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"INTERACCIONES MATERNO-EMBRIONARIAS DURANTE EL DESARROLLO TEMPRANO DE EMBRIONES BOVINOS"

TESIS DOCTORAL

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Justificación

Oviedo, 20 de febrero de 2015

El Director del Departamento de BIOQUIMICA Y BIOLOGIA MOLECULAR

Fdo.: PEDRO SÁNCHEZ LAZO

Contra la presente resolución podrá interponer recurso de alzada ente el Excmo. Sr. Rector Magfco. de esta Universidad en el plazo de un mes a contar desde el siguiente a la recepción de la presente resolución, de acuerdo con lo previsto en el artículo 114 de la Ley 30/92, de 26 de noviembre, del Régimen Jurídico de las Administraciones Públicas y Procedimiento Administrativo Común (B.O.E. de 27 de noviembre), modificada por la Ley 4/1999, de 13 de enero (B.OE. de 14 de enero)

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: Interacciones materno-embrionarias durante el desarrollo temprano de embriones bovinos	Inglés: Embryo-maternal interactions during the early development of bovine embryos

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RESUMEN (en español)

La comunicación recíproca entre el embrión y el endometrio es esencial para la implantación embrionaria y la preñez a término en mamíferos. La ausencia de factores involucrados en esta comunicación durante el cultivo *in vitro* merma la eficiencia de las técnicas de reproducción asistida. Esta tesis estudia proteínas candidatas a ejercer un papel embriotrófico durante el desarrollo temprano bovino (Día 5 - Día 8) y que podrían añadirse al medio de cultivo para mejorar el desarrollo y la calidad del embrión bovino. Con este objetivo caracterizamos la expresión en el endometrio y embrión de varios factores identificados previamente en el fluido uterino (FU) de vacas preñadas. En concreto, las proteínas seleccionadas fueron: las citoquinas, TNF e IL1B; el factor de crecimiento HDGF; y los receptores TNFR2 e IL1R1. Además, analizamos la influencia del sexo del embrión en la comunicación materno-embrionaria mediante la comparación del perfil proteómico de FUs con embriones macho y hembra. Por último, realizamos un ensayo funcional de HDGF en el cultivo *in vitro* de embriones bovinos. Según nuestros resultados, los embriones aumentan la expresión proteica de TNF, TNFR2 e IL1B en determinadas estructuras celulares del endometrio, mientras que los niveles de mRNA no se ven afectados. Al mismo tiempo, los embriones inducen una disminución de leucocitos endometriales en el epitelio glandular funcional y en el estroma subepitelial, lo que concuerda con la regulación a la baja de NFKB y el ambiente de tolerancia inmunológica reportado previamente durante el desarrollo temprano. Por otro lado, el pasaje temporal por el tracto materno induce una regulación a la baja de la transcripción de *TNFR2* y *HDGF* en el embrión. Además, hemos determinado que el endometrio reconoce el sexo del embrión. Hemos identificado 23 proteínas diferencialmente expresadas en los FUs con embriones macho y hembra, así como diferencias en varias vías metabólicas que incluyen procesamiento de antígeno, glicolisis, biosíntesis, antioxidación, apoptosis y propiedades funcionales del FU. Por último, la adición de una proteína recombinante del HDGF al medio de cultivo sin proteína de mórulas de Día 6, en grupo e individuales, aumenta la proliferación celular y el desarrollo embrionario. Estos resultados contribuyen a dilucidar los procesos que controlan el desarrollo embrionario temprano y pueden mejorar el cultivo *in vitro* de embriones bovinos. Estudios futuros podrán caracterizar con mayor detalle las proteínas y vías moleculares descritas en esta tesis con el objetivo de mejorar el cultivo de embriones, teniendo en cuenta también el sexo del embrión.

RESUMEN (en Inglés)

The reciprocal communication between embryo and endometrium is essential for embryo implantation and successful pregnancy to term in mammals. Deficiency in factors involved in this communication during the *in vitro* culture may hampers the efficiency of reproductive technology procedures. This thesis studies several proteins that likely exert an embryotrophic role during the early bovine development (Day 5 - Day 8) and that may be added to the *in vitro* culture media to enhance embryo development and quality. To this end, we analyzed the endometrial and embryonic expression of several factors previously identified in the bovine uterine fluid (UF) of pregnant animals. Specifically, the selected proteins were: the cytokines TNF and IL1B; the growth factor HDGF; and the receptors TNFR2 and IL1R1. Furthermore, we analyze if early embryo-maternal communication is subjected to sexual dimorphism by comparing the proteomic profiles of UFs containing male and female embryos. Our results show that embryos increase the protein expression of TNF, TNFR2 and IL1B in some endometrial cells, while their mRNA is not affected. At the same time embryos induce a decrease in the number of leucocytes in the functional glandular epithelium and subepithelial stroma, which is in concordance with the NFKB down-regulation and the maternal immune tolerance towards the embryo reported during early development. On the other hand, after uterine passage, embryos show down-regulation of *TNFR2* and *HDGF* mRNA. Furthermore, we have determined that endometrium recognizes the embryonic sex. We have identified 23 proteins that are differentially expressed in male and female UFs, as well as differences in several metabolic pathways that include regulation in antigen processing, biosynthesis, antioxidation, apoptosis, glycolysis, and functional properties of the UF. Finally, adding a recombinant protein of HDGF to defined culture medium of Day 6 morulae, both in groups and individuals, improves embryo development and cell counts. These results may help to elucidate the molecular mechanisms that govern early development and could improve the *in vitro* culture of bovine embryos. Candidate proteins and pathways uncovered in this thesis can be pursued in future studies with the goal of improvement the culture of embryos, considering also the importance of sex-specific protocols.

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Abreviaturas y símbolos

ACTB	beta actina	actin, beta
AI	índice apoptótico	apoptotic index
AP1	proteína activadora 1	activating protein 1
BCS	calificación de la condición corporal	body condition score
BSA	albúmina sérica bovina	bovine serum albumin
BVA	análisis de la variación biológica	biological variation analysis
C20RF29	cromosoma 11 marco de lectura abierto, C2orf29 humano	chromosome 11 open reading frame, human C2orf29
cDNA	DNA complementario	complementary DNA
CID	disociación inducida por colisión	collision-induced dissociation
CL	cuerpo lúteo	yelow body
CNRQ	cantidades relativas normalizadas	normalized relative quantities
COCs	complejos *cumulus*-ovocito	*cumulus*-oocyte complexes
CPI	índice de proliferación celular	cell proliferation index
CSF2 (GMCSF2)	factor estimulador de colonias 2 (granulocitos-macrófagos)	colony stimulating factor 2 (granulocyte-macrophage)
Ct	ciclo umbral	threshold cycle
DAB	diaminobencidina	diaminobenzidine
DDAH2	dimetilarginina dimetilamino hidrolasa 2	dimethylarginine dimethylaminohydrolase 2
DE	expresada diferencialmente	differential expressed
DIGE (2D-DIGE)	electroforesis bidimensional diferencial en gel	two-dimensional difference gel electrophoresis
EGF	factor de crecimiento epidérmico	epidermal growth factor
EMT	transición epitelio-mesénquima	epithelial-mesenchymal transition
ENO	enolasa	enolase
ERK	MAP quinasa regulada por señales extracelulares	extracellular regulated MAP kinase
ET	transferencia de embriones	embryo transfer
Etk	proteína tirosina quinasa	tyrosine-protein kinase
FCS	suero fetal bovino	fetal calf serum
FDR	tasa de descubrimientos falsos	false discovery rate
FGF	factor de crecimiento fibroblástico	fibroblast growth factor
FIV	fertilización *in vitro*	*in vitro* fertilization
FSH	hormona foliculoestimulante	follicle-stimulating hormone
GAPD	gliceraldehído-3-fosfato deshidrogenasa	glyceraldehyde 3-phosphate dehydrogenase
GF	factor de crecimiento	growth factor
GLM	modelo lineal generalizado	generalized lineal model
GnRH	hormona liberadora de gonadotropinas	gonadotropin-releasing hormone
GO	ontología génica	gene ontology
GPI	glucosa-6-fosfato isomerasa	glucose-6-phosphate isomerase
GSTP1	glutatión S-transferasa pi 1	glutathione S-transferase pi 1
HDGF	factor de crecimiento derivado del hepatoma	hepatoma-derived growth factor
HGF	factor de crecimiento de hepatocitos	hepatocyte growth factor

HRP	peroxidasa del rábano	horseradish peroxidase
HSPA5	proteína de choque térmico 5	heat shock protein 5
HS	heparan sulfato	heparan sulfate
I-20S	subunidad del inmunoproteasoma 20S (β5i)	immunoproteasome subunit 20S (β5i)
ICM	masa celular interna	inner cell mass
IFNT	interferón tau	interferón tau
IGF	factor de crecimiento insulínico	insulin-like growth factor
IGFBP	proteína de unión a IGF	insulin-like growth factor binding protein
IGG	inmunoglobulina G	immunoglobulin G
IHC	inmunohistoquímica	immunohistochemistry
IKBA	inhibidor alfa de NFKB	NFKB inhibitor alpha
IL1B	interleuquina 1 beta	interleukin 1, beta
IL1R1	receptor de interleuquina 1, tipo I	interleukin 1 receptor, type I
IL1R2	receptor de interleuquina 1, tipo II	interleukin 1 receptor, type II
IL1RAP	proteína accesoria del receptor de IL1	interleukin 1 receptor accessory protein
IL1RN	antagonista del receptor de interleuquina 1	interleukin 1 receptor antagonist
IP	inmunoproteasoma	immunoproteasome
IPA	análisis de las redes de interacciones Ingenuity	Ingenuity pathway analysis network
IRAK	quinasa asociada al receptor de IL1	interleukin-1 receptor-associated kinase
ISG	genes estimulados por interferon tau	interferon-*stimulated genes*
IVC	cultivo *in vitro*	*in vitro* culture
IVEP	producción de embriones *in vitro*	*in vitro* embryo production
IVP	producido *in vitro*	*in vitro* produced
JNK	quinasa c-Jun N-terminal	c-Jun N-terminal kinase
LEDGF	factor de crecimiento derivado del cristalino	Lens Epithelium-derived Growth factor
LH	hormona luteinizante	luteinizing hormone
LIF	factor inhibidor de la leucemia	leukemia inhibitory factor
LSMs	medias de cuadrados mínimos	least squares means
MAPKs	proteínas quinasas activadas por mitógenos	mitogen activated kinase-like protein
MET	epitelial c-met proto-oncogén	epithelial c-met proto-oncogene
MIV	maduración *in vitro*	*in vitro*maturation
MS	espectrometría de masas	mass spectrometry
mSOF	SOF modificado	modified SOF
Myd88	factor de diferenciación mieloide 88	myeloid differentiation primary response 88
NFKB	factor nuclear kappa B	nuclear factor kappa B
NFs	factores de normalización	normalization factors
NGS	suero normal de cabra	normal goat serum
NIK	quinasa inductora de NFKB	NFKB inducing kinase
OXT	oxitocina	oxytocin
OXTR	receptor de oxitocina	oxytocin receptor
P/IP	proteasoma/inmunoproteasoma	proteasome/immunoproteasome
P4	progesterona (pregn-4-en-3,20-diona)	progesterone (pregn-4-en-3,20-diona)
PA28A	subunidad 1 activadora del proteasoma	proteasome activator subunit 1

(PSME1)		
PAF	factor activador de plaquetas	platelet-activating factor
PARK7 (DJ1)	proteína de parkinson 7	parkinson protein 7
PBS	tampón fosfato salino	phosphate buffered saline
PGAM1	fosfoglicerato mutasa	phosphoglycerate mutase 1
PGE2	prostaglandina E2	prostaglandin E2
PGF2A	prostaglandina F2 alfa	prostaglandin F2 alpha
PGR	receptor de progesterona	progesterone receptor
PI3K/AKT	fosfoinositol 3-quinasa/B-quinasa	phosphatidylinositol 3-kinase/B-kinase
PNP	purina nucleósido fosforilasa	purine nucleoside phosphorylase
PVA	polivinil alcohol	polyvinyl alcohol
PVDF	fluoruro de polivinilideno	Polyvinylidene fluoride
PVP	polivinilpirrolidona	polyvinylpyrrolidone
rHDGF	HDGF recombinante	recombinant HDGF
RPL19	proteína ribosómica L19	ribosomal protein L19
RT-qPCR	PCR cuantitativa a tiempo real	real time-quantitative PCR
SDHA	subunidad A del complejo succinato deshidrogenasa	succinate dehydrogenase complex, subunit A
SDS	dodecilsulfato sódico	sodium dodecyl sulfate
SDS-PAGE	electroforesis en gel de poliacrilamida con SDS	SDS-polyacrylamide gel electrophoresis
SEM	error estándar de la media	standard error of the mean
SLC30A6	miembro 6 de la familia 30 del portador del soluto	solute carrier family 30 (zinc transporter), member 6
SOF	fluido oviductal sintético	synthetic oviductal fluid
ST	transferencia sin embriones	sham transfer
TAB	proteína de unión a TAK1	Tak1-binding protein
TAK1	quinasa 1 activada por TGFB	TGFB activated kinase 1
TALP	tyrode, albúmina, lactato, piruvato	tyrode, albumin, lactate, pyruvate
TCM 199	medio de cultivo tisular 199	tissue culture medium 199
TE	trofoblasto	trophectoderm
TGFB	factor de crecimiento transformante beta	transforming growth factor, beta
TIR	receptor de IL1/Toll	Toll/interleukin-1 receptor
TLR	receptores tipo toll	Toll-like receptor
TNF	factor de necrosis tumoral alfa	tumor necrosis factor
TNFRSF1A (TNFR1)	miembro 1A de la superfamilia de receptores de TNF	TNF receptor superfamily, member 1A
TNFRSF1B (TNFR2)	miembro 1B de la superfamilia de receptores de TNF	TNF receptor superfamily, member 1B
Tollip	proteína de interacción con Toll	toll interacting protein
TRAF	factor asociado al receptor de TNF	TNF receptor associated factor
TUNEL	Marcado del extremo libre por dUTP	TdT – mediated dUTP Nick – end labelling
TXN	tiorredoxina	thioredoxin
FU/UF	fluido uterino	uterine fluid
VEGF	factor de crecimiento endotelial vascular	vascular endothelial growth factor
VEGFR	receptor de VEGF	VEGF receptor

WB	western blot	western blot
XCI	inactivación del cromosoma X	X Chromosome Inactivation
YWHAZ	proteína 14-3-3 zeta/delta	14-3-3 protein zeta/delta
ZP	zona pelúcida	pellucid zone

TABLA DE CONTENIDOS

CAPÍTULO 1 INTRODUCCIÓN GENERAL

ANTECEDENTES

La biotecnología reproductiva es una herramienta esencial para la sostenibilidad de la ganadería (Wu and Zan 2012). Entre las técnicas reproductivas disponibles, destaca la producción de embriones *in vitro*, (IVEP) seguida de transferencia a hembras receptoras (Hasler 2014). Las ventajas de la IVEP frente a otras técnicas, tales como la superovulación e inseminación artificial, son numerosas. Entre ellas, se encuentran el solventar problemas de infertilidad de vacas con alto potencial genético; incrementar la eficiencia de la producción de embriones; aumentar la intensidad de la selección genética en el tiempo; y preseleccionar el sexo del embrión mediante el uso de semen sexado. Además de su notable interés comercial, la investigación a partir de embriones bovinos producidos *in vitro* proporciona información muy valiosa acerca del desarrollo embrionario y otros procesos celulares en mamíferos (Lonergan 2007). En este sentido, el modelo bovino (*Bos primigenius taurus* o *Bos taurus*; Linneo, 1758) es un buen referente para la investigación en humanos. Ambas especies comparten varias características reproductivas importantes, como son la ovulación de un único folículo (Malhi et al. 2005); el metabolismo embrionario (Baumann et al. 2007); o la expresión de ciertos genes relacionados principalmente con la actividad hormonal (Barreto et al. 2011).

Tras el inicio de las primeras transferencias embrionarias en 1970 y el nacimiento del primer ternero fruto de la fecundación *in vitro* en 1982 (Brackett et al. 1982), se han producido importantes avances en la biotecnología reproductiva durante las últimas cuatro décadas (Hasler 2014). No obstante, solamente un 30 - 40 % de los ovocitos, recuperados a partir de vacas de matadero o usando la técnica *ovum pickup* en animales vivos, alcanzan el estadio de blastocisto, en el cual los embriones pueden ser transferidos a receptoras o criopreservados para su uso futuro. Por otro lado, la calidad de los EPIV es menor que la de los embriones recogidos *in vivo* (Rizos et al. 2002), o incluso que la de los embriones fertilizados *in vitro* pero cultivados en el tracto reproductivo materno durante un período de tiempo (Enright et al. 2000). Esta merma se detecta en términos de criotolerancia (Leibo and Loskutoff 1993), expresión génica (Corcoran et al. 2007), capacidad para establecer preñez (Hasler 2000) y cambios epigenéticos (Urrego et al. 2014). Estas evidencias han llevado a concluir que el período de cultivo *in vitro*, comprendido entre la fertilización y el estadio de blastocisto, es

especialmente sensible a factores que afectan a la calidad embrionaria (Lonergan et al. 2006).

Uno de los retos actuales en Biotecnología Reproductiva consiste en diseñar condiciones de cultivo *in vitro* similares al ambiente uterino en el que se desarrolla el embrión temprano. Este ambiente es el resultado de una compleja interacción entre el endometrio y el embrión, esencial para el posterior establecimiento y mantenimiento de la preñez (Lonergan and Forde 2014). Por tanto, el estudio e identificación de proteínas que intervienen en la comunicación materno-embrionaria durante el período de formación del blastocisto en el útero (de Día 5 a Día 8) puede ayudar a mejorar las técnicas de cultivo de embriones *in vitro*. Para avanzar un paso más, se puede tener en cuenta la influencia del sexo del embrión en la expresión de estas proteínas, dado el creciente interés en la producción de embriones con semen sexado (Wheeler et al. 2006) y la existencia de notables diferencias metabólicas entre embriones machos y hembras. Así se ha demostrado que los embriones bovinos macho y hembra presentan diferencias durante su desarrollo, en términos de expresión génica (Bermejo-Álvarez et al. 2010, Gutiérrez-Adán et al. 2000), metabolismo (Muñoz et al. 2014, Sturmey et al. 2010), regulación epigenética (Bermejo-Álvarez et al. 2008, Dobbs et al. 2013) y respuesta a factores embriotróficos secretados por el endometrio (Dobbs et al. 2014). Asimismo, el endometrio presenta diferentes patrones de expresión génica en respuesta a embriones producidos mediante diferentes técnicas de manipulación *in vitro* o a embriones de diferente calidad (Beltman et al. 2013, Mansouri-Attia et al. 2009a, Sandra et al. 2011). Por tanto, es plausible que el endometrio también exprese diferentes proteínas en función del sexo del embrión.

