (*Papio anubis*) endometrium: regulation during the menstrual cycle and early pregnancy. *Hum. Reprod.* **3**, 553–9.

Ferrara, N. & Davis-Smyth, T. (1997). The biology of vascular endothelial growth factor. *Endocr. Rev.* **18**, 4–25.

Fleming, T.P., Kwong, W.Y., Porter, R., Ursell, E., Fesenko, I., Wilkins, A., Miller, D.J., Watkins, A.J. & Eckert, J.J. (2004). The embryo and its future. *Biol. Reprod.* **71**, 1046–54.

Fukuda, M.N., Sato, T., Nakayama, J., Klier, G., Mikami, M., Aoki, D. & Nozawa, S. (1995). Trophinin and tastin, a novel cell adhesion molecule complex with potential involvement in embryo implantation. *Genes Dev.* **9**, 1199–210.

Godornes, C., Leader, B.T., Molini, B.J., Centurion-Lara, A. & Lukehart, S.A. (2007). Quantitation of rabbit cytokine mRNA by real-time RT-PCR. *Cytokine* **38**, 1–7.

Grippo, A.A., S. H. Anderson, S.H., Chapman, D.A., Henault, M.A. & Killian, G.J. (1994). Cholesterol, phospholipid and phospholipase activity of ampullary and isthmic fluid from the bovine oviduct. *J. Reprod. Fertil.* **102**, 87–93.

Henault, M.A. & Killian, G.J. (1993). Synthesis and secretion of lipids by bovine oviduct mucosal explants. *J. Reprod. Fertil.* **98**, 431–43.

Hoffman, L.H., Olson, G.E., Carson, D.D. & Chilton, B.S. (1998). Progesterone and implanting blastocysts regulate Muc1 expression in rabbit uterine epithelium. *Endocrinology* **139**, 266–71.

Illera, M.J., Lorenzo, P.L., Gui, L.T., Beyler, S.A., Apparao, K.B.C. & Lessey, B.A. (2003). A role for $\alpha_V\beta_3$ integrin during implantation in the rabbit model. *Biol. Reprod.* **68**, 766–71.

Kay, E. & Feigelson, M. (1972). An estrogen modulated protein in rabbit oviductal fluid. *Biochim. Biophys. Acta* **271**, 436–41.

Killian, G.J. (2004). Evidence for the role of oviduct secretions in sperm function, fertilization and embryo development. *Anim. Reprod. Sci.* **82–83**, 141–53.

Krishnan, R. S. & Daniel, J.C. (1967). Blastokinin: inducer and regulator of blastocyst development in the rabbit uterus. *Science* **158**, 490–2.

Kopu, H.T., Hemminki, S.M., Torkkeli, T.K. & Janne, O.A. (1979). Hormonal control of uteroglobin secretion in rabbit uterus: inhibition of uteroglobin synthesis and messenger ribonucleic acid accumulation by oestrogen and anti-oestrogen administration. *Biochem. J.* **180**, 491–500.

Krusche, C.A., Vloet, T.D., Herrler, A., Black, S. & Beier, H.M. (2002). Functional and structural regression of the rabbit corpus luteum is associated with altered luteal immune cell phenotypes and cytokine expression patterns. *Histochem. Cell. Biol.* **118**, 479–89.

Lai, Y.M., Wang, H.S., Lee, C.L., Lee, J.D., Huang, H.Y., Chang, F.H., Lee, J.F. & Soong, Y.K. (1996). Insulin-like growth factor-binding proteins produced by Vero cells, human oviductal cells and human endometrial cells, and the role of insulin-like growth factor-binding protein-3 in mouse embryo co-culture systems. *Hum. Reprod.* **11**, 1281–6.

Lee, K.Y. & DeMayo, F.J. (2004). Animal models of implantation. *Reproduction* **128**, 679–95.

Leese, H.J. (1988). The formation and function of oviduct fluid. *J. Reprod. Fertil.* **82**, 843–56.

Lessey, B.A. (1998). Endometrial integrins and the establishment of uterine receptivity. *Hum. Reprod.* **13**, 247–58.

Lessey, B.A., Ilesanmi, A.O., Sun, J., Lessey, M.A., Harris, J. & Chwalisz, K. (1996). Luminal and glandular endometrial epithelium express integrins differentially throughout the menstrual cycle: implications for implantation, contraception, and infertility. *Am. J. Reprod. Immunol.* **35**, 195–204.

Llobat, L., Marco-Jiménez, F., Peñaranda, D.S., Thieme, R., Navarrete-Santos, A. & Vicente, J.S. (2012a). mRNA expression in rabbit blastocyst and endometrial tissue of candidate gene involved in gestational losses. *Reprod. Domest. Anim.* **47**, 281–7.

Llobat, L., Marco-Jiménez, F., Peñaranda, D.S., Saenz-de-Juano, M.D. & Vicente, J.S. (2012b). Effect of embryonic genotype on reference gene selection for RT-qPCR normalization. *Reprod. Domest. Anim.* **47**, 629–34.