Numerosos estudios han analizado los cambios que tienen lugar en el transcriptoma del endometrio bovino en respuesta a embriones durante la preimplantación (Bauersachs and Wolf 2013, Forde and Lonergan 2012, Klein et al. 2006, Mansouri-Attia et al. 2009b, Spencer et al. 2013). Sin embargo, los cambios en la expresión génica no siempre son seguidos de cambios en la expresión de proteínas (Kyama et al. 2006), que son las moléculas realmente efectoras. Por tanto, la presente tesis se ha enmarcado en el análisis proteómico de la comunicación materno-embrionaria. En bovino, se han reportado cambios en el proteoma del endometrio o del fluido uterino durante el desarrollo preimplantacional (Beltman et al. 2014, Berendt et al. 2005, Forde et al.

2014, Muñoz et al. 2012, Okumu et al. 2014), aunque hasta la fecha no se habían abordado en función del sexo del embrión.

COMUNICACIÓN MATERNO-EMBRIONARIA EN BOVINO

Señales del embrión y respuesta uterina

La síntesis y secreción de citoquinas y factores de crecimiento (GF), tanto por la parte materna como por el lado embrionario, es esencial para crear un ambiente adecuado que favorezca el establecimiento y mantenimiento de la preñez (Bazer et al. 2010). Estas moléculas actúan sobre receptores presentes en las células diana, de forma autocrina o paracrina (Figura 1.1). Entre estas citoquinas destaca el interferón tau (IFNT), la principal señal para el reconocimiento materno de preñez en rumiantes (Betteridge et al. 1980, Northey and French 1980, Spencer and Bazer 2004). El IFNT es producido y secretado por el trofectodermo (Farin et al. 1989), alcanzando la máxima concentración celular alrededor del Día 16 de preñez en la vaca (Charlier et al. 1989, Godkin et al. 1982, Hansen et al. 1988, Martal et al. 1979). Curiosamente, de forma simultánea a la implantación del blastocisto, entre los Días 19 y 21, los niveles de proteína y mRNA de IFNT decaen bruscamente (Ealy and Yang 2009, Guillomot and Guay 1982). La inducción de la secreción de IFNT podría estar determinada genéticamente ya que esta citoquina es secretada por blastocistos producidos completamente *in vitro* (Hernandez-Ledezma et al. 1993, Stojkovic et al. 1999). No obstante, el endometrio parece ejercer un papel fundamental en el control de la secreción del IFNT, posiblemente mediante la secreción de otras citoquinas y GF (Emond et al. 2004, Imakawa et al. 1993, Imakawa et al. 1995, Ko et al. 1991). El IFNT puede actuar de forma autocrina, regulando la elongación del blastocisto (Wang et al. 2013), o de forma paracrina, previniendo la lisis del cuerpo lúteo y manteniendo la síntesis de progesterona (Robinson et al. 2008) (Figura 1.1).

Los genes del endometrio estimulados por IFNT (ISG) se activan principalmente en el estroma y en el epitelio glandular (Choi et al. 2003, Johnson et al. 2002, Song et al. 2007). La transcripción de los ISG y de otros genes regulados por la progesterona activan diversas vías moleculares implicadas en la regulación de la receptividad uterina y la implantación del embrión (Forde et al. 2011a, Forde et al. 2009, Spencer et al. 2008). Todos estos mecanismos muestran la complejidad de la comunicación materno-embrionaria, que implica la interacción de diferentes tipos celulares mediante la secreción de numerosas proteínas.

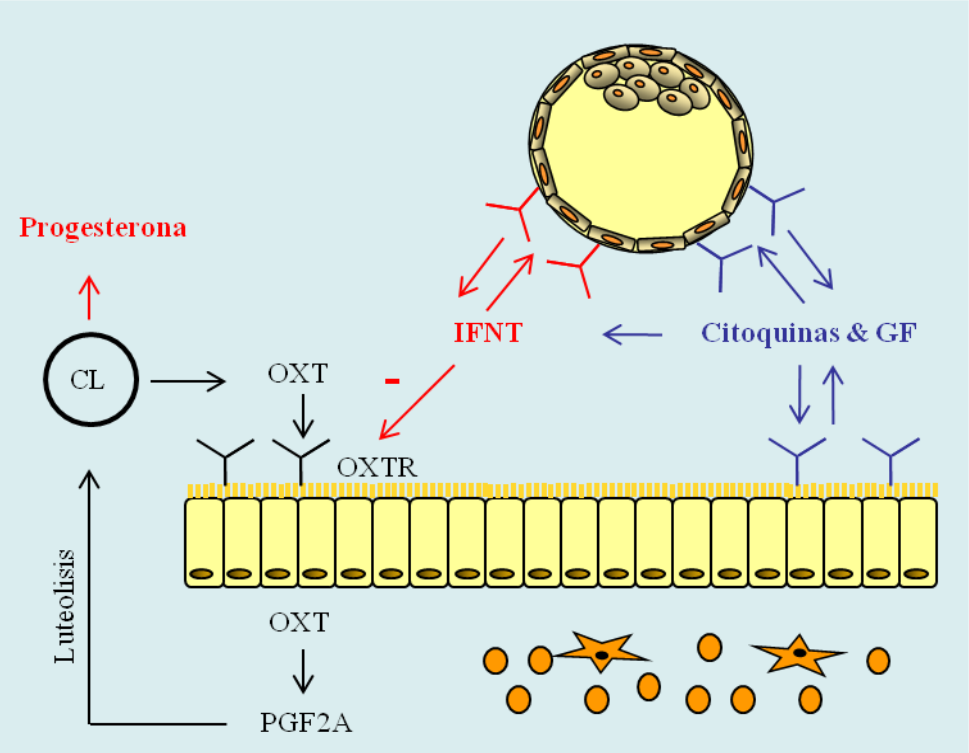

Figura 1.1 Esquema de la comunicación materno-embrionaria mediante citoquinas y factores de crecimiento (GF). Se detalla el papel del IFNT en la inhibición de la luteolisis. La oxitocina (OXT) secretada por el cuerpo lúteo (CL), se une a sus receptores uterinos (OXTR) induciendo la expresión de la prostaglandina F2 alfa (PGF2A). IFNT inhibe este proceso mediante el bloqueo de la expresión de OXTR. Así, se mantiene la producción de progesterona. A su vez, la producción de IFNT puede ser regulada por otras citoquinas y GF.

La proteómica y la comunicación materno-embrionaria

La biotecnología reproductiva se está beneficiando considerablemente de los métodos que analizan cambios globales en el DNA (genómica), o en la expresión de genes (transcriptómica), proteínas (proteómica) y metabolitos (metabolómica), de un tejido o medio biológico. Estos métodos permiten obtener una visión global de las complejas interacciones que tienen lugar durante el desarrollo embrionario (Egea et al. 2014, Seli et al. 2010, Upadhyay et al. 2013, Wolf et al. 2003). En la especie bovina, numerosos estudios han desvelado la existencia de genes que se expresan diferencialmente en el endometrio durante el ciclo estral (Bauersachs et al. 2008, Bauersachs et al. 2005, Forde et al. 2011a, Mitko et al. 2008), en respuesta a la preñez durante estadios preimplantacionales (Bauersachs et al. 2006, Bauersachs et al. 2012, Forde et al. 2009, Forde et al. 2011b, Forde et al. 2012, Forde et al. 2013, Mansouri-Attia et al. 2009b, Spencer et al. 2013, Walker et al. 2010) e incluso en respuesta a embriones de diferente origen o viabilidad (Beltman et al. 2010, Beltman et al. 2013, Mansouri-Attia et al. 2009a, Sandra et al. 2011). Estos estudios aportan información muy valiosa sobre rutas

moleculares que podrían estar implicadas en el establecimiento de la receptividad uterina o en el desarrollo embrionario. Si además la información transcriptómica del embrión y el endometrio se combinan, identificando ligandos y receptores en ambos tejidos (Carter et al. 2010, Mamo et al. 2012), el análisis de la comunicación materno-embrionaria es más completo.

Sin embargo, los cambios en el mRNA no siempre se corresponden con cambios en las proteínas, que son las moléculas realmente efectoras (Kyama et al. 2006, Stephens et al. 2010). Así, existen numerosos pasos a lo largo del desarrollo embrionario en los que la transcripción *de novo* está silenciada (Kotini et al. 2011, Monk 1995) y también numerosos mecanismos de control postranscripcional y postraduccional. Entre ellos figuran la maduración, transporte, almacenamiento, degradación y traducción del mRNA (Mitchell and Tollervey 2000, Moore 2005, Orphanides and Reinberg 2002), así como la activación y fosforilación de proteínas (Landry et al. 2014). Por todo ello, la proteómica resulta más útil que la transcriptómica en el estudio de la comunicación materno-embrionaria. En contraste con la transcriptómica, los análisis proteómicos son mucho menos abundantes debido a que requieren instrumental y personal más especializado y por tanto, un coste superior (Conrotto and Souchelnytskyi 2008). En bovino, solamente se han analizado cambios en el proteoma del endometrio en respuesta a la preñez en Día 18 (Berendt et al. 2005), aunque en otras especies, especialmente en humano, existen más estudios (Al-Gubory et al. 2014, Chen et al. 2009, DeSouza et al. 2005, Dominguez et al. 2009, Rai et al. 2010).

El análisis de biopsias endometriales presenta ciertas limitaciones ya que éstas no representan a la totalidad del endometrio sino a una pequeña parte del mismo. Esto dificulta la comparación de los resultados procedentes de diferentes estudios (Cheong et al. 2013). El análisis proteómico del fluido uterino puede solventar estos inconvenientes puesto que éste representa una integración de todos los compartimentos endometriales. Además, el fluido uterino puede considerarse un reflejo de los componentes que son secretados por el endometrio o el embrión, y que podrían ejercer un papel en la comunicación materno-embrionaria (Salamonsen et al. 2013). En bovino, existen análisis proteómicos del fluido uterino durante el ciclo estral (Faulkner et al. 2012, Mullen et al. 2012), durante la formación del blastocisto (Día 5 - Día 8) (Muñoz et al. 2012), en respuesta a embriones de diferente calidad en Día 7 (Beltman et al. 2014) y también en respuesta a la preñez, durante el reconocimiento materno de la preñez

(alrededor del Día 16) (Forde et al. 2014). El estudio realizado por Muñoz y colaboradores, ha sentado la base de parte de la presente tesis, por lo que a continuación se resumirán los resultados más relevantes.

El fluido uterino procedente de novillas a las que se les transfirió medio con y sin embriones, se analizó mediante electroforesis bidimensional diferencial en gel (2D-DIGE) acoplada a espectrometría de masas. Así, se identificaron 38 proteínas diferencialmente reguladas en el fluido uterino con y sin embriones. Tres de ellas, la Twinfilina, la proteína SYNCRIP, que interacciona con el RNA citoplásmático y se une a la sinaptotagmina, y el GF derivado del hepatoma (HDGF) no habían sido identificadas nunca en el útero de mamíferos. La agrupación funcional de las proteínas reguladas diferencialmente mediante la aplicación Ingenuity Pathway Analysis de QIAGEN (IPA®, QIAGEN Redwood City,www.qiagen.com/ingenuity), mostró una red de interacciones relacionada con el complejo proteico NFKB (factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas). En concreto, mediante western blot, se demostró una regulación a la baja de la subunidad p65 del complejo NFKB, lo cual contribuiría a explicar el privilegio inmunológico del embrión durante el desarrollo temprano. Algunas de las proteínas reguladas diferencialmente en el fluido uterino en función de la presencia de embriones, fueron seleccionadas para su estudio en la presente tesis: el factor de necrosis tumoral alfa (TNF), la interleuquina 1 beta (IL1B), potentes activadores de NFKB; y HDGF.

TNF

TNF es una citoquina inflamatoria con múltiples funciones, incluyendo la mediación en procesos reproductivos (Aggarwal et al. 2012, Haider and Knöfler 2009). La proteína fue aislada por primera vez en el suero de ratón como un factor secretado por macrófagos y causante de necrosis en células tumorales (Carswell et al. 1975). Pertenece a la superfamilia de TNF, compuesta por otros 18 ligandos y 29 receptores. El TNF se une a dos receptores, el receptor de TNF 1 (TNFR1) y el receptor de TNF 2 (TNFR2) (Locksley et al. 2001). Tanto el ligando como sus receptores son homotrímeros, que pueden formar estructuras transmembrana (Chan et al. 2000, Kriegler et al. 1988, Tang et al. 1996) o bien dar lugar a formas solubles tras su escisión de la membrana (Black et al. 1997, Wallach et al. 1990). La forma soluble de los receptores podría neutralizar al TNF, aunque su afinidad por él es mucho menor que la de los receptores de membrana (Ahn et al. 2010, Moosmayer et al. 1996).

TNFR1 se expresa constitutivamente en la mayoría de tejidos, mientras que TNFR2 está sujeto a una fuerte regulación y se encuentra predominantemente en las células inmunes y endoteliales (Grell et al. 1995). La activación de TNFR2 por el TNF, puede desencadenar varias vías de señalización (Haider and Knöfler 2009) (Figura 1.2).

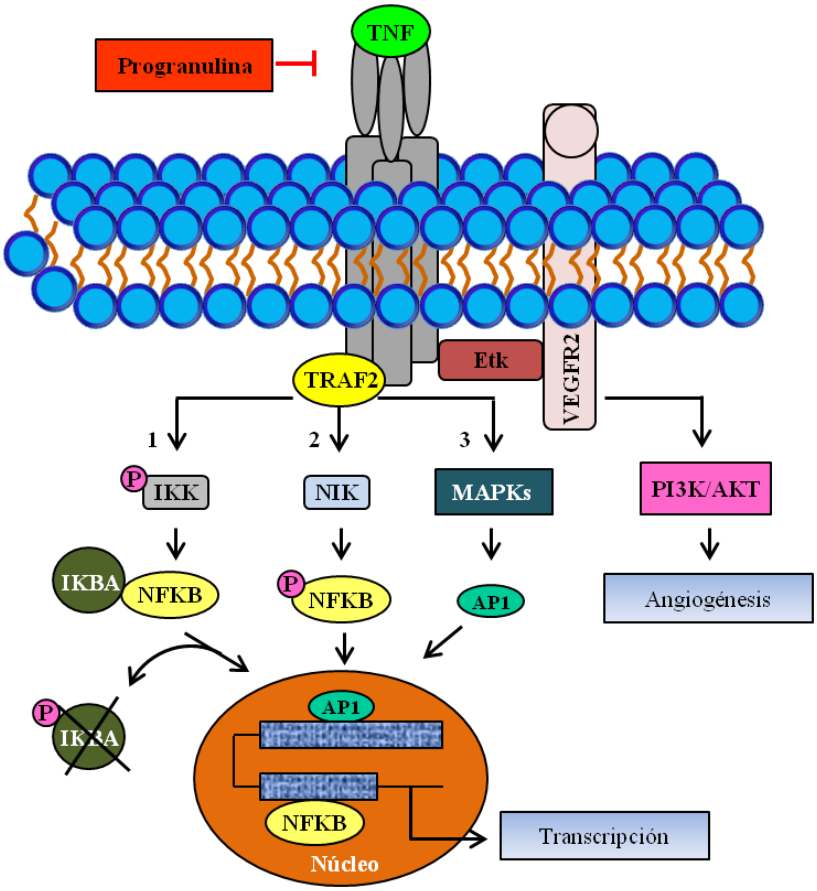

Figura 1.2 Vías de señalización del TNF tras su unión a TNFR2. Modificada de Moelants et al. (2013). La progranulina es un antagonista de TNF. TNFR2, mediante su unión a TRAF2 (factor 2 asociado al receptor de TNF), puede activar tres vías de señalización: 1) Activación clásica de NFKB mediante el complejo IKK que actúa degradando al inhibidor alfa de NFKB (IKBA); 2) Activación alternativa de NFKB mediante la quinasa inductora de NFKB (NIK), la cual fosforila y activa a NFKB; 3) Activación de la proteína activadora 1 (AP1) mediante las quinasas activadas por mitógenos (MAP3K y JNK). Ambos factores activos, NFKB y AP1, regulan la transcripción promoviendo la supervivencia, proliferación, adhesión y migración celular. Además, TNFR2, mediante su unión a la proteína adaptadora Etk (tirosina quinasa epitelial/endotelial) y a VEGFR2 (receptor del factor de crecimiento endotelial vascular), puede activar la vía PI3K/AKT (fosfoinositol 3-quinasa/B-quinasa) promoviendo la angiogénesis. TNF también puede unirse a TNFR1, el cual activa las vías 1, 2 y 3, aunque también puede promover la apoptosis mediante la activación de caspasas (no representado).

El efecto final de TNF parece depender de la interacción entre la concentración de TNF, el tipo celular y tisular, la distribución de TNFR2 y la duración del estímulo provocado por el TNF (Beutler et al. 1985, Männel and Echtenacher 2004). El TNF suele actuar mediante la vía habitual ligando-receptor, pero también puede utilizar una señalización inversa, consistente en que la señal es transmitida del receptor a la célula que porta el ligando transmembrana (Horiuchi et al. 2010, Watts et al. 1999, Wiley et al. 1996).

Numerosos estudios han investigado los patrones de expresión del TNF y TNFR2 en el tracto reproductivo de diversas especies. Ambos han sido detectados en el endometrio, el embrión preimplantacional y el fluido uterino (sólo TNF) en bovino (El-Sayed et al. 2007, Groebner et al. 2010, Mamo et al. 2012, Muñoz et al. 2012, Okuda et al. 2010) y en otras especies (Boomsma et al. 2009, Galvão et al. 2013, Hunt et al. 1996, Payan-Carreira et al. 2011). Además, el TNF y TNFR2 han sido localizados en la placenta de humano, ratón, rata y perro (Ben-Yair et al. 1997, Chen et al. 1991, Payan-Carreira et al. 2011, Yelavarthi and Hunt 1993, Yelavarthi et al. 1991), donde el TNF podría tener un efecto en el control de la proliferación del trofoblasto e invasión (Haider and Knöfler 2009).

En el endometrio bovino, la expresión de TNF y TNFR2 ha sido estudiada a lo largo del ciclo estral (mRNA y proteína) y en los Días 12, 15 y 18 de preñez (mRNA) (Groebner et al. 2010, Okuda et al. 2010). Durante el ciclo estral, TNF y TNFR2 están presentes en el epitelio luminal, epitelio glandular, endotelio y estroma, aunque débilmente en este último (Okuda et al. 2010). La expresión de TNF y TNFR2 varía a lo largo del ciclo en bovino y en otras especies (Galvão et al. 2013, Groebner et al. 2010, Okuda et al. 2010, Payan-Carreira et al. 2011, Roby and Hunt 1994, Tabibzadeh et al. 1995), lo que indica que el TNF podría estar implicado en la regulación de la receptividad uterina. De hecho, las células endometriales bovinas cultivadas *in vitro* responden a la presencia de TNF incrementando la expresión de la prostaglandina E2 (PGE2) y de la prostaglandina F2 alpha (PGF2A) (Murakami et al. 2001, Skarzynski et al. 2000). Además, se ha demostrado que el TNF puede modular la función del cuerpo lúteo *in vivo*, de forma dependiente a su concentración (Korzekwa et al. 2008, Skarzynski et al. 2007). La regulación de la expresión de TNF en las células endometriales *in vitro* puede ser mediada por el estradiol-17 beta, la progesterona (Hansen 1994), y por otras citoquinas (interleuquina 1 alfa, IL1A, y el propio TNF) (Okuda et al. 2010). De forma similar, la expresión de TNFR2 en las células endometriales está regulada por la oxitocina (Okuda et al. 2010).

El blastocisto bovino contiene la proteína TNF (Muñoz et al. 2012) y el mRNA de *TNFR2* (Groebner et al. 2010). Al igual que las células endometriales, las células embrionarias pueden responder a la presencia de TNF. La adición de TNF al medio de maduración de los ovocitos bovinos disminuye la proporción de ovocitos que alcanzan el estadio de blastocisto en Día 8 (Soto et al. 2003). Por otro lado, la adición de TNF al

medio de cultivo tras la fertilización *in vitro*, puede no tener efecto sobre el desarrollo o bien disminuir el porcentaje de blastocistos en Día 7 (Jackson et al. 2012, Soto et al. 2003). Curiosamente, los efectos del TNF en el embrión parecen estar mediados por las prostaglandinas, al igual que en el endometrio (Jackson et al. 2012). En todo caso, los efectos de TNF sobre el desarrollo *in vitro* parecen depender de la concentración de TNF, el momento en el que se añade e incluso del tipo de medio de cultivo.

La presencia de TNF en el fluido uterino (Boomsma et al. 2009, Muñoz et al. 2012, von Wolff et al. 1999) indica que éste puede actuar como mediador en la comunicación materno-embrionaria. Tanto el endometrio como el embrión humanos, producen y secretan TNF *in vitro* (Tabibzadeh et al. 1995, Witkin et al. 1991). En bovino, el TNF se encuentra diferencialmente regulado en el fluido uterino por la presencia de embriones (Muñoz et al. 2012). Además, en humano, los niveles de TNF en el fluido uterino previamente a la transferencia embrionaria se correlacionan positivamente con las tasas de preñez (Boomsma et al. 2009).

IL1B

IL1B fue identificada por primera vez en 1980 al estudiar la respuesta inflamatoria aguda (Smith et al. 1980). Actualmente se considera que esta citoquina podría tener un papel clave en generar la inmunotolerancia necesaria para la implantación y en la aparición de la viviparidad placentaria (Paulesu et al. 2008). La señalización mediada por IL1B depende de la expresión de otros miembros del sistema IL1. Éste consta de dos agonistas: IL1A e IL1B; dos receptores: interleuquina 1 receptor, tipo I (IL1R1) e interleuquina 1 receptor, tipo II (IL1R2); una proteína accesoria del receptor (IL1RAP); y una proteína antagonista del receptor (IR1RN) (Dinarello 1994). IL1B se expresa como un precursor inactivo de 31 kDa, que una vez procesado intracelularmente por la caspasa-1 da lugar a su forma madura bioactiva de 17 kDa (Martinon et al. 2009, Thornberry et al. 1992). Ambas proteínas, IL1B y su precursor pueden ser secretadas, utilizando una vía de secreción no clásica (Keller et al. 2008, Nickel 2003).

Una vez secretada, IL1B se une al receptor de membrana IL1R1 presente en las células diana (Vigers et al. 1997). La activación del receptor induce la unión de IL1RAP al complejo IL1R1/IL1B, lo cual es esencial para la transducción de señal (Wesche H 1997). El IL1R1 es portador de un dominio IL1 tipo toll (TIR) en su parte citoplasmática, común con los receptores tipo toll (TLR) y con el receptor de IL18

(O'neill 2000). A este dominio se une un complejo de múltiples proteínas que activa al NFKB y a varias proteínas quinasas activadas por mitógenos (MAPKs) (Figura 1.3).

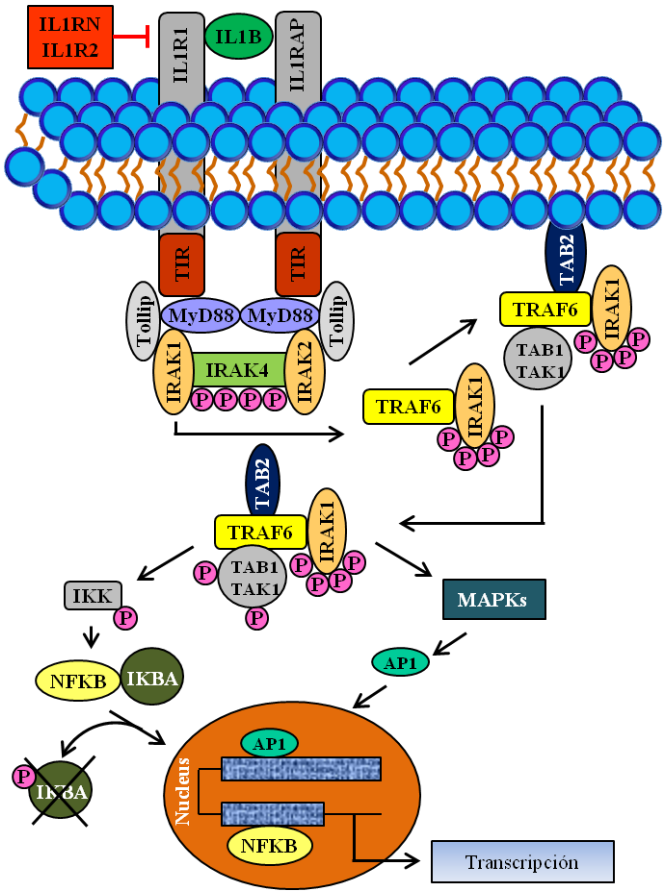

Figura 1.3 Vías de señalización de IL1B mediante su unión a IL1R1. Modificada de Multhoff et al. (2011). Existen dos inhibidores de IL1B, el antagonista IL1RN, y el *decoy receptor* IL1R2. Una vez que se ha unido IL1B y la proteina accesoria (IL1RAP), la forma heterodímera del receptor comienza la señalización. A su dominio TIR se une un complejo de múltiples proteínas, que incluye a las proteínas adaptadoras, Myd88 y Tollip, y a varias quinasas asociadas a IL1R (IRAK1, IRAK2 e IRAK4). La hiperfosforilación resultante lleva a la disociación de IRAK1 y TRAF6 (factor 6 asociado al TNFR) y a la formación de un nuevo complejo intermediario de membrana que también incluye a proteínas activadas por el factor de crecimiento transformante beta (TAB1, TAB2 y TAK1). En el citoplasma, este complejo puede activar a NFKB a través de las quinasas IKK, o a la proteína activadora 1 (AP1) a través de varias MAPKs. Finalmente, en el núcleo se regula la transcripción promoviendo la supervivencia, proliferación, adhesión y migración celular.

La activación de IL1R1 conlleva la estabilización del mRNA y la regulación de la transcripción génica (Weber et al. 2010). Existen al menos dos reguladores de la actividad de IL1B. Uno de ellos, IL1RN, actúa como antagonista de IL1B, uniéndose a IL1R1 (Eisenberg et al. 1990). El complejo IL1R1/IL1RN impide la unión de la proteína accesoria y por tanto no se produce señalización (Greenfeder et al. 1995). El segundo regulador es el receptor ILR2, que actúa como un *decoy receptor* (McMahan et al. 1991). Su dominio extracelular es similar al de ILR1 y por tanto presenta una alta afinidad por IL1B. Sin embargo, su dominio citoplasmático es mucho más corto y por

tanto no puede mediar ninguna señal biológica. La forma de membrana de IL1R2 puede ser liberada de la superficie celular por medio de la misma metaloproteínasa que libera al TNF (Orlando et al. 1997).

IL1B parece desempeñar un importante papel en el desarrollo embrionario, regulando la implantación y la comunicación materno-embrionaria (Bazer and Johnson 2014, Krüssel et al. 2003, Lindhard et al. 2002). Varios miembros del sistema IL1 (IL1B, IL1R1 y IL1RN) están presentes en el tracto reproductivo y embriones de humano (De los Santos et al. 1996, Hunt et al. 1992, Simón et al. 1996, Simón et al. 1993), ratón (Kruessel et al. 1997, McMaster et al. 1992, Takacs and Kauma 1996) y cerdo (Kruessel et al. 1997). Además, IL1R2 se encuentra también en el endometrio humano (Boucher et al. 2001). En bovino, se ha localizado IL1B en células endometriales durante el ciclo estral (Paula-Lopes et al. 1999, Tanikawa et al. 2009), en el embrión (Muñoz et al. 2012) y en el fluido uterino (Davidson et al. 1995, Muñoz et al. 2012); e *IL1R1*en células endometriales durante el ciclo estral (Tanikawa et al. 2005, Tanikawa et al. 2009).

En el endometrio bovino, la proteína IL1B se expresa en el epitelio luminal, el epitelio glandular, el estroma y el endotelio. La expresión en el epitelio luminal es más intensa el Día del celo (Día 0) que en la fase luteal media (Día 7), mientras que la expresión en el epitelio glandular permanece constante a lo largo del ciclo (Paula-Lopes et al. 1999). El mRNA de *IL1R1* se expresa en mayor abundancia en la fase luteal tardía del ciclo estral (Tanikawa et al. 2005) y se encuentra presente tanto en las células epiteliales como estromales (Tanikawa et al. 2009). De forma similar al efecto de TNF sobre las células endometriales cultivadas *in vitro,* IL1B incrementa la producción de las prostaglandinas PGE2 y PGF2A en las células endometriales o estromales, dependiendo de la fase del ciclo estral (Betts and Hansen 1992, Davidson et al. 1995, Tanikawa et al. 2009). IL1B también inhibe el crecimiento de las células estromales, aunque no de las epiteliales (Davidson et al. 1995). La citoquina está además implicada en la degradación de la matriz extracelular mediante la producción de plasminógeno en las células epiteliales (Tanikawa et al. 2009). En humano, IL1B induce la expresión endometrial de varias proteínas relacionadas con la implantación y receptividad uterina (Geisert et al. 2012).

En el blastocisto bovino, solamente se ha localizado la proteína IL1B (Muñoz et al. 2012), pero la expresión de su receptor IL1R1 no ha sido investigada. No obstante, las

células embrionarias bovinas también responden a IL1B. Se ha demostrado que la adición de IL1B a las 8 - 10 horas postinseminación, incrementa el porcentaje de ovocitos que alcanzan el estadio de blastocisto en Día 9. Curiosamente, la respuesta embrionaria resultó ser dependiente del periodo de cultivo (la adición de IL1B en Día 5 no tuvo ningún efecto), de la dosis (la mejor concentración fue 0.1 ng/mL) y del número de embriones cultivados por gota (los efectos beneficiosos se observaron con una mayor densidad de blastocistos) (Paula-Lopes et al. 1998).

La presencia de la IL1B en el fluido uterino bovino es coherente con la participación de la citoquina en la comunicación materno-embrionario bovina durante el ciclo estral y la preñez (Davidson et al. 1995, Muñoz et al. 2012, Paula-Lopes et al. 1999). Además, los niveles de IL1B en el fluido uterino bovino están influenciados por la presencia de embriones (Muñoz et al. 2012). Diversos estudios en humano y cerdo apuntan al embrión como el principal responsable en la regulación de la secreción de IL1B, que actuaría de forma autocrina o paracrina. Así, el nivel de secreción de IL1B por los embriones humanos *in vitro* puede correlacionarse positivamente con el número de blastómeros y con el éxito de la preñez (Barañao et al. 1997, Taskin et al. 2012). Además, la secreción de IL1B por el embrión porcino se asocia temporalmente con el período de elongación del blastocisto y con el momento del reconocimiento materno de la preñez (Ross et al. 2003, Tuo et al. 1996).

HDGF

HDGF regula el crecimiento y diferenciación celular (Everett et al. 2000, Kishima et al. 2002, Oliver and Al-Awqati 1998). Fue identificado por primera vez en el medio de cultivo condicionado de hepatocitos humanos (Nakamura et al. 1994). Sus propiedades mitogénicas han sido demostradas en numerosos tipos celulares, incluyendo hepatocitos, fibroblastos (Nakamura et al. 1994), músculo liso (Everett et al. 2001) y endotelio (Oliver and Al-Awqati 1998). El HDGF puede actuar como agente tumorigénico (Tao et al. 2014, Wang et al. 2014), angiogénico (Li et al. 2013, Thirant et al. 2012), neurotrófico (Zhou et al. 2004), represor de la apoptosis (Lee et al. 2010), y mediador de la transición epitelio-mesenquimal (Song et al. 2014). La familia a la que pertenece, denominada HRPs (proteínas relacionadas con el HDGF), presenta seis homólogos que incluyen el HDGF, el primer miembro identificado y prototipo, HRP-1, HRP-2, HRP-3, HRP-4, y el GF derivado del cristalino (LEDGF). Todos los miembros de esta familia presentan un dominio de 100 residuos altamente conservado en el

extremo N-terminal, denominado dominio HATH o PWWP (proline-tryptophan-tryptophan-proline). Por el contrario, su extremo C-terminal presenta notables diferencias en longitud y carga (Dietz et al. 2002). A pesar de que HDGF no presenta la secuencia peptídica clásica de secreción celular, el péptido puede ser secretado por otras vías independientes de la secreción clásica mediada por el retículo endoplasmático. Un estudio reciente ha demostrado que el HDGF endógeno es transportado al exterior de las células HEK293 mediante diversos mecanismos, como son el procesamiento N-terminal de los primeros 10 residuos, la fosforilación del residuo S 165 y la unión intramolecular disulfato entre los residuos C12 y C 108 (Thakar et al. 2010).

El mecanismo de señalización celular desencadenado por HDGF aún no se ha aclarado completamente. HDGF se localiza principalmente en el núcleo, aunque en algunas células se encuentra también en el citoplasma (Everett et al. 2000, Nakamura et al. 1994, Oliver and Al-Awqati 1998). Recientemente se ha propuesto un modelo de señalización para HDGF, por el cual este GF, a través de su dominio HATH, se une a receptores o a proteoglicanos heparan sulfato (HS) presentes en la superficie celular (Wang et al. 2011) (Figura 1.4). Un posible regulador del HDGF es el GF de hepatocitos (HGF), el cual induce la expresión de HDGF en células cancerígenas (Lee et al. 2010). Curiosamente, HGF es una progestamedina que activa numerosos genes uterinos implicados en el crecimiento, morfogénesis o en la síntesis de enzimas y sus inhibidores, previamente a la adhesión del trofoblasto en el epitelio uterino (Bazer et al. 2009, Bazer et al. 2010, Geisert et al. 2012).

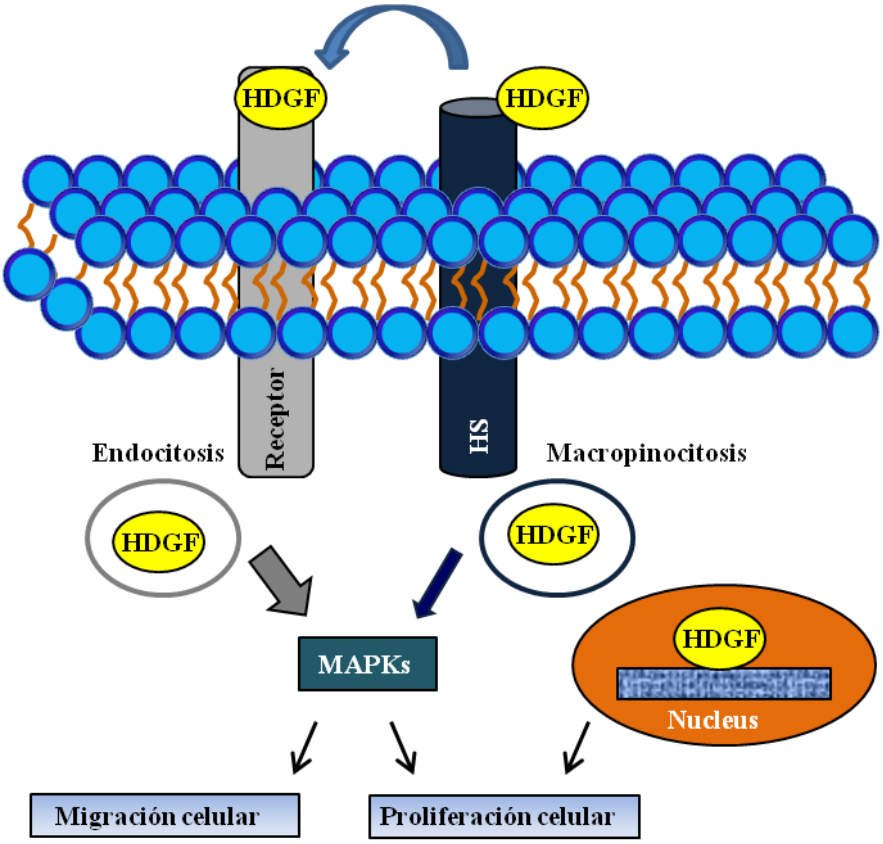

Figura 1.4 Vías de señalización del HDGF mediante su unión a receptores y proteoglicanos heparan sulfatos (HS), presentes en la superficie celular. Modificada de Wang et al. (2011). La unión de HDGF a los receptores se favorece mediante los HS. Cuando HDGF se une a los receptores, sufre un proceso de internalización por endocitosis mediada por caveolina, mientras que cuando se une a HS se internaliza por macropinocitosis. Una vez en el citoplasma, HDGF activa a la familia MAPK, principalmente mediante el mecanismo de internalización por endocitosis, promoviendo la proliferación y migración celular. HDGF también puede localizarse en el núcleo, donde regula la proliferación celular.