Lloyd, R.E., Romar, R., Matas, C., Gutierrez-Adan, A., Holt, W.V. & Coy, P. (2009). Effects of oviductal fluid on the development, quality, and gene expression of porcine blastocysts produced *in vitro*. *Reproduction* **137**, 679–87.

Lonergan, P., Rizos, D., Kanka, J., Nemcova, L., Mbaye, A.M., Kingston, M., Wade, M., Duffy, P. & Boland, M.P. (2003). Temporal sensitivity of bovine embryos to culture environment after fertilization and the implications for blastocyst quality. *Reproduction* **126**, 337–46.

Mamo, S., Gal, A.B., Polgar, Z. & Dinnyes, A. (2008). Expression profiles of the pluripotency marker gene *POU5f1* and validation of reference genes in rabbit oocytes and preimplantation stage embryos. *BMC Mol. Biol.* **9**, 67.

Marco-Jiménez, F., Vicente, J.S., Lavara, R., Balasch, S. & Viudes-de-Castro, M.P. (2010). Poor prediction value of sperm head morphometry for fertility and litter size in rabbit. *Reprod. Domest. Anim.* **45**, 118–23.

McLaren, A. & Michie, D. (1954). Studies on the transfer of fertilized mouse eggs to uterine foster-mothers. *J. Exp. Biol.* **33**, 394–416.

Minami, N., Kato, H., Inoue, Y., Yamada, M., Utsumi, K. & Iritani, A. (1994). Nonspecies-specific effects of mouse oviducts on the development of bovine IVM/IVF embryos by a serum free co-culture. *Theriogenology* **41**, 1435–45.

Mocé, M.L., Santacreu, M.A. & Climent, A. (2002). The effect of divergent selection for uterine capacity on progesterone, estradiol and cholesterol levels around implantation time in rabbits. *World Rabbit Sci.* **10**, 89–97.

Mukherjee, A.B., Zhang, Z. & Chilton, B.S. (2007). Uteroglobin: a steroid-inducible immunomodulatory protein that founded the secretoglobin superfamily. *Endocr. Rev.* **28**, 707–25.

Muller, H. & Beato, M. (1980). RNA synthesis in rabbit endometrial nuclei. Hormonal regulation of transcription of the uteroglobin gene. *Eur. J. Biochem.* **112**, 235–41.

Murata, T., Hori, M., Lee, S., Nakamura, A., Kohama, K., Karaki, H. & Ozaki, H. (2005). Vascular endothelium has a local anti-adenovirus vector system and glucocorticoid optimizes its gene transduction. *Arterioscler. Thromb. Vasc. Biol.* **25**, 1796–803.

Muscettola, M., Massai, L., Lodi, L., Briganti, F., Fontani, G. & Lupo, C. (2003). IFN-gamma production in rabbits: behavioral and endocrine correlates. *Life Sci.* **72**, 1331–43.

Naturil-Alfonso, C., Vicente, J.S., Peñaranda, D.S. & Marco-Jiménez, F. (2013). Up-regulation of insulin-like growth factor I and uteroglobin in *in vivo*-developed parthenogenetic embryos. *Reprod. Domest. Anim.* **48**, 126–30.

Navarrete-Santos, A., Ramin, N., Tonack, S. & Fischer, B. (2008). Cell lineage-specific signaling of insulin and insulin-like growth factor I in rabbit blastocysts. *Endocrinology* **149**, 515–24.

Paria, B.C., Song, H. & Dey, S.K. (2001). Implantation: molecular basis of embryo–uterine dialogue. *Int. J. Dev. Biol.* **45**, 597–606.

Peri, A., Dubin, N.H., Dhanireddy, R. & Mukherjee, A.B. (1995). Uteroglobin gene expression in the rabbit uterus throughout gestation and in the fetal lung. Relationship between uteroglobin and eicosanoid levels in the developing fetal lung. *J. Clin. Invest.* **96**, 343–53.

Platt, J.S. & Hunt, J.S. (1998). Interferon-gamma gene expression in cycling and pregnant mouse uterus: temporal aspects and cellular localization. *J. Leukoc. Biol.* **64**, 393–400.

Pollard, J.W., Hunt, J.S., Wiktor-Jedrzejczak, W. & 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**, 273–83.

Psychoyos, A. (1986). Uterine receptivity for nidation. *Ann. N Y Acad. Sci.* **476**, 36–42.

Psychoyos, A. & Nikas, G. (1994). Uterine pinopodes as markers of uterine receptivity. *Assisted Reprod. Rev.* **4**, 26–32.

Riffo, M., Diaz-Gonzalez, K. & Nieto, A., (2007). Uteroglobin induces the development and cellular proliferation of the mouse early embryo. *J. Exp. Zool.* **307**, 28–34.

Rizos, D., Carter, F., Besenfelder, U., Havlicek, V. & Lonergan, P. (2010). Contribution of the female reproductive tract to low fertility in postpartum lactating dairy cows. *J. Dairy Sci.* **93**, 1022–9.