El posible papel de HDGF en la reproducción permanece desconocido. Recientemente, se ha descrito por primera vez la presencia de HDGF en el tracto reproductivo bovino. En concreto, este GF se encuentra en el fluido uterino bovino, donde es regulado positivamente por la presencia de embriones (Muñoz et al. 2012). Varios factores de crecimiento producidos por el tracto materno y que comparten vías de señalización y secreción con el HDGF, mejoran el desarrollo embrionario *in vitro* y la supervivencia a término tras transferencia embrionaria (Block et al. 2011, Bonilla et al. 2011, Fields et al. 2011, Lim et al. 2007, Sakagami et al. 2012). Por tanto, el HDGF es un buen candidato para su ensayo en el cultivo *in vitro* de embriones bovinos.

ANATOMÍA Y FISIOLOGÍA REPRODUCTIVA DE LA VACA

El éxito de los primeros eventos reproductivos y del posterior desarrollo del embrión depende en gran medida del estado del aparato reproductor femenino. En concreto, el útero, donde comienza el proceso de formación del blastocisto (Betteridge and Fléchon 1988), es fundamental para garantizar el establecimiento de la preñez (Horcajadas et al. 2007, Ulbrich et al. 2013).

Útero y endometrio

El útero presenta las siguientes funciones principales:

- Participa en el transporte de los espermatozoides (Suarez 2006).
- Regula la funcionalidad del cuerpo lúteo, glándula endocrina productora de progesterona y esencial para el mantenimiento de la gestación (Robinson et al. 2008).
- Produce y secreta factores necesarios para la nutrición del embrión (Bazer 1975).
- Desarrolla la parte materna de la placenta y modifica su tamaño y estructura para adaptarse a las necesidades del feto (Ferrell 1991).

En la especie bovina el útero es de tipo bicorne (Hafez and Hafez 1993) (Figura 1.5A). Consta de un cuello o cérvix, que lo comunica con la vagina; un cuerpo; y dos cuernos uterinos. Cada cuerno está conectado mediante el oviducto al ovario, órgano en el cual se encuentran las células germinales femeninas u ovocitos. Dependiendo de su posición respecto al ovario que presenta el cuerpo lúteo, los cuernos uterinos se denominan ipsilateral (mismo lado) o contralateral (lado contrario). Cada cuerno uterino puede dividirse en tres partes: caudal, media y craneal. (Bauersachs et al. 2005). La parte craneal es la que recibe al embrión desde el oviducto y donde se realiza la transferencia de embriones.

Al igual que la mayoría de órganos huecos, la pared uterina consta de una capa externa serosa o de perimetrio, una capa intermedia muscular o miometrio y una línea interna de membrana mucosa o endometrio (Figura 1.5B) (Peters and Ball 2004). En rumiantes, el endometrio consta de dos áreas diferentes tanto estructural como biológicamente: (1) las carúnculas, pequeñas prominencias aglandulares de origen estromal y muy

vascularizadas, que darán lugar a la parte maternal de la placenta una vez que ocurra la implantación embrionaria; y (2) las intercarúnculas, grandes áreas endometriales donde se concentran las glándulas, cuyas secreciones son cruciales para el mantenimiento del embrión (Atkinson et al. 1984, Gray et al. 2001).

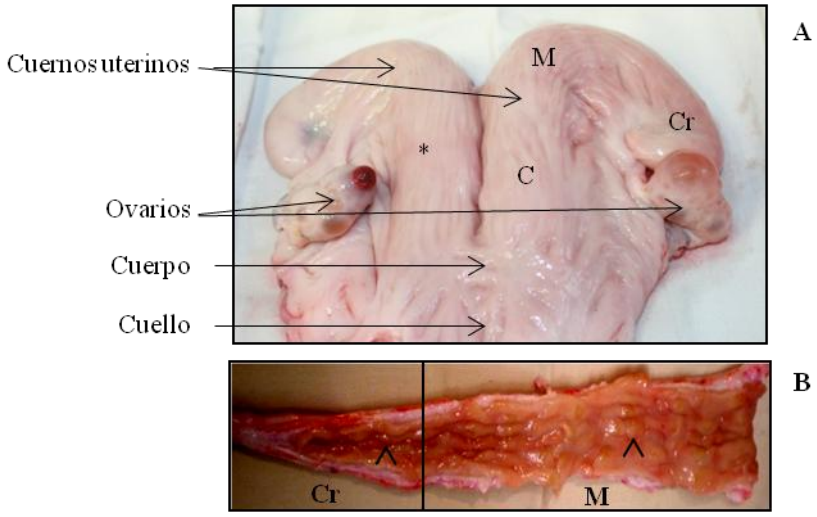

Figura 1.5A) Útero y ovarios bovinos, cara dorsal externa. El asterisco marca el cuerno uterino ipsilateral. El cuerno uterino presenta tres partes: caudal (C), media (M) y craneal (Cr). B) Cuerno uterino, abierto longitudinalmente y mostrando el endometrio. Se aprecian las carúnculas (cabeza de flecha), tanto en el tercio medio (M) como craneal (Cr).

Histología del endometrio

En el endometrio se pueden distinguir tres capas histológica y funcionalmente distintas (Figura 1.6). La más profunda o basal, el estrato basal adyacente al miometrio, apenas sufre cambios durante el ciclo estral y está formada por un tejido conectivo laxo. La ancha capa intermedia se caracteriza por un estroma de aspecto esponjoso, por lo que se llama estrato esponjoso. La capa superficial, la más delgada, tiene un estroma de aspecto compacto, por lo que se conoce como estrato compacto. Las capas esponjosa y compacta sufren una descamación cíclica seguida de regeneración durante el ciclo estral, por lo que en conjunto también se las llama estrato funcional (Young and Heath 2000). Distribuidos en estas tres capas se encuentran los diferentes tipos celulares que conforman el endometrio: el epitelio luminal, el epitelio glandular (ausente en las carúnculas), el estroma, los leucocitos endometriales y los vasos sanguíneos. (Figura 1.6a) (Young and Heath 2000).

El epitelio luminal es la primera estructura materna en contacto con el embrión que accede al útero. Se trata de un epitelio cilíndrico pseudoestratificado, formado por células provistas de microvellosidades en su borde apical, y algunas presentan cilios

(Figura 1.6b). Numerosos estudios indican que en las células del epitelio luminal se regula la receptividad uterina mediante la expresión diferencial de determinadas proteínas o sus receptores durante el ciclo estral o gestación (Bai et al. 2014, McCarthy et al. 2012, Ohta et al. 2014, Okumu et al. 2014). También pueden secretar proteínas al lumen uterino, aunque en menor medida que las glándulas (Uzumcu et al. 1998).

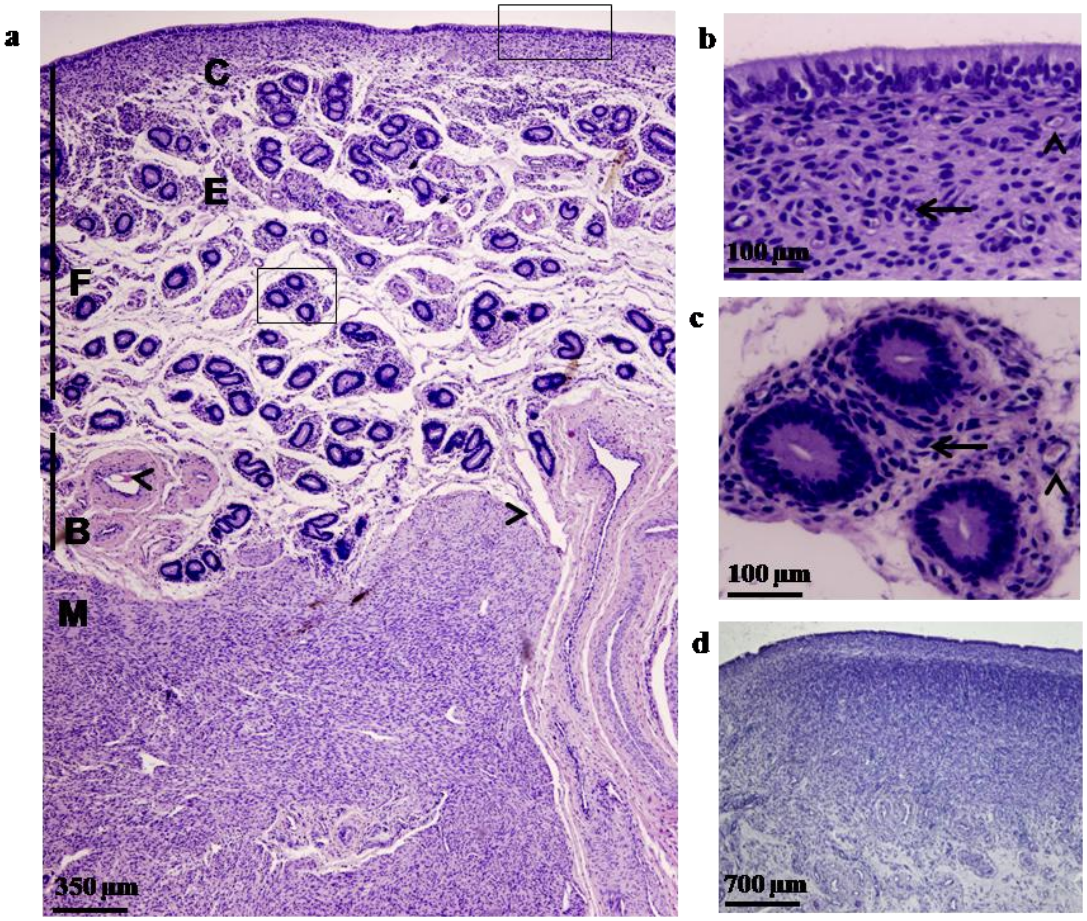

Figura1.6 Imágenes representativas del endometrio teñido con hematoxilina-eosina a) Histología general de una región intercaruncular, mostrando los estratos compacto (C), esponjoso (E), funcional (F) y basal (B), y el miometrio (M). Los rectángulos indican zonas fotografiadas a mayor aumento b) Epitelio luminal c) Epitelio glandular. Las flechas muestran células del estroma y las cabezas de flecha, vasos sanguíneos d) Carúncula.

Las glándulas uterinas tienen un epitelio cilíndrico simple uniforme en toda su extensión (Figura 1.6c). Forman ramificaciones tubulares simples, más o menos sinuosas, especialmente hacia su extremo. Las células glandulares transportan o sintetizan y secretan proteínas en la luz uterina, incluyendo iones, glucosa, citoquinas, factores de crecimiento, hormonas, y proteasas y sus inhibidores (Amoroso 1952, Bazer 1975, Forde et al. 2013). Estas proteínas son esenciales para el reconocimiento de la preñez y para el desarrollo del feto en diversas especies con implantación

sinepiteliocorial o no invasiva, como la bovina (Bazer et al. 2011, Geisert et al. 1992, Gray et al. 2002, Gray et al. 2001, Song et al. 2006).

El estroma es un tejido conectivo formado mayoritariamente por células de tipo fibroblástico, encargadas de secretar la mayor parte de la matriz extracelular. Las células estromales también pueden secretar proteínas y establecer interacciones con las células glandulares (Evron et al. 2011, Lessey et al. 2002).

Los leucocitos endometriales incluyen a los linfocitos T, macrófagos y granulocitos (Cobb and Watson 1995). Los linfocitos T y los macrófagos participan en la inmunosupresión, y los macrófagos también en la presentación de antígeno (Hansen 2007). Los granulocitos modulan la invasión trofoblástica *in vitro*, aunque su presencia en el endometrio de especies con implantación invasiva sugiere que presentan además otras funciones que quizás estén conservadas (Bulmer and Lash 2005). El número de leucocitos endometriales disminuye por acción de la progesterona (Majewski and Hansen 2002, Padua et al. 2005) y por la presencia de embriones durante la preimplantación (Groebner et al. 2011). Los leucocitos se han encontrado en tres localizaciones endometriales: intraepitelial, intersticial y en agregados linfoides en la parte basal del endometrio (Groebner et al. 2011, Leung et al. 2000).

A través del estroma endometrial discurre una compleja red vascular. En el miometrio, las arterias arqueadas se forman a partir de las arterias uterinas y ováricas, las cuales a su vez dan lugar a las arterias radiales. Después de cruzar la unión miometrio-endometrial, las arterias radiales se ramifican para formar las arterias basales y espirales en el endometrio. Las primeras irrigan la capa basal del endometrio, mientras que las segundas bañan la capa funcional. Las arterias espirales se ramifican nuevamente a lo largo de la capa funcional, para dar lugar a un prominente plexo subepitelial, el cual drena en senos venosos (Padykula 1989). A lo largo del ciclo estral, la red vascular endometrial presenta ciclos de crecimiento y regresión regulados por factores locales como el GF endotelial vascular (VEGF) y citoquinas (Banerjee et al. 2013). En humanos, un buen flujo sanguíneo en el endometrio se considera un marcador de receptividad uterina (Ng et al. 2004).

Ciclo estral bovino

La función del ciclo estral es la preparación del endometrio para una posible gestación, independientemente de la presencia de embriones. El ciclo estral típico en bovino se

sucede periódicamente a lo largo de todo el año en ausencia de fecundación y tiene una duración media de 21 Días en vacas y de 20 en novillas (Phillips 2008). El ciclo estral puede dividirse en tres fases (Peters 1985): fase luteal, estro, y fase folicular (Figura 1.7).

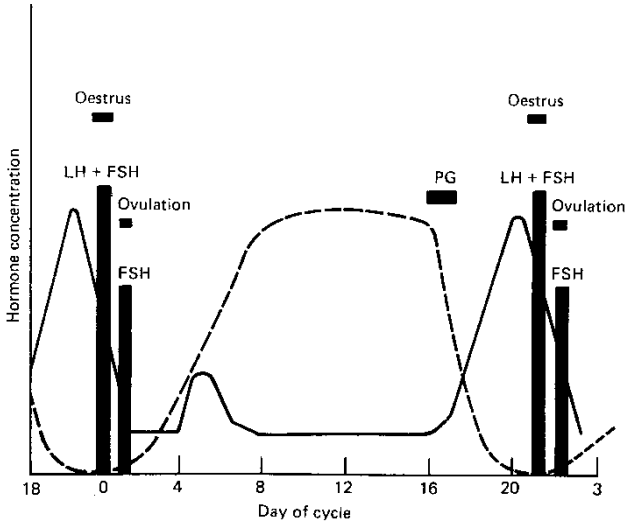

Figura 1.7 Esquema representativo de los cambios en los niveles hormonales durante el ciclo estral bovino. La línea sólida representa la concentración de estradiol; la línea punteada representa la concentración de progesterona (Peters 1985).

Durante la fase folicular (Día 19 - Día 21), la hormona foliculoestimulante (FSH) induce el crecimiento folicular y la maduración del folículo preovulatorio, el cual comienza a secretar estradiol. En ausencia de progesterona, el estradiol actúa sobre el sistema nervioso central para inducir el estro (Día 0), o período de receptividad sexual, que dura de 12 a 16 horas y marca el inicio del ciclo. Al final del estro se alcanza el pico máximo de las hormonas luteinizante (LH) y FSH, así como la ruptura del folículo destinado a ovular o folículo dominante. La ovulación (Día 1) trae consigo la formación del cuerpo lúteo, el cual sufre un proceso de maduración a lo largo de la fase luteal (Día 2 - Día 17). El cuerpo lúteo es una estructura endocrina temporal involucrada en la producción de altos niveles de progesterona y de niveles moderados de estradiol e inhibina A. La progesterona inhibe la liberación de la hormona liberadora de gonadotropinas (GnRH) por el hipotálamo, y por lo tanto de LH y FSH. Además, la progesterona es responsable de la preparación del útero para permitir la implantación del embrión y de mantener la gestación. Al final de la fase luteal se produce la lisis del cuerpo lúteo, dando pie al inicio de la fase folicular. La luteolisis ocurre como consecuencia de la secreción de PGF2A por el epitelio luminal y glandular superficial, lo que conlleva un brusco descenso en los niveles de progesterona. Tres hormonas

ováricas son las principales responsables de la secreción endometrial pulsátil de PGF2A: progesterona, estrógeno y oxitocina.

Cambios en el endometrio a lo largo del ciclo

La variación hormonal a lo largo del ciclo provoca una serie de modificaciones histológicas, secretoras e intracelulares en el endometrio (Dockery and Rogers 1989, Gargett et al. 2008).

Modificaciones histológicas y secretoras

Durante la fase folicular ocurre una descamación seguida de regeneración en las capas superficiales del endometrio, descrita principalmente en primates y humanos (Gargett et al. 2008, Mihm et al. 2011). En bovino, este proceso no ha sido tan estudiado, aunque recientemente se ha propuesto que la proliferación y apoptosis celular en el endometrio bovino está sujeta a variaciones cíclicas (Arai et al. 2013). Bajo la influencia del estradiol, aumentan el número de mitosis, el endometrio se engrosa (de 3 a5 mm) y el epitelio recubre de nuevo toda la superficie endometrial. Los tubos glandulares se alargan y el número de células ciliadas y de vasos sanguíneos se incrementan (Li et al. 1988).

Durante la fase luteal, la progesterona induce la síntesis y secreción de proteínas (Bazer et al. 2010). Por tanto, el endometrio no se engrosa más, pero las glándulas se alargan volviéndose tortuosas y alcanzan su máxima capacidad secretora. Los vasos sanguíneos también presentan circunvoluciones. En torno a los núcleos de las células secretoras aparecen gránulos de glucógeno que migran hacia la superficie celular y vierten su contenido a la luz glandular por exocitosis (Dockery et al. 1988). Entre las proteínas secretadas por el endometrio y que presentan un efecto sobre la función uterina o el desarrollo del embrión destacan cuatro factores de crecimiento:

- El GF epidérmico (EGF) tiene una acción paracrina y autocrina sobre el crecimiento celular e induce a los receptores de estradiol. En presencia de estrógenos se produce un aumento rápido del mRNA de *EGF* y sus receptores (Katagiri and Takahashi 2004).

Los GFs de fibroblastos (FGFs) participan en diversos eventos celulares como la proliferación, migración, diferenciación, angiogénesis y supervivencia celular (Powers et al. 2000). Algunos FGFs parecen tener un importante papel en la comunicación

materno-embrionaria ya que FGF2 estimula la producción de IFNT por el embrión bovino (Michael et al. 2006). Además, la expresión endometrial de varios FGFs varía a lo largo del ciclo y de la preñez temprana bovina (Día 7 - Día 19). Esta modulación está relacionada con los niveles de expresión de los receptores en el embrión (Okumu et al. 2014).

- El GF insulínico tipo 1 y tipo 2 (IGF1 e IGF2). Estas proteínas tienen un papel en la proliferación y diferenciación del tejido uterino y embrionario en bovino. (Keller 1998). Además, la expresión de ambas proteínas y sus factores de unión (IGFBP2 e IGFBP6) es regulada en el útero bovino durante la preñez o en función de los niveles de progesterona (McCarthy et al. 2012).
- El GF de transformación beta, TGFB. Actúa sobre la diferenciación celular, aumentando la concentración de los receptores de progesterona (Sugawara et al. 2010).

Modificaciones intracelulares: modificación de los receptores de esteroides

La cantidad y distribución de los receptores hormonales fluctúan a lo largo del ciclo estral. El estradiol, durante la fase folicular, estimula la síntesis y el aumento de concentración de los receptores de estrógenos y de progesterona (Meikle et al. 2001a, Spencer and Bazer 1995). La progesterona, durante la fase luteal, inhibe la expresión de sus receptores (PGR) en los epitelios luminal y glandular, mientras que los PGR en el estroma se mantienen. La unión de la progesterona a los PGR en las células estromales induce la expresión de HGF y de FGF por estas células. A su vez estos GFs actúan de forma paracrina en el epitelio adyacente (Chen et al. 2000a, Chen et al. 2000b, Koji et al. 1994, Spencer and Bazer 2002, Spencer et al. 2004b). Existe una clara asociación espacio-temporal entre la desaparición de los PGR en el epitelio y la receptividad uterina para la implantación (Geisert et al. 2005, Spencer and Bazer 2004). De hecho, la regulación a la baja de los PGR en el epitelio es un evento conservado entre mamíferos asociado con la apertura de la denominada ventana de implantación (Meikle et al. 2001b).

DESARROLLO EMBRIONARIO BOVINO

La reproducción en mamíferos se caracteriza por la fertilización, junto con un desarrollo embrionario temprano en el tracto reproductivo materno, seguido de la implantación del embrión en la pared del útero. Esto permite el desarrollo de la placenta, que aportará al feto los nutrientes y el oxígeno necesario (Bazer et al. 2009, Spencer et al. 2004a). (Figura 1.8).

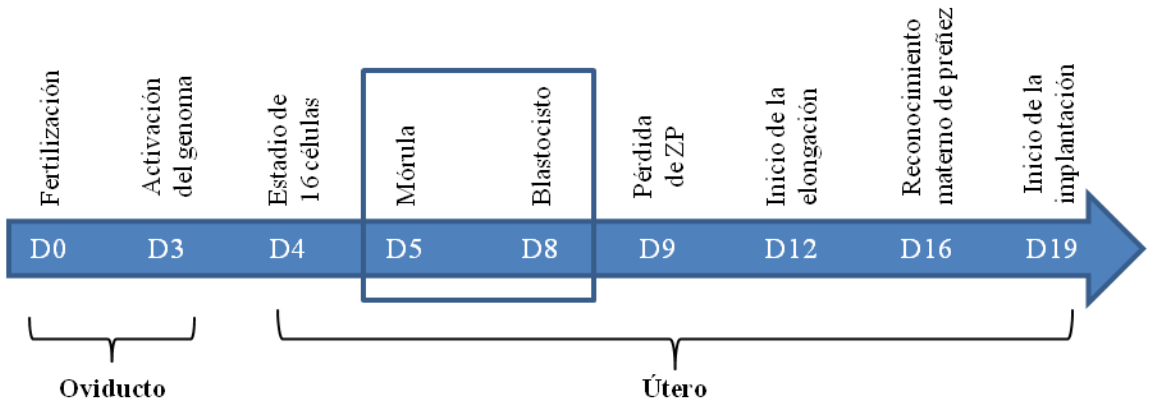

Figura 1.8 Principales eventos durante el desarrollo temprano bovino. El rectángulo muestra el período de estudio de la presente tesis. ZP: zona pelúcida.

Preimplantación

El período preimplantacional en bovino comienza con la fertilización y finaliza con la elongación del blastocisto. La fertilización del ovocito (Día 0) tiene lugar en el oviducto, donde el zigoto recién formado inicia las primeras divisiones nucleares y la activación del genoma embrionario tiene lugar (Día 3). Entre el Día 4 y el Día 5, el embrión entra en el útero, donde continúa dividiéndose A partir del quinto ciclo de división celular (32 células), que se corresponde con el Día 5 aproximadamente, el embrión comienza a compactarse (Bazer et al. 2009, Van Soom et al. 1997). La compactación consiste en la formación de íntimas uniones intercelulares, que junto con una disminución en el tamaño de los blastómeros, da lugar a la formación de un embrión más redondo y cohesionado. A su vez, se observa un aumento del espacio perivitelino, especialmente notable en el embrión desarrollado enteramente *in vivo* (Rizos et al. 2002). Durante el proceso de compactación, el embrión es denominado mórula temprana, mientras que una vez que ha finalizado la compactación se denomina mórula o mórula compacta (Van Soom et al. 1997) (Figura 1.9A).

De forma paralela a la fase final de la compactación de la mórula, ocurre la diferenciación del blastocisto (Día 6) (De Loof 1992). El blastocisto se caracteriza por la presencia de una cavidad central, el blastocele, completamente rodeada por una capa de células diferenciadas denominada trofectodermo o trofoblasto (TE). Justo debajo del TE se encuentra un pequeño grupo de células indiferenciadas denominadas masa celular interna (ICM) (Figuras 1.9B, 1.9C). Ambos tipos celulares, TE y ICM, forman diferentes linajes con un destino y papel específico en el desarrollo futuro del embrión. El TE será responsable de la elongación del blastocisto, implantación y formación de los anejos extra-embrionarios. Por otro lado, la ICM será el origen de las diferentes hojas embrionarias (endodermo, ectodermo y mesodermo), que llevarán finalmente a la diferenciación de los tejidos y órganos del embrión (Marikawa and Alarcón 2009). Por tanto, en el estadio de blastocisto, los dos tipos celulares presentan diferentes patrones de expresión de genes (Berg et al. 2011, Herrmann et al. 2013, Nagatomo et al. 2013, Ozawa et al. 2012). La expresión génica en el TE está dirigida principalmente a la comunicación materno-embrionaria, el transporte de fluido y macromoléculas, al metabolismo, a la organización del citoesqueleto de actina, y a la eliminación de la zona pelúcida. Por otro lado, la ICM presenta un mayor nivel de expresión de genes relacionados con la respuesta inmune, la pluripotencia, y la especificación o migración celular (Ozawa et al. 2012).

En las células del TE el sistema de filamentos intermedios intracelulares se desarrolla y genera un aumento de los contactos intercelulares que sella el embrión. Este sellado es permeable al transporte selectivo desde el exterior al interior del embrión. Así, el transporte activo de iones determina una composición iónica diferente en el interior del blastocisto y en el suero, lo que explica la entrada de agua al interior (Kidder and Watson 2005, Watson 1992). Además, las células del TE permiten la entrada de macromoléculas mediante endocitosis (Heyner et al. 1989, Pemble and Kaye 1986). Este fenómeno es particularmente importante en las especies con implantación tardía como la bovina o porcina, donde el útero presenta una alta actividad secretora (Baumbach et al. 1990). Por último, el TE elabora mensajes embrionarios (secreción de proteínas, expresión de antígenos de superficie) esenciales para la comunicación con el endometrio. A medida que prosigue el desarrollo, la expresión génica relacionada con la comunicación materno-embrionaria es cada vez más específica de la especie (Walters et al. 2000).

Según va creciendo la presión osmótica en el interior del blastocele, el embrión comienza a expandirse (Día 7). La expansión conlleva el aumento del diámetro del embrión, el adelgazamiento de la zona pelúcida (ZP) y la compactación de la ICM. Finalmente, se produce la rotura de la ZP y la eclosión del blastocisto (Día 8-Día 9) (Figuras 1.9D, 1.9E). Dependiendo de la especie, la eclosión se puede producir por la presión del blastocisto en crecimiento o por la lisis enzimática mediante enzimas uterinas o embrionarias (Demeestere et al. 1996, Fléchon and Renard 1978). En bovino, no existe una evidencia clara de la ruptura de la ZP por enzimas endometriales ya que ésta se ha encontrado vacía en los lavados uterinos *in vivo*, tras la eclosión del blastocisto (Betteridge et al. 1980). La zona pelúcida actúa como mediador en la unión de los gametos y la respuesta inmune materna. También previene el contacto y la adhesión del trofectodermo embrionario al epitelio luminal (Clark 2010).

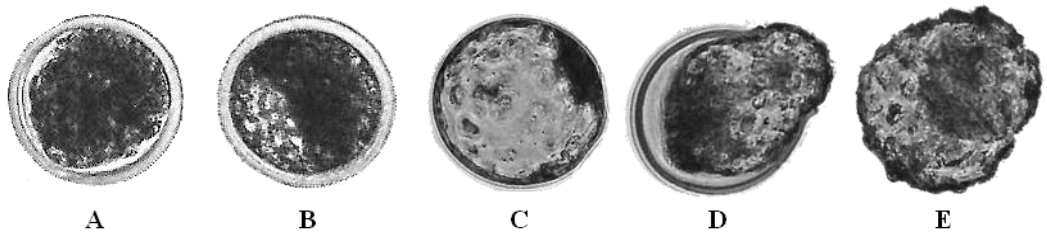

Figura 1.9 Diferentes estadios de embriones bovinos producidos *in vitro* A) Mórula B) Blastocisto temprano C) Blastocisto expandido D) Blastocisto eclosionando E) Blastocisto eclosionado.

La pérdida de la ZP marca el inicio de la implantación en roedores, equinos, humanos y primates. Sin embargo, en rumiantes y cerdos el embrión comienza a sufrir una serie de cambios morfológicos significativos (Allen and Stewart 2001, Guillomot et al. 1991). El blastocisto eclosionado da lugar a un embrión de forma ovoide (Día 10) y comienza el proceso de elongación (Día 12 - Día 19), durante el cual las células del TE proliferan con rapidez. Durante este período el embrión es completamente dependiente de las secreciones uterinas. Esto ha sido demostrado en el modelo ovino, en el que la ablación perinatal de las glándulas uterinas bloquea la elongación del blastocisto (Gray et al. 2001). De forma similar, el blastocisto bovino es incapaz de elongarse correctamente *in vitro* (Alexopoulos et al. 2006, Brandão et al. 2004), aunque sí lo hará si es transferido a hembras sincronizadas (Clemente et al. 2009).

A partir del Día 14, el embrión filamentoso está posicionado en la luz uterina, donde tiene lugar una íntima asociación entre las membranas del trofectodermo y del epitelio luminal. No obstante, el embrión todavía puede ser recuperado intacto mediante lavado

uterino (Guillomot et al. 1991). Las carúnculas se convierten en edematosas y su superficie comienza a plegarse, formando criptas. Además, las células epiteliales carunculares desarrollan protrusiones citoplasmáticas, que son sitios de endocitosis y que en otras especies se denominan pinópodos (ratón, rata, humano y conejo). Estos cambios son progresivos y no ocurren simultáneamente en todas las carúnculas. Las glándulas uterinas se convierten también en sitios de aposición. El trofoblasto desarrolla en las zonas intercarunculares papilas que penetran en las bocas de los conductos glandulares superficiales (Día 15 - Día 18). Estas papilas ayudan en la absorción de las secreciones glandulares por el embrión y la formación de interacciones adhesivas más robustas.

Implantación y placentación

La implantación en bovino comienza el Día 19, cuando el TE comienza a adherirse firmemente al epitelio luminal. Poco después (Día 20 - Día 29), células del trofectodermo especializadas se fusionan con el epitelio luminal uterino, formando un sincitio multinucleado (Hoffman and Wooding 1993, King et al. 1980, Spencer et al. 2004a, Wooding 1992). Este tipo de placenta se denomina sinepiteliocorial, y es intermedia entre la epiteliocorial del cerdo, donde trofectodermo y epitelio interaccionan someramente y la hemocorial humana, donde el trofectodermo invade el endometrio y entra en contacto con los vasos sanguíneos maternales. También se denomina placenta múltiple cotiledonaria, haciendo referencia a su peculiar organización, en la que cada cotiledón fetal se asocia con una carúncula materna (Peter 2013).

Dimorfismo sexual

El dimorfismo sexual en mamíferos se atribuye mayoritariamente a diferencias hormonales en el feto y tejidos adultos (Ford and D'Occhio 1989). Sin embargo, previamente a la formación de las gónadas, ya se observan diferencias fenotípicas asociadas con el sexo que dependen únicamente de la composición cromosómica, XX o XY, del embrión (Gutiérrez-Adán et al. 1996). Así, los genes ligados al cromosoma Y se expresan exclusivamente en machos (Bermejo-Álvarez et al. 2010, Gutiérrez-Adán et al. 1997, Kobayashi et al. 2006). Por otro lado, los genes ligados al cromosoma X se presentan en doble dosis en las hembras, antes de que ocurra la inactivación del cromosoma X (XCI) (Bermejo-Álvarez et al. 2010, Kobayashi et al. 2006). El estadio

en el que tiene lugar la XCI no está claramente establecido y parece variar entre las diferentes especies (Okamoto and Heard 2009). En bovino, una gran parte de las células sufre XCI entre la eclosión del blastocisto y el reconocimiento materno de la preñez (Día 7-Día 14) (Bermejo-Álvarez et al. 2011b). Esta diferencia en los niveles de transcripción de genes codificados en los cromosomas sexuales afecta también a la transcripción de genes autosómicos y a la regulación epigenética (Bermejo-Álvarez et al. 2008, Itoh and Arnold 2014).

La transcripción sujeta a dimorfismo sexual puede afectar a diferentes procesos biológicos. Entre ellos se encuentran el metabolismo de la glucosa y de proteínas (Bermejo-Álvarez et al. 2010, Tiffin et al. 1991), la tasa de reemplazo de aminoácidos (Sturmey et al. 2010) y la tasa de actividad mitocondrial (Mittwoch 2004). Finalmente, estos procesos podrían determinar la susceptibilidad al estrés ambiental y desviar la proporción de sexos (Gutiérrez-Adán et al. 2006). Por ejemplo, la diferencia sexual en la velocidad de desarrollo *in vitro* observada en algunos casos (Nedambale et al. 2004, Rubessa et al. 2011) podría explicarse por un exceso de glucosa, que afectaría diferencialmente a hembras y machos (Bredbacka and Bredbacka 1996, Jiménez et al. 2003, Kimura et al. 2005). También se ha sugerido que determinados parámetros maternales como la dieta, niveles de glucosa y testosterona, o la exposición a factores de riesgo pueden sesgar la proporción de sexos, aunque los mecanismos biológicos subyacentes no han sido identificados (Bermejo-Álvarez et al. 2011a, Gutiérrez-Adán et al. 2006). Recientemente, se ha propuesto que podrían estar implicados algunos factores embriotróficos, como el factor estimulante de colonias-2 o CSF2, cuya secreción maternal varía en función del ambiente y además afecta diferencialmente al desarrollo de embriones hembra y macho *in vitro* (Dobbs et al. 2014).

Colectivamente, estas evidencias sugieren que el sexo del embrión ha de ser tenido en cuenta a la hora de diseñar los medios de cultivo *in vitro* o de analizar la comunicación materno-embrionaria. Dada la sensibilidad del endometrio frente a embriones con diferente potencial de desarrollo (Beltman et al. 2013, Mansouri-Attia et al. 2009a, Sandra et al. 2011), es plausible que el tracto reproductivo también pueda reconocer y reaccionar a embriones de diferente sexo. De hecho, existe dimorfismo sexual en la producción de moléculas relacionadas con el reconocimiento materno de la preñez, como el IFNT, tanto *in vitro* como *in vivo* (Kimura et al. 2004a, Kimura et al. 2004b, Larson et al. 2001).

OBJETIVOS Y ESTRUCTURA DEL TRABAJO

El objetivo general de esta tesis es la identificación, localización y estudio funcional de proteínas candidatas a ejercer un papel embriotrófico durante el desarrollo bovino temprano (de Día 5 a Día 8). Mediante el estudio de estas proteínas pretendemos mejorar el cultivo *in vitro* de embriones bovinos.

En el **capítulo 2** estudiamos el posible papel de TNF y su receptor TNFR2 en la comunicación materno-embrionaria bovina. Mediante RT-qPCR, inmunohistoquímica y western blot, analizamos muestras de endometrio procedentes de animales con o sin embriones, y embriones cultivados parcialmente en el tracto materno o enteramente cultivados *in vitro*. Específicamente, evaluamos las siguientes hipótesis:

2.1. TNF y TNFR2 (mRNA y proteína) están presentes en el endometrio y embrión durante el estadio de blastocisto.

2.2. La expresión de TNF y TNFR2 (mRNA y proteína) está regulada recíprocamente por el endometrio y el embrión durante el estadio de blastocisto.

2.3. La presencia de embriones afecta a la expresión endometrial de leucocitos CD45.

En el **capítulo 3** realizamos un estudio similar al planteado en el capítulo 2, para IL1By su receptor IL1R1, atendiendo a las siguientes hipótesis:

3.1. IL1B e IL1R1 (mRNA y proteína) están presentes en el endometrio y embrión durante el estadio de blastocisto.

3.2. La expresión de IL1B e IL1R1 (mRNA y proteína) está regulada recíprocamente por el endometrio y el embrión durante el estadio de blastocisto.

En el **capítulo 4**, analizamos el papel de HDGF en la comunicación materno - embrionaria bovina utilizando un diseño experimental similar al de los capítulos 3 y 4, para contrastar las siguientes hipótesis:

4.1. HDGF (mRNA y proteína) está presente en el endometrio y en el embrión durante el estadio de blastocisto.

4.2. La expresión de HDGF (mRNA y proteína) está regulada recíprocamente por el endometrio y el embrión durante el estadio de blastocisto.

4.3. La adición de HDGF recombinante al medio de cultivo de embriones producidos *in vitro* tiene un efecto sobre la capacidad de desarrollo embrionario; la proliferación y diferenciación celular; y los índices de apoptosis y necrosis.

En el **capítulo 5** investigamos el dimorfismo sexual en la comunicación materno - embrionaria bovina. A partir de novillas transferidas con embriones macho o hembra en Día 5, analizamos y comparamos el proteoma de su fluido uterino en Día 8, para comprobar las siguientes hipótesis:

5.1. Existen proteínas reguladas diferencialmente en el fluido uterino en función de la presencia de embriones macho o hembra.