Saenz-de-Juano, M.D., Peñaranda, D.S., Marco-Jiménez, F., Llobat, L. & Vicente, J.S. (2011). Differential mRNA expression in rabbit *in vivo* preimplantation embryos. *Reprod. Dom. Anim.* **46**, 567–72.

Saenz-de-Juano, M.D., Marco-Jiménez, F., Peñaranda, D.S., Joly, T. & Vicente, J.S. (2012). Effects of slow freezing procedure on late blastocyst gene expression and survival rate in rabbit. *Biol. Reprod.* **87**, 91.

Salilew-Wondim, D., Schellander, K., Hoelker, M. & Tesfaye, D. (2012). Oviductal, endometrial and embryonic gene expression patterns as molecular clues for pregnancy establishment. *Anim. Reprod. Sci.* **134**, 9–18.

Sharkey, A. (1998). Cytokines and implantation. *Rev. Reprod.* **3**, 52–61.

Shen, X.Z., Tsai, M.J., Bullock, D.W. & Woo, S.L. (1983). Hormonal regulation of rabbit uteroglobin gene transcription. *Endocrinology* **112**, 871–6.

Snead, R., Day, L., Chandra, T., Mace, Jr, M., Bullock, D.W. & Woo, S.L. (1981). Mosaic structure and mRNA precursors of uteroglobin, a hormone regulated mammalian gene. *J. Biol. Chem.* **256**, 11911–6.

Spencer, T.E., Burghardt, R.C., Johnson, G.A. & Bazer, F.W. (2004). Conceptus signals for establishment and maintenance of pregnancy. *Anim. Reprod. Sci.* **82–83**, 537–50.

Stewart, C.L., Kaspar, P., Brunet, L.J., Bhatt, H., Gadi, I., Köntgen, F. & Abbondanzo, J.S. (1992). Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature* **359**, 76–9.

Sutherland, A.E., Calarco, P.G. & Damsky, C.H. (1993). Developmental regulation of integrin expression at the time of implantation in the mouse embryo. *Development* **119**, 1175–86.

Szekeres-Bartho, J. (2002). Immunological relationship between the mother and the fetus. *Int. Rev. Immunol.* **21**, 471–95.

Tesfaye, D., Salilew-Wondim, D. & Schellander, K. (2011). The role of endometrium in bovine pregnancy establishment. In *27th Annual Meeting AETE, Chester, England, 9–10 September* 2011 (eds AETE) pp. 75–86. Chester, UK: AETE.

Torry, D.S., Leavenworth, J., Chang, M., Maheshwari, V., Groesch, K., Ball, E.R. & Torry, R.J. (2007). Angiogenesis in implantation. *J. Assist. Reprod. Genet.* **24**, 303–15.

van Mourik, M.S.M., Macklon, N.S. & Heijnen, C.J. (2009). Embryonic implantation: cytokines, adhesion molecules, and immune cells in establishing an implantation environment. *J. Leukoc. Biol.* **85**, 4–19.

Vecchio, D., Neglia, G., Di Palo, R., Campanile, G., Balestrieri, M., Giovane, A., Killian, G., Zicarelli, L. & Gasparrini, B. (2010). Ion, protein, phospholipid and energy substrate content of oviduct fluid during the oestrous cycle of buffalo (*Bubalus bubalis*). *Reprod. Domest. Anim.* **45**, e32–9.

Viudes-De-Castro, M.P. & Vicente, J.S. (1997). Effect of sperm count on the fertility and prolificity rates of meat rabbits. *Anim. Reprod. Sci.* **46**, 313–9.

Weitlauf, H.M. (1994). Biology of implantation. In *The Physiology of Reproduction* (eds E. Knobil & J.D. Neill) pp. 391–440. New York: Raven Press.

Weltzien, F.A., Pasqualini, C., Vernier, P. & Dufour, S. (2005). A quantitative real-time RT-PCR assay for European eel tyrosine hydroxylase. *Gen. Comp. Endocrinol.* **142**, 134–42.

Wijayagunawardane, M.P., Kodithuwakku, S.P., Yamamoto, D. & Miyamoto, A. (2005). Vascular endothelial growth factor system in the cow oviduct: a possible involvement in the regulation of oviductal motility and embryo transport. *Mol. Reprod. Dev.* **72**, 511–20.

Winterhager, E., Mulholland, J. & Glasser, S.R. (1994). Morphological and immunohistochemical differentiation patterns of rabbit uterine epithelium *in vitro*. *Anat. Embryol.* **189**, 71–9.

Yadav, P.S., Saini, A., Kumar, A. & Jain, G.C. (1998). Effect of oviductal cell co-culture on cleavage and development of goat IVF embryos. *Anim. Reprod. Sci.* **51**, 301–6.

Yang, X. & Foote, R. (1987). Production of identical twin rabbits by micromanipulation of embryos. *Biol. Reprod.* **37**, 1007–14.

Zhu, L.J., Bagchi, M.K. & Bagchi, I.C. (1998). Attenuation of calcitonin gene expression in pregnant rat uterus leads to a block in embryonic implantation. *Endocrinology* **139**, 330–9.