5.2. Dichas proteínas reguladas diferencialmente en el fluido uterino se localizan en las células endometriales.

5.3. El efecto del fluido uterino sobre el desarrollo *in vitro* de embriones depende del sexo.

Finalmente, en el **capítulo 6** discutimos los resultados obtenidos, dando una visión global del papel de las proteínas estudiadas en la comunicación materno - embrionaria, y resumimos las conclusiones principales.

REFERENCIAS

Aggarwal, B.B., Gupta, S.C., Kim, J.H., 2012. Historical perspectives on tumor necrosis factor and its superfamily: 25 years later, a golden journey. Blood. 119, 651-665.

Ahn, I.E., Ju, J.H., Kang, K.Y., Park, S.-H., Kim, H.-Y., 2010. The silent progression of metastatic malignancy during the treatment with soluble tumor necrosis factor receptor. Clinical Rheumatology. 29, 225-227.

Al-Gubory, K.H., Arianmanesh, M., Garrel, C., Bhattacharya, S., Cash, P., Fowler, P.A., 2014. Proteomic analysis of the sheep caruncular and intercaruncular endometrium reveals changes. Reproduction.8, 599-614.

Alexopoulos, N.I., Vajta, G., Maddox-Hyttel, P., French, A.J., Trounson, A.O., 2006. Stereomicroscopic and histological examination of bovine embryos following extended in vitro culture. Reproduction, Fertility and Development. 17, 799-808.

Allen, W., Stewart, F., 2001. Equine placentation. Reproduction, Fertility and Development. 13, 623-634.

Amoroso, E., 1952. Placentation. Marshall's Physiology of Reproduction. AS Parkes, Editor. London, Longmans, Green & Co., Inc.

Arai, M., Yoshioka, S., Tasaki, Y., Okuda, K., 2013. Remodeling of bovine endometrium throughout the estrous cycle. Animal Reproduction Science. 142, 1-9.

Atkinson, B., King, G., Amoroso, E., 1984. Development of the caruncular and intercaruncular regions in the bovine endometrium. Biology of Reproduction. 30, 763 - 774.

Bai, R., Bai, H., Kuse, M., Ideta, A., Aoyagi, Y., Fujiwara, H., Okuda, K., Imakawa, K., Sakurai, T., 2014. Involvement of VCAM1 in the bovine conceptus adhesion to the uterine endometrium. Reproduction. 148, 119-127.

Banerjee, P., Ghosh, S., Dutta, M., Subramani, E., Khalpada, J., Roychoudhury, S., Chakravarty, B., Chaudhury, K., 2013. Identification of key contributory factors responsible for vascular dysfunction in idiopathic recurrent spontaneous miscarriage. PloS One. 8, e80940.

Barañao, R., Piazza, A., Rumi, L., Fried, E., 1997. Determination of IL-1 and IL-6 Levels in Human Embryo Culture-Conditioned Media. American Journal of Reproductive Immunology. 37, 191-194.

Barreto, R.S.N., Bressan, F.F., Oliveira, L.J., Pereira, F.T.V., Perecin, F., Ambrósio, C.E., Meirelles, F.V., Miglino, M.A., 2011. Gene expression in placentation of farm animals: An overview of gene function during development. Theriogenology. 76, 589-597.

Bauersachs, S., Mitko, K., Ulbrich, S., Blum, H., Wolf, E., 2008. Transcriptome studies of bovine endometrium reveal molecular profiles characteristic for specific stages of estrous cycle and early pregnancy. Experimental and clinical endocrinology & diabetes. 116, 371-384.

Bauersachs, S., Ulbrich, S., Gross, K., Schmidt, S., Meyer, H., Einspanier, R., Wenigerkind, H., Vermehren, M., Blum, H., Sinowatz, F., Wolf, E., 2005. Gene expression profiling of bovine endometrium during the oestrous cycle: detection of

molecular pathways involved in functional changes. Journal of Molecular Endocrinology. 34, 889 - 908.

Bauersachs, S., Ulbrich, S., Gross, K., Schmidt, S., Meyer, H., Wenigerkind, H., Vermehren, M., Sinowatz, F., Blum, H., Wolf, E., 2006. Embryo-induced transcriptome changes in bovine endometrium reveal species-specific and common molecular markers of uterine receptivity. Reproduction. 132, 319 - 331.

Bauersachs, S., Ulbrich, S.E., Reichenbach, H.-D., Reichenbach, M., Büttner, M., Meyer, H.H., Spencer, T.E., Minten, M., Sax, G., Winter, G., 2012. Comparison of the effects of early pregnancy with human interferon, alpha 2 (IFNA2), on gene expression in bovine endometrium. Biology of Reproduction. 86, 46-61.

Bauersachs, S., Wolf, E., 2013. Immune aspects of embryo-maternal cross-talk in the bovine uterus. Journal of Reproductive Immunology. 97, 20-26.

Baumann, C.G., Morris, D.G., Sreenan, J.M., Leese, H.J., 2007. The quiet embryo hypothesis: molecular characteristics favoring viability. Molecular Reproduction and Development. 74, 1345-1353.

Baumbach, G.A., Bartley, N.G., Kattesh, H.G., Godkin, J.D., 1990. Immunolocalization and endocytosis of the uterine secretory protein, uteroferrin, in pre-implantation pig trophectoderm on day 11 of pregnancy. Anatomy and Embryology. 182, 563-568.

Bazer, F., Spencer, T., Johnson, G., Burghardt, R., 2011. Uterine receptivity to implantation of blastocysts in mammals. Frontiers in bioscience (Scholar edition). 3, 745-767.

Bazer, F.W., 1975. Uterine protein secretions: relationship to development of the conceptus. Journal of Animal Science. 41, 1376-1382.

Bazer, F.W., Johnson, G.A., 2014. Pig blastocyst - uterine interactions. Differentiation. 87, 52-65.

Bazer, F.W., Spencer, T.E., Johnson, G.A., Burghardt, R.C., Wu, G., 2009. Comparative aspects of implantation. Reproduction. 138, 195-209.

Bazer, F.W., Wu, G., Spencer, T.E., Johnson, G.A., Burghardt, R.C., Bayless, K., 2010. Novel pathways for implantation and establishment and maintenance of pregnancy in mammals. Molecular Human Reproduction. 16, 135-152.

Beltman, M.E., Forde, N., Furney, P., Carter, F., Roche, J., Lonergan, P., Crowe, M., 2010. Characterisation of endometrial gene expression and metabolic parameters in beef heifers yielding viable or non-viable embryos on Day 7 after insemination. Reproduction, Fertility and Development. 22, 987-999.

Beltman, M.E., Forde, N., Lonergan, P., Crowe, M.A., 2013. Altered endometrial immune gene expression in beef heifers with retarded embryos. Reproduction, Fertility and Development. 25, 966-970.

Beltman, M.E., Mullen, M., Elia, G., Hilliard, M., Diskin, M., Evans, A., Crowe, M., 2014. Global proteomic characterization of uterine histotroph recovered from beef heifers yielding good quality and degenerate day 7 embryos. Domestic Animal Endocrinology. 46, 49-57.

Ben-Yair, E., Less, A., Lev, S., Ben-Yehoshua, L., Tartakovsky, B., 1997. Tumour necrosis factor alpha binding to human and mouse trophoblast. Cytokine. 9, 830-836.

Berendt, F.J., Fröhlich, T., Schmidt, S.E.M., Reichenbach, H.-D., Wolf, E., 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.

Berg, D.K., Smith, C.S., Pearton, D.J., Wells, D.N., Broadhurst, R., Donnison, M., Pfeffer, P.L., 2011. Trophectoderm lineage determination in cattle. developmental Cell. 20, 244-255.

Bermejo-Álvarez, P., Rizos, D., Lonergan, P., Gutierrez-Adan, A., 2011a. Transcriptional sexual dimorphism during preimplantation embryo development and its consequences for developmental competence and adult health and disease. Reproduction. 141, 563-570.

Bermejo-Álvarez, P., Rizos, D., Lonergan, P., Gutierrez-Adan, A., 2011b. Transcriptional sexual dimorphism in elongating bovine embryos: implications for XCI and sex determination genes. Reproduction. 141, 801-808.

Bermejo-Álvarez, P., Rizos, D., Rath, D., Lonergan, P., Gutierrez-Adan, A., 2008. Epigenetic differences between male and female bovine blastocysts produced in vitro. Physiological Genomics. 32, 264-272.

Bermejo-Álvarez, P., Rizos, D., Rath, D., Lonergan, P., Gutierrez-Adan, A., 2010. Sex determines the expression level of one third of the actively expressed genes in bovine blastocysts. Proceedings of the National Academy of Sciences. 107, 3394-3399.

Betteridge, K., Eaglesome, M., Randall, G., Mitchell, D., 1980. Collection, description and transfer of embryos from cattle 10 - 16 days after oestrus. Journal of Reproduction and Fertility. 59, 205-216.

Betteridge, K., Fléchon, J.-E., 1988. The anatomy and physiology of pre-attachment bovine embryos. Theriogenology. 29, 155-187.

Betts, J., Hansen, P., 1992. Regulation of prostaglandin secretion from epithelial and stromal cells of the bovine endometrium by interleukin-1 beta, interleukin-2, granulocyte-macrophage colony stimulating factor and tumor necrosis factor-alpha. Life Sciences. 51, 1171-1176.

Beutler, B., Greenwald, D., Hulmes, J., Chang, M., Pan, Y.-C., Mathison, J., Ulevitch, R., Cerami, A., 1985. Identity of tumour necrosis factor and the macrophage-secreted factor cachectin.

Black, R., Rauch, C., Kozlosky, C., Peschon, J., Slack, J., Wolfson, M., Al, E., 1997 A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. Nature. 385, 729-733.

Block, J., Hansen, P., Loureiro, B., Bonilla, L., 2011. Improving post-transfer survival of bovine embryos produced *in vitro*: Actions of insulin-like growth factor-1, colony stimulating factor-2 and hyaluronan. Theriogenology. 76, 1602-1609.

Bonilla, A., Ozawa, M., Hansen, P., 2011. Timing and dependence upon mitogen-activated protein kinase signaling for pro-developmental actions of insulin-like growth factor 1 on the preimplantation bovine embryo. Growth Hormone and IGF Research. 21, 107-111.

Boomsma, C.M., Kavelaars, A., Eijkemans, M.J.C., Lentjes, E.G., Fauser, B.C.J.M., Heijnen, C.J., Macklon, N.S., 2009. Endometrial secretion analysis identifies a cytokine profile predictive of pregnancy in IVF. Human Reproduction. 24, 1427-1435.

Boucher, A., Kharfi, A., Al-Akoum, M., Bossu, P., Akoum, A., 2001. Cycle-dependent expression of interleukin-1 receptor type II in the human endometrium. Biology of Reproduction. 65, 890 - 898.

Brackett, B., Bousquet, D., Boice, M., Donawick, W., Evans, J., Dressel, M., 1982. Normal development following in vitro fertilization in the cow. Biology of Reproduction. 27, 147-158.

Brandão, D.O., Maddox-Hyttel, P., Løvendahl, P., Rumpf, R., Stringfellow, D., Callesen, H., 2004. Post hatching development: a novel system for extended in vitro culture of bovine embryos. Biology of Reproduction. 71, 2048-2055.

Bredbacka, K., Bredbacka, P., 1996. Glucose controls sex-related growth rate differences of bovine embryos produced in vitro. Journal of Reproduction and Fertility. 106, 169-172.

Bulmer, J.N., Lash, G.E., 2005. Human uterine natural killer cells: a reappraisal. Molecular Immunology. 42, 511-521.

Carswell, E., Old, L.J., Kassel, R., Green, S., Fiore, N., Williamson, B., 1975. An endotoxin-induced serum factor that causes necrosis of tumors. Proceedings of the National Academy of Sciences. 72, 3666-3670.

Carter, F., Rings, F., Mamo, S., Holker, M., Kuzmany, A., Besenfelder, U., Havlicek, V., Mehta, J., Tesfaye, D., Schellander, K., 2010. Effect of elevated circulating progesterone concentration on bovine blastocyst development and global transcriptome following endoscopic transfer of in vitro produced embryos to the bovine oviduct. Biology of Reproduction. 83, 707-719.

Clark, G.F., 2010. The Mammalian Zona Pellucida: A Matrix That Mediates Both Gamete Binding and Immune Recognition? Systems Biology in Reproductive Medicine. 56, 349-364.

Clemente, M., De La Fuente, J., Fair, T., Al Naib, A., Gutierrez-Adan, A., Roche, J., Rizos, D., Lonergan, P., 2009. Progesterone and conceptus elongation in cattle: a direct effect on the embryo or an indirect effect via the endometrium? Reproduction. 138, 507-517.

Cobb, S., Watson, E., 1995. Immunohistochemical study of immune cells in the bovine endometrium at different stages of the oestrous cycle. Research in Veterinary Science. 59, 238-241.

Conrotto, P., Souchelnytskyi, S., 2008. Proteomic approaches in biological and medical sciences: principles and applications. Experimental Oncology. 30, 171-180.

Corcoran, D., Rizos, D., Fair, T., Evans, A.C., Lonergan, P., 2007. Temporal expression of transcripts related to embryo quality in bovine embryos cultured from the two-cell to blastocyst stage in vitro or in vivo. Molecular Reproduction and Development. 74, 972-977.

Chan, F.K.-M., Chun, H.J., Zheng, L., Siegel, R.M., Bui, K.L., Lenardo, M.J., 2000. A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. Science. 288, 2351-2354.

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

Chen, C., Spencer, T.E., Bazer, F.W., 2000a. Expression of hepatocyte growth factor and its receptor c-metin the ovine uterus. Biology of Reproduction. 62, 1844-1850.

Chen, C., Spencer, T.E., Bazer, F.W., 2000b. Fibroblast growth factor-10: a stromal mediator of epithelial function in the ovine uterus. Biology of Reproduction. 63, 959-966.

Chen, H.-L., Yang, Y., Hu, X.-L., Yelavarthi, K.K., Fishback, J.L., Hunt, J.S., 1991. Tumor necrosis factor alpha mRNA and protein are present in human placental and uterine cells at early and late stages of gestation. American Journal of Pathology. 139, 327-335.

Chen, J.I., Hannan, N.J., Mak, Y., Nicholls, P.K., Zhang, J., Rainczuk, A., Stanton, P.G., Robertson, D.M., Salamonsen, L.A., Stephens, A.N., 2009. Proteomic characterization of midproliferative and midsecretory human endometrium. Journal of Proteome Research. 8, 2032-2044.

Cheong, Y., Boomsma, C., Heijnen, C., Macklon, N., 2013. Uterine secretomics: a window on the maternal-embryo interface. Fertility and Sterility. 99, 1093-1099.

Choi, Y., Johnson, G., Spencer, T., Bazer, F., 2003. Pregnancy and interferon tau regulate major histocompatibility complex class I and beta2-microglobulin expression in the ovine uterus. Biology of Reproduction. 68, 1703 - 1710.

Davidson, J., Tiemann, U., Betts, J., Hansen, P., 1995. DNA synthesis and prostaglandin secretion by bovine endometrial cells as regulated by interleukin-1. Reproduction, Fertility and Development. 7, 1037-1043.

De Loof, A., 1992. Problems and paradigms. All animals develop from a blastula: Consequences of an undervalued definition for thinking on development. Bioessays. 14, 573-575.

De Los Santos, M.J., Mercader, A., Francés, A., Portoles, E., Remohí, J., Pellicer, A., Simon, C., 1996. Role of endometrial factors in regulating secretion of components of the immunoreactive human embryonic interleukin-1 system during embryonic development. Biology of Reproduction. 54, 563-574.

Demeestere, I., Barlow, P., Leroy, F., 1996. Hardening of zona pellucida of mouse oocytes and embryos in vivo and in vitro. International Journal of Fertility and Women's Medicine. 42, 219-222.

Desouza, L., Diehl, G., Yang, E.C., Guo, J., Rodrigues, M.J., Romaschin, A.D., Colgan, T.J., Siu, K., 2005. Proteomic analysis of the proliferative and secretory phases of the human endometrium: protein identification and differential protein expression. Proteomics. 5, 270-281.

Dietz, F., Franken, S., Yoshida, K., Nakamura, H., Kappler, J., Gieselmann, V., 2002. The family of hepatoma-derived growth factor proteins: characterization of a new member HRP-4 and classification of its subfamilies. Biochem. J. 366, 491-500.

Dinarello, C.A., 1994. The interleukin-1 family: 10 years of discovery. The FASEB Journal. 8, 1314-1325.

Dobbs, K., Rodriguez, M., Sudano, M., Ortega, M., Pj., H., 2013. Dynamics of DNA methylation during early development of the preimplantation bovine embryo. PloS One. 8, e66230.

Dobbs, K.B., Gagné, D., Fournier, E., Dufort, I., Robert, C., Block, J., Sirard, M.A., Bonilla, L., Ealy, A.D., Loureiro, B., Hansen, P.J., 2014. Sexual dimorphism in developmental programming of the bovine preimplantation embryo caused by colony stimulating factor 2. Biology of Reproduction. 91, 80-92.

Dockery, P., Li, T., Rogers, A., Cooke, I., Lenton, E., 1988. The ultrastructure of the glandular epithelium in the timed endometrial biopsy. Human Reproduction. 3, 826-834.

Dockery, P., Rogers, A., 1989. 2 The effects of steroids on the fine structure of the endometrium. Bailliere's Clinical Obstetrics and Gynaecology. 3, 227-248.

Dominguez, F., Garrido-Gomez, T., Lopez, J., Camafeita, E., Quinonero, A., Pellicer, A., Simon, C., 2009. Proteomic analysis of the human receptive versus non-receptive endometrium using differential in-gel electrophoresis and MALDI-MS unveils stathmin 1 and annexin A2 as differentially regulated. Human Reproduction. 24, 2607-2617.

Ealy, A.D., Yang, Q.E., 2009. Review article: control of interferon-tau expression during early pregnancy in ruminants. American Journal of Reproductive Immunology. 61, 95-106.

Egea, R.R., Puchalt, N.G., Escrivá, M.M., Varghese, A.C., 2014. OMICS: Current and future perspectives in reproductive medicine and technology. Journal of Human Reproductive Sciences. 7, 73-92.

Eisenberg, S.P., Evans, R.J., Arend, W.P., Verderber, E., Brewer, M.T., Hannum, C.H., Thompson, R.C., 1990. Primary structure and functional expression from complementary DNA of a human interleukin-1 receptor antagonist. Nature. 343, 341-346.

El-Sayed, A., Hoelker, M., Rings, F., Salilew, D., Jennen, D., Tholen, E., Sirard, M.-A., Schellander, K., Tesfaye, D., 2007. Large-scale transcriptional analysis of bovine embryo biopsies in relation to pregnancy success after transfer to recipients. Physiological Genomics. 28, 84-96.

Emond, V., Maclaren, L.A., Kimmins, S., Arosh, J.A., Fortier, M.A., 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-τ. Biology of Reproduction. 70, 54-64.

Enright, B., Lonergan, P., Dinnyes, A., Fair, T., Ward, F., Yang, X., Boland, M., 2000. Culture of in vitro produced bovine zygotes in vitro vs in vivo: implications for early embryo development and quality. Theriogenology. 54, 659-673.

Everett, A.D., Lobe, D.R., Matsumura, M.E., Nakamura, H., Mcnamara, C.A., 2000. Hepatoma-derived growth factor stimulates smooth muscle cell growth and is expressed in vascular development. The Journal of clinical investigation. 105, 567-575.

Everett, A.D., Stoops, T., Mcnamara, C.A., 2001. Nuclear targeting is required for hepatoma-derived growth factor-stimulated mitogenesis in vascular smooth muscle cells. Journal of Biological Chemistry. 276, 37564-37568.

Evron, A., Goldman, S., Shalev, E., 2011. Effect of primary human endometrial stromal cells on epithelial cell receptivity and protein expression is dependent on menstrual cycle stage. Human Reproduction. 26, 176-190.

Farin, C., Imakawa, K., Roberts, R., 1989. In situ localization of mRNA for the interferon, ovine trophoblast protein-1, during early embryonic development of the sheep. Molecular Endocrinology. 3, 1099-1107.

Faulkner, S., Elia, G., Mullen, M.P., O'boyle, P., Dunn, M.J., Morris, D., 2012. A comparison of the bovine uterine and plasma proteome using iTRAQ proteomics. Proteomics. 12, 2014-2023.

Ferrell, C.L., 1991. Maternal and fetal influences on uterine and conceptus development in the cow: I. Growth of tissues of the gravid uterus. Journal of Animal Science. 69, 1945-1953.

Fields, S.D., Hansen, P.J., Ealy, A.D., 2011. Fibroblast growth factor requirements for in vitro development of bovine embryos. Theriogenology. 75, 1466-1475.

Fléchon, J.-E., Renard, J.-P., 1978. A scanning electron microscope study of the hatching of bovine blastocysts in vitro. Journal of Reproduction and Fertility. 53, 9-12.

Ford, J., D'occhio, M., 1989. Differentiation of sexual behavior in cattle, sheep and swine. Journal of Animal Science. 67, 1816-1823.

Forde, N., Beltman, M.E., Duffy, G., Duffy, P., Mehta, J., Ó'gaora, P., Roche, J., Lonergan, P., Crowe, M., 2011a. Changes in the endometrial transcriptome during the bovine estrous cycle: effect of low circulating progesterone and consequences for conceptus elongation. Biology of Reproduction. 84, 266-278.

Forde, N., Carter, F., Fair, T., Crowe, M., Evans, A., Spencer, T., Bazer, F., Mcbride, R., Boland, M., O'gaora, P., 2009. Progesterone-regulated changes in endometrial gene expression contribute to advanced conceptus development in cattle. Biology of Reproduction. 81, 784-794.

Forde, N., Carter, F., Spencer, T., Bazer, F., Sandra, O., Mansouri-Attia, N., Okumu, L., Mcgettigan, P., Mehta, J., Mcbride, R., 2011b. Conceptus-induced changes in the endometrial transcriptome: how soon does the cow know she is pregnant? Biology of Reproduction. 85, 144-156.

Forde, N., Duffy, G.B., Mcgettigan, P.A., Browne, J.A., Mehta, J.P., Kelly, A.K., Mansouri-Attia, N., Sandra, O., Loftus, B.J., Crowe, M.A., Fair, T., Roche, J.F., Lonergan, P., Evans, A.C.O., 2012. Evidence for an early endometrial response to pregnancy in cattle: both dependent upon and independent of interferon tau. Physiological Genomics. 44, 799-810.

Forde, N., Lonergan, P., 2012. Transcriptomic analysis of the bovine endometrium: What is required to establish uterine receptivity to implantation in cattle? J Reprod Dev. 58, 189-195.

Forde, N., Mcgettigan, P.A., Mehta, J.P., O'hara, L., Mamo, S., Bazer, F.W., Spencer, T., Lonergan, P., 2014. Proteomic analysis of uterine fluid during the pre-implantation period of pregnancy in cattle. Reproduction.

Forde, N., Mehta, J.P., Mcgettigan, P.A., Mamo, S., Bazer, F.W., Spencer, T.E., Lonergan, P., 2013. Alterations in expression of endometrial genes coding for proteins secreted into the uterine lumen during conceptus elongation in cattle. BMC Genomics. 14, 321-334.

Galvão, A., Valente, L., Skarzynski, D.J., Szóstek, A., Piotrowska-Tomala, K., Rebordão, M.R., Mateus, L., Ferreira-Dias, G., 2013. Effect of cytokines and ovarian steroids on equine endometrial function: an in vitro study. Reproduction, Fertility and Development. 25, 985-997.

Gargett, C.E., Chan, R.W., Schwab, K.E., 2008. Hormone and growth factor signaling in endometrial renewal: role of stem/progenitor cells. Molecular and Cellular Endocrinology. 288, 22-29.

Geisert, R., Fazleabas, A., Lucy, M., Mathew, D., 2012. Interaction of the conceptus and endometrium to establish pregnancy in mammals: role of interleukin 1β. Cell and Tissue Research. 349, 825-838.

Geisert, R., Morgan, G., Short, E., Zavy, M., 1992. Endocrine events associated with endometrial function and conceptus development in cattle. Reproduction, Fertility and Development. 4, 301-305.

Geisert, R., Ross, J., Ashworth, M., White, F., Johnson, G., Desilva, U., 2005. Maternal recognition of pregnancy signal or endocrine disruptor: the two faces of oestrogen during establishment of pregnancy in the pig. Society of Reproduction and Fertility supplement. 62, 131-145.

Godkin, J., Bazer, F., Moffatt, J., Sessions, F., Roberts, R., 1982. Purification and properties of a major, low molecular weight protein released by the trophoblast of sheep blastocysts at day 13-21. Journal of Reproduction and Fertility. 65, 141-150.

Gray, C., Burghardt, R., Johnson, G., Bazer, F., Spencer, T., 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., Spencer, T.E., 2001. Endometrial glands are required for preimplantation conceptus elongation and survival. Biology of Reproduction. 64, 1608-1613.

Greenfeder, S.A., Varnell, T., Powers, G., Lombard-Gillooly, K., Shuster, D., Mcintyre, K.W., Ryan, D.E., Levin, W., Madison, V., Ju, G., 1995. Insertion of a structural domain of interleukin (IL)-1β confers agonist activity to the IL-1 receptor antagonist. Implications for IL-1 bioactivity. Journal of Biological Chemistry. 270, 22460-22466.

Grell, M., Douni, E., Wajant, H., Löhden, M., Clauss, M., Maxeiner, B., Georgopoulos, S., Lesslauer, W., Kollias, G., Pfizenmaier, K., 1995. The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. Cell. 83, 793-802.

Groebner, A.E., Schulke, K., Schefold, J.C., Fusch, G., Sinowatz, F., Reichenbach, H.D., Wolf, E., Meyer, H.H.D., Ulbrich, S.E., 2011. Immunological mechanisms to establish embryo tolerance in early bovine pregnancy. Reproduction, Fertility and Development. 23, 619-632.

Groebner, A.E., Schulke, K., Unterseer, S., Reichenbach, H.D., Reichenbach, M., Büttner, M., Wolf, E., Meyer, H.H.D., Ulbrich, S.E., 2010. Enhanced proapoptotic gene expression of XAF1, CASP8 and TNFSF10 in the bovine endometrium during early pregnancy is not correlated with augmented apoptosis. Placenta. 31, 168-177.

Guillomot, M., Flechon, J., Leroy, F., 1991. [Blastocyst development and implantation].

Guillomot, M., Guay, P., 1982. Ultrastructural features of the cell surfaces of uterine and trophoblastic epithelia during embryo attachment in the cow. The Anatomical Record. 204, 315-322.

Gutiérrez-Adán, A., Behboodi, E., Andersen, G., Medrano, J., Murray, J., 1996. Relationship between stage of development and sex of bovine IVM-IVF embryos cultured in vitro versus in the sheep oviduct. Theriogenology. 46, 515-525.

Gutiérrez-Adán, A., Behboodi, E., Murray, J., Anderson, G., 1997. Early transcription of the SRY gene by bovine preimplantation embryos. Molecular Reproduction and Development. 48, 246-250.

Gutiérrez-Adán, A., Perez-Crespo, M., Fernandez-Gonzalez, R., Ramirez, M., Moreira, P., Pintado, B., Lonergan, P., Rizos, D., 2006. Developmental consequences of sexual dimorphism during pre-implantation embryonic development. Reproduction in Domestic Animals. 41, 54-62.

Gutiérrez-Adán, A., Oter, M., Martínez-Madrid, B., Pintado, B., De La Fuente, J., 2000. Differential expression of two genes located on the X chromosome between male and female in vitro–produced bovine embryos at the blastocyst stage. Molecular Reproduction and Development. 55, 146-151.

Hafez, E., Hafez, E., 1993. Anatomy of female reproduction. Reproduction in farm animals.

Haider, S., Knöfler, M., 2009. Human Tumour Necrosis Factor: Physiological and Pathological Roles in Placenta and Endometrium. Placenta. 30, 111-123.

Hansen, P., 1994. Interactions between the immune system and the ruminant conceptus. Journal of Reproduction and Fertility. Supplement. 49, 69-82.

Hansen, P.J., 2007. Regulation of immune cells in the uterus during pregnancy in ruminants. Journal of Animal Science. 85, E30-E31.

Hansen, T.R., Imakawa, K., Polites, H., Marotti, K., Anthony, R., Roberts, R., 1988. Interferon RNA of embryonic origin is expressed transiently during early pregnancy in the ewe. Journal of Biological Chemistry. 263, 12801-12804.

Hasler, J.F., 2000. In-vitro production of cattle embryos: problems with pregnancies and parturition. Human Reproduction. 15, 47-58.

Hasler, J.F., 2014. Forty years of embryo transfer in cattle: A review focusing on the journal *Theriogenology*, the growth of the industry in North America, and personal reminisces. Theriogenology. 81, 152-169.

Hernandez-Ledezma, J.J., Mathialagan, N., Villanueva, C., Sikes, J.D., Roberts, R.M., 1993. Expression of bovine trophoblast interferons by in vitro-derived blastocysts is correlated with their morphological quality and stage of development. Molecular Reproduction and Development. 36, 1-6.

Herrmann, D., Dahl, J.A., Lucas-Hahn, A., Collas, P., Niemann, H., 2013. Histone modifications and mRNA expression in the inner cell mass and trophectoderm of bovine blastocysts. Epigenetics. 8, 281.

Heyner, S., Rao, L., Jarett, L., Smith, R.M., 1989. Preimplantation mouse embryos internalize maternal insulin via receptor-mediated endocytosis: pattern of uptake and functional correlations. Developmental Biology. 134, 48-58.

Hoffman, L.H., Wooding, F., 1993. Giant and binucleate trophoblast cells of mammals. Journal of Experimental Zoology. 266, 559-577.

Horcajadas, J., Pellicer, A., Simon, C., 2007. Wide genomic analysis of human endometrial receptivity: new times, new opportunities. Human Reproduction Update. 13, 77-86.

Horiuchi, T., Mitoma, H., Harashima, S.-I., Tsukamoto, H., Shimoda, T., 2010. Transmembrane TNF-α: structure, function and interaction with anti-TNF agents. Rheumatology. 49, 1215-1228.

Hunt, J.S., Chen, H.-L., Miller, L., 1996. Tumor necrosis factors: pivotal components of pregnancy? Biology of Reproduction. 54, 554-562.

Hunt, J.S., Chen, H.L., Hu, X.L., Tabibzadeh, S., 1992. Tumor necrosis factor-alpha messenger ribonucleic acid and protein in human endometrium. Biology of Reproduction. 47, 141-147.

Imakawa, K., Helmer, S.D., Nephew, K.P., Meka, C.S., Christenson, R.K., 1993. A novel role for GM-CSF: enhancement of pregnancy specific interferon production, ovine trophoblast protein-1. Endocrinology. 132, 1869-1871.

Imakawa, K., Tamura, K., Mcguire, W., Khan, S., Harbison, L., Stanga, J., Helmer, S., Christenson, R., 1995. Effect of interleukin-3 on ovine trophoblast interferon during early conceptus development. Endocrine. 3, 511-517.

Itoh, Y., Arnold, A.P., 2014. X chromosome regulation of autosomal gene expression in bovine blastocysts. Chromosoma. 123, 481-489.

Jackson, L., Farin, C., Whisnant, S., 2012. Tumor necrosis factor alpha inhibits in vitro bovine embryo development through a prostaglandin mediated mechanism. Journal of Animal Science and Biotechnology. 3, 7-11.

Jiménez, A., Madrid-Bury, N., Fernandez, R., Pérez-Garnelo, S., Moreira, P., Pintado, B., De La Fuente, J., Gutiérrez-Adán, A., 2003. Hyperglycemia-induced apoptosis affects sex ratio of bovine and murine preimplantation embryos. Molecular Reproduction and Development. 65, 180-187.

Johnson, G., Joyce, M., Yankey, S., Hansen, T., Ott, T., 2002. The Interferon Stimulated Genes (ISG) 17 and Mx have different temporal and spatial expression in the ovine uterus suggesting more complex regulation of the Mx gene. Journal of Endocrinology. 174, R7-R11.

Katagiri, S., Takahashi, Y., 2004. Changes in EGF concentrations during estrous cycle in bovine endometrium and their alterations in repeat breeder cows. Theriogenology. 62, 103-112.

Keller, M., Rüegg, A., Werner, S., Beer, H.D., 2008. Active caspase-1 is a regulator of unconventional protein secretion. Cell. 132, 818-831.

Kidder, G.M., Watson, A.J., 2005. Roles of Na, K-ATPase in early development and trophectoderm differentiation. Seminars in Nephrology. 25, 352-355.

Kimura, K., Spate, L., Green, M., Roberts, R.M., 2005. Effects of D-glucose concentration, D-fructose, and inhibitors of enzymes of the pentose phosphate pathway on the development and sex ratio of bovine blastocysts. Molecular Reproduction and Development. 72, 201-207.

Kimura, K., Spate, L.D., Green, M.P., Murphy, C.N., Seidel, G.E., Michael Roberts, R., 2004a. Sexual dimorphism in interferon-τ production by in vivo-derived bovine embryos. Molecular Reproduction and Development. 67, 193-199.

Kimura, K., Spate, L.D., Green, M.P., Roberts, R.M., 2004b. Effects of oxidative stress and inhibitors of the pentose phosphate pathway on sexually dimorphic production of IFN-τ by bovine blastocysts. Molecular Reproduction and Development. 68, 88-95.

King, G., Atkinson, B., Robertson, H., 1980. Development of the bovine placentome from days 20 to 29 of gestation. Journal of Reproduction and Fertility. 59, 95-100.

Kishima, Y., Yamamoto, H., Izumoto, Y., Yoshida, K., Enomoto, H., Yamamoto, M., Kuroda, T., Ito, H., Yoshizaki, K., Nakamura, H., 2002. Hepatoma-derived growth factor stimulates cell growth after translocation to the nucleus by nuclear localization signals. Journal of Biological Chemistry. 277, 10315-10322.

Klein, C., Bauersachs, S., Ulbrich, S., Einspanier, R., Meyer, H., Schmidt, S., Reichenbach, H.-D., Vermehren, M., Sinowatz, F., Blum, H., Wolf, E., 2006. Monozygotic twin model reveals novel embryo-induced transcriptome changes of bovine endometrium in the preattachment period. Biology of Reproduction. 74, 253 - 264.

Ko, Y., Lee, C.Y., Ott, T.L., Davis, M.A., Simmen, R.C., Bazer, F.W., Simmen, F.A., 1991. Insulin-like growth factors in sheep uterine fluids: concentrations and relationship to ovine trophoblast protein-1 production during early pregnancy. Biology of Reproduction. 45, 135-142.

Kobayashi, S., Isotani, A., Mise, N., Yamamoto, M., Fujihara, Y., Kaseda, K., Nakanishi, T., Ikawa, M., Hamada, H., Abe, K., 2006. Comparison of gene expression in male and female mouse blastocysts revealed imprinting of the X-linked gene, Rhox5/Pem, at preimplantation stages. Current Biology. 16, 166-172.

Koji, T., Chedid, M., Rubin, J.S., Slayden, O.D., Csaky, K.G., Aaronson, S.A., Brenner, R.M., 1994. Progesterone-dependent expression of keratinocyte growth factor mRNA in stromal cells of the primate endometrium: keratinocyte growth factor as a progestomedin. The Journal of cell biology. 125, 393-401.

Korzekwa, A., Murakami, S., Wocławek-Potocka, I., Bah, M.M., Okuda, K., Skarzynski, D.J., 2008. The influence of tumor necrosis factor α (TNF) on the secretory function of bovine corpus luteum: TNF and its receptors expression during the estrous cycle. Reproductive Biology. 8, 245-262.

Kotini, A.G., Mpakali, A., Agalioti, T., 2011. Dnmt3a1 upregulates transcription of distinct genes and targets chromosomal gene clusters for epigenetic silencing in mouse embryonic stem cells. Molecular and Cellular Biology. 31, 1577-1592.

Kriegler, M., Perez, C., Defay, K., Albert, I., Lu, S., 1988. A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF. Cell. 53, 45-53.

Kruessel, J., Huang, H.-Y., Wen, Y., Kloodt, A., Bielfeld, P., Polan, M., 1997. Different pattern of interleukin-1 beta-(IL-1 beta), interleukin-1 receptor antagonist- (IL-1ra) and interleukin-1 receptor type I- (IL-1R tI) mRNA-expression in single preimplantation mouse embryos at various developmental stages. Journal of Reproductive Immunology. 34, 103-120.

Krüssel, J.-S., Bielfeld, P., Polan, M.L., Simón, C., 2003. Regulation of embryonic implantation. European Journal of Obstetrics & Gynecology and Reproductive Biology. 110, S2-S9.

Kyama, C.M., T'jampens, D., Mihalyi, A., Simsa, P., Debrock, S., Waelkens, E., Landuyt, B., Meuleman, C., Fulop, V., Mwenda, J.M., 2006. ProteinChip technology is a useful method in the pathogenesis and diagnosis of endometriosis: a preliminary study. Fertility and Sterility. 86, 203-209.

Landry, C.R., Freschi, L., Zarin, T., Moses, A.M., 2014. Turnover of protein phosphorylation evolving under stabilizing selection. Frontiers in genetics. 5, 245-251.

Larson, M.A., Kimura, K., Kubisch, H.M., Roberts, R.M., 2001. Sexual dimorphism among bovine embryos in their ability to make the transition to expanded blastocyst and in the expression of the signaling molecule IFN-τ. Proceedings of the National Academy of Sciences. 98, 9677-9682.

Lee, K.H., Choi, E.Y., Kim, M.K., Lee, S.H., Jang, B.I., Kim, T.N., Kim, S.W., Kim, S.W., Song, S.K., Kim, J.-R., 2010. Hepatoma-derived growth factor regulates the bad-mediated apoptotic pathway and induction of vascular endothelial growth factor in stomach cancer cells. Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics. 19, 67-76.

Leibo, S., Loskutoff, N., 1993. Cryobiology of in vitro-derived bovine embryos. Theriogenology. 39, 81-94.

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

Leung, S., Derecka, K., Mann, G., Flint, A., Wathes, D., 2000. Uterine lymphocyte distribution and interleukin expression during early pregnancy in cows. Journal of Reproduction and Fertility. 119, 25-33.

Li, S.-Z., Zhao, Y.-B., Cao, W.-D., Qu, Y., Luo, P., Zhen, H.-N., Chen, X.-Y., Yan, Z.-F., Fei, Z., 2013. The expression of hepatoma-derived growth factor in primary central nervous system lymphoma and its correlation with angiogenesis, proliferation and clinical outcome. Medical Oncology. 30, 1-7.

Li, T., Rogers, A., Dockery, P., Lenton, E., Cooke, I., 1988. A new method of histologic dating of human endometrium in the luteal phase. Fertility and Sterility. 50, 52-60.

Lim, K.T., Jang, G., Ko, K.H., Lee, W.W., Park, H.J., Kim, J.J., Lee, S.H., Hwang, W.S., Lee, B.C., Kang, S.K., 2007. Improved in vitro bovine embryo development and increased efficiency in producing viable calves using defined media. Theriogenology. 67, 293-302.

Lindhard, A., Bentin-Ley, U., Ravn, V., Islin, H., Hviid, T., Rex, S., Bangsbøl, S., Steen, S., 2002. Biochemical evaluation of endometrial function at the time of implantation. Fertility and Sterility. 78, 221-233.

Locksley, R.M., Killeen, N., Lenardo, M.J., 2001. The TNF and TNF receptor superfamilies-integrating mammalian biology. Cell. 104, 487-501.

Lonergan, P., 2007. State-of-the-art embryo technologies in cattle. Society of Reproduction and Fertility supplement. 64, 315-325.

Lonergan, P., Fair, T., Corcoran, D., Evans, A.C.O., 2006. Effect of culture environment on gene expression and developmental characteristics in IVF-derived embryos. Theriogenology. 65, 137-152.

Lonergan, P., Forde, N., 2014. Maternal-embryo interaction leading up to the initiation of implantation of pregnancy in cattle. animal. 8, 64-69.

Majewski, A.C., Hansen, P.J., 2002. Progesterone Inhibits Rejection of Xenogeneic Transplants in the Sheep Uterus. Hormone Research in Paediatrics. 58, 128-135.

Malhi, P.S., Adams, G.P., Singh, J., 2005. Bovine model for the study of reproductive aging in women: follicular, luteal, and endocrine characteristics. Biology of Reproduction. 73, 45-53.

Mamo, S., Mehta, J.P., Forde, N., Mcgettigan, P., Lonergan, P., 2012. Conceptus-endometrium crosstalk during maternal recognition of pregnancy in cattle. Biology of Reproduction. 87, 6, 1-9.

Männel, D.N., Echtenacher, B., 2004. TNF in the inflammatory response. Chemical Immunology. 7, 141-161.

Mansouri-Attia, N., Sandra, O., Aubert, J., Degrelle, S., Everts, R., Giraud-Delville, C., Heyman, Y., Galio, L., Hue, I., Yang, X., 2009a. Endometrium as an early sensor of in vitro embryo manipulation technologies. Proceedings of the National Academy of Sciences. 106, 5687 - 5692.

Mansouri-Attia, N.R., Aubert, J., Reinaud, P., Giraud-Delville, C., Taghouti, G.R., Galio, L., Everts, R.E., Degrelle, S.V., Richard, C., Hue, I., Yang, X., Tian, X.C., Lewin, H.A., Renard, J.-P., Sandra, O., 2009b. Gene expression profiles of bovine caruncular and intercaruncular endometrium at implantation. Physiological Genomics. 39, 14-27.

Marikawa, Y., Alarcón, V.B., 2009. Establishment of trophectoderm and inner cell mass lineages in the mouse embryo. Molecular Reproduction and Development. 76, 1019-1032.

Martal, J., Lacroix, M.-C., Loudes, C., Saunier, M., Wintenberger-Torres, S., 1979. Trophoblastin, an antiluteolytic protein present in early pregnancy in sheep. Journal of Reproduction and Fertility. 56, 63-73.

Martinon, F., Mayor, A., Tschopp, J., 2009. The inflammasomes: guardians of the body. Annual Review of Immunology. 27, 229-265.

Mccarthy, S.D., Roche, J.F., Forde, N., 2012. Temporal changes in endometrial gene expression and protein localization of members of the IGF family in cattle: effects of progesterone and pregnancy. Physiol. Genomics. 44, 130-140.

Mcmahan, C.J., Slack, J.L., Mosley, B., Cosman, D., Lupton, S., Brunton, L., Grubin, C., Wignall, J., Jenkins, N., Brannan, C., 1991. A novel IL-1 receptor, cloned from B cells by mammalian expression, is expressed in many cell types. The EMBO journal. 10, 2821-2832.

Mcmaster, M., Newton, R., Dey, S., Andrews, G., 1992. Activation and distribution of inflammatory cells in the mouse uterus during the preimplantation period. The Journal of Immunology. 148, 1699-1705.

Meikle, A., Garófalo, E., Rodríguez-Piñón, M., Tasende, C., Sahlin, L., 2001a. Regulation by gonadal steroids of estrogen and progesterone receptors along the reproductive tract in female lambs. Acta Veterinaria Scandinavica. 42, 161-170.

Meikle, A., Sahlin, L., Ferraris, A., Masironi, B., Blanc, J., RodríGuez-Irazoqui, M., RodríGuez-Piñón, M., Kindahl, H., Forsberg, M., 2001b. Endometrial mRNA expression of oestrogen receptor α, progesterone receptor and insulin-like growth factor-I (IGF-I) throughout the bovine oestrous cycle. Animal Reproduction Science. 68, 45-56.

Michael, D.D., Álvarez, I.M., Ocon, O.M., Powell, A.M., Talbot, N.C., Johnson, S.E., Ealy, A.D., 2006. Fibroblast growth factor-2 is expressed by the bovine uterus and stimulates interferon-τ production in bovine trophectoderm. Endocrinology. 147, 3571-3579.

Mihm, M., Gangooly, S., Muttukrishna, S., 2011. The normal menstrual cycle in women. Animal Reproduction Science. 124, 229-236.

Mitchell, P., Tollervey, D., 2000. mRNA stability in eukaryotes. Current Opinion in Genetics & Development. 10, 193-198.

Mitko, K., Ulbrich, S., Wenigerkind, H., Sinowatz, F., Blum, H., Wolf, E., Bauersachs, S., 2008. Dynamic changes in messenger RNA profiles of bovine endometrium during the oestrous cycle. Reproduction. 135, 225 - 240.

Mittwoch, U., 2004. The elusive action of sex-determining genes: mitochondria to the rescue? Journal of Theoretical Biology. 228, 359-365.

Moelants, E.a.V., Mortier, A., Van Damme, J., Proost, P., 2013. Regulation of TNF-[alpha] with a focus on rheumatoid arthritis. Immunology and Cell Biology. 91, 393-401.

Monk, M., 1995. Epigenetic programming of differential gene expression in development and evolution. Dev Genet.17, 188-197.

Moore, M.J., 2005. From birth to death: the complex lives of eukaryotic mRNAs. Science. 309, 1514-1518.

Moosmayer, D., Wajant, H., Gerlach, E., Schmidt, M., Brocks, B., Pfizenmaier, K., 1996. Characterization of different soluble TNF receptor (TNFR80) derivatives: positive influence of the intracellular domain on receptor/ligand interaction and TNF neutralization capacity. Journal of Interferon & Cytokine Research. 16, 471-477.

Multhoff, G., Molls, M., Radons, J., 2011. Chronic inflammation in cancer development. Frontiers in Immunology. 2.

Mullen, M.P., Elia, G., Hilliard, M., Parr, M.H., Diskin, M.G., Evans, A.C.O., Crowe, M.A., 2012. Proteomic characterization of histotroph during the preimplantation phase of the estrous cycle in cattle. Journal of Proteome Research. 11, 3004-3018.

Muñoz, M., Corrales, F.J., Caamaño, J.N., Díez, C., Trigal, B., Mora, M.I., Martín, D., Carrocera, S., Gómez, E., 2012. Proteome of the early embryo-maternal dialogue in the cattle uterus. Journal of Proteome Research. 11, 751-766.

Muñoz, M., Uyar, A., Correia, E., Díez, C., Fernandez-Gonzalez, A., Caamaño, J.N., Trigal, B., Carrocera, S., Seli, E., Gomez, E., 2014. Non-invasive assessment of embryonic sex in cattle by metabolic fingerprinting of in vitro culture medium. Metabolomics. 10, 443-451.

Murakami, S., Miyamoto, Y., Skarzynski, D., Okuda, K., 2001. Effects of tumor necrosis factor-alpha on secretion of prostaglandin E2 and F2alpha in bovine endometrium throughout the estrous cycle. Theriogenology. 55, 1667-1678.

Nagatomo, H., Kagawa, S., Kishi, Y., Takuma, T., Sada, A., Yamanaka, K.-I., Abe, Y., Wada, Y., Takahashi, M., Kono, T., 2013. Transcriptional wiring for establishing cell lineage specification at the blastocyst stage in cattle. Biology of Reproduction. 88, 158-168.

Nakamura, H., Izumoto, Y., Kambe, H., Kuroda, T., Mori, T., Kawamura, K., Yamamoto, H., Kishimoto, T., 1994. Molecular cloning of complementary DNA for a novel human hepatoma-derived growth factor. Its homology with high mobility group-1 protein. Journal of Biological Chemistry. 269, 25143-25149.

Nedambale, T.L., Dinnyés, A., Yang, X., Tian, X.C., 2004. Bovine blastocyst development in vitro: timing, sex, and viability following vitrification. Biology of Reproduction. 71, 1671-1676.

Ng, E.H.Y., Chan, C.C.W., Tang, O.S., Yeung, W.S.B., Ho, P.C., 2004. Comparison of endometrial and subendometrial blood flow measured by three-dimensional power Doppler ultrasound between stimulated and natural cycles in the same patients. Human Reproduction. 19, 2385-2390.

Nickel, W., 2003. The mystery of nonclassical protein secretion. European Journal of Biochemistry. 270, 2109-2119.

Northey, D., French, L., 1980. Effect of embryo removal and intrauterine infusion of embryonic homogenates on the lifespan of the bovine corpus luteum. Journal of Animal Science. 50, 298-302.

O'neill, L., 2000. The Toll/interleukin-1 receptor domain: a molecular switch for inflammation and host defence. Biochemical Society Transactions. 28, 557-563.

Ohta, T., Koshi, K., Ushizawa, K., Hosoe, M., Takahashi, T., Yamaguchi, T., Kizaki, K., Hashizume, K., 2014. Expression profiles of perforin, granzyme B and granulysin genes during the estrous cycle and gestation in the bovine endometrium. Animal Science Journal. 85, 763-769.

Okamoto, I., Heard, E., 2009. Lessons from comparative analysis of X-chromosome inactivation in mammals. Chromosome Research. 17, 659-669.

Okuda, K., Sakumoto, R., Okamoto, N., Acosta, T.J., Abe, H., Okada, H., Sinowatz, F., Skarzynski, D.J., 2010. Cellular localization of genes and proteins for tumor necrosis factor-a (TNF), TNF receptor types I and II in bovine endometrium. Molecular and Cellular Endocrinology. 330, 41-48.

Okumu, L.A., Forde, N., Mamo, S., Mcgettigan, P., Mehta, J.P., Roche, J.F., Lonergan, P., 2014. Temporal regulation of fibroblast growth factors and their receptors in the endometrium and conceptus during the pre-implantation period of pregnancy in cattle. Reproduction. 147, 825-834.

Oliver, J.A., Al-Awqati, Q., 1998. An endothelial growth factor involved in rat renal development. Journal of Clinical Investigation. 102, 1208-1219.

Orlando, S., Sironi, M., Bianchi, G., Drummond, A.H., Boraschi, D., Yabes, D., Mantovani, A., 1997. Role of metalloproteases in the release of the IL-1 type II decoy receptor. Journal of Biological Chemistry. 272, 31764-31769.

Orphanides, G., Reinberg, D., 2002. A unified theory of gene expression. Cell. 108, 439-451.

Ozawa, M., Sakatani, M., Yao, J., Shanker, S., Yu, F., Yamashita, R., Wakabayashi, S., Nakai, K., Dobbs, K.B., Sudano, M.J., 2012. Global gene expression of the inner cell mass and trophectoderm of the bovine blastocyst. BMC Developmental Biology. 12, 33-46.

Padua, M.B., Tekin, Ş., Spencer, T.E., Hansen, P.J., 2005. Actions of progesterone on uterine immunosuppression and endometrial gland development in the uterine gland knockout (UGKO) ewe. Molecular Reproduction and Development. 71, 347-357.

Padykula, H.A., 1989. Regeneration in the primate uterus. Biology of the Uterus. Springer. 279-288.

Paula-Lopes, F.F., De Moraes, A.a.S., Edwards, J.L., Justice, J.E., Hansen, P.J., 1998. Regulation of Preimplantation Development of Bovine Embryos by Interleukin-1β. Biology of Reproduction. 59, 1406-1412.

Paula-Lopes, F.F., Moraes, A.a.D., Hansen, P., 1999. The presence of interleukin-1beta in the bovine reproductive tract. Journal of Interferon & Cytokine Research. 19, 279-285.

Paulesu, L., Jantra, S., Ietta, F., Brizzi, R., Bigliardi, E., 2008. Interleukin-1 in reproductive strategies. Evolution & development. 10, 778-788.

Payan-Carreira, R., Pires, M.A., Ström Holst, B., Rodriguez-Martinez, H., 2011. Tumour Necrosis Factor in the Canine Endometrium: An Immunohistochemical Study. Reproduction in Domestic Animals. 46, 410-418.

Pemble, L., Kaye, P., 1986. Whole protein uptake and metabolism by mouse blastocysts. Journal of Reproduction and Fertility. 78, 149-157.

Peter, A.T., 2013. Bovine placenta: a review on morphology, components, and defects from terminology and clinical perspectives. Theriogenology. 80, 693-705.

Peters, A., 1985. Studies of hormone patterns during the oestrous cycle of beef cows. Reproduction Nutrition Développement. 25, 919-927.

Peters, A., Ball, P., 2004. Reproduction in cattle.

Phillips, C., 2008. Cattle behaviour and welfare John Wiley & Sons.

Powers, C., Mcleskey, S., Wellstein, A., 2000. Fibroblast growth factors, their receptors and signaling. Endocrine-Related Cancer. 7, 165-197.

Rai, P., Kota, V., Sundaram, C.S., Deendayal, M., Shivaji, S., 2010. Proteome of human endometrium: identification of differentially expressed proteins in proliferative and secretory phase endometrium. Proteomics-Clinical Applications. 4, 48-59.

Rizos, D., Ward, F., Duffy, P., Boland, M.P., Lonergan, P., 2002. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. Molecular Reproduction and Development. 61, 234-248.

Robinson, R.S., Hammond, A.J., Wathes, D.C., Hunter, M.G., Mann, G.E., 2008. Corpus Luteum-Endometrium-Embryo interactions in the dairy cow: underlying mechanisms and clinical relevance. Reproduction in Domestic Animals. 43, 104-112.

Roby, K.F., Hunt, J.S., 1994. Mouse endometrial tumor necrosis factor-alpha messenger ribonucleic acid and protein: localization and regulation by estradiol and progesterone. Endocrinology. 135, 2780-2789.

Ross, J.W., Malayer, J.R., Ritchey, J.W., Geisert, R.D., 2003. Characterization of the interleukin-1β system during porcine trophoblastic elongation and early placental attachment. biology of reproduction. 69, 1251-1259.

Rubessa, M., Boccia, L., Campanile, G., Longobardi, V., Albarella, S., Tateo, A., Zicarelli, L., Gasparrini, B., 2011. Effect of energy source during culture on *in vitro* embryo development, resistance to cryopreservation and sex ratio. Theriogenology. 76, 1347-1355.

Sakagami, N., Umeki, H., Nishino, O., Uchiyama, H., Ichikawa, K., Takeshita, K., Kaneko, E., Akiyama, K., Kobayashi, S., Tamada, H., 2012. Normal calves produced after transfer of embryos cultured in a chemically defined medium supplemented with epidermal growth factor and insulin-like growth factor I following ovum pick up and in vitro fertilization in Japanese Black cows. Journal of Reproduction and Development. 58, 140-146.

Salamonsen, L.A., Edgell, T., Rombauts, L.J.F., Stephens, A.N., Robertson, D.M., Rainczuk, A., Nie, G., Hannan, N.J., 2013. Proteomics of the human endometrium and uterine fluid: a pathway to biomarker discovery. Fertility and Sterility. 99, 1086-1092.

Sandra, O., Mansouri-Attia, N., Lea, R.G., 2011. Novel aspects of endometrial function: a biological sensor of embryo quality and driver of pregnancy success. Reproduction, Fertility and Development. 24, 68-79.

Seli, E., Robert, C., Sirard, M.-A., 2010. OMICS in assisted reproduction: possibilities and pitfalls. Molecular Human Reproduction. 16, 513-530.

Simón, C., Mercader, A., Frances, A., Gimeno, M.J., Polan, M.L., Remohí, J., Pellicer, A., 1996. Hormonal regulation of serum and endometrial IL-1α, IL-1β and IL-1ra: IL-1 endometrial microenvironment of the human embryo at the apposition phase under physiological and supraphysiological steroid level conditions. Journal of Reproductive Immunology. 31, 165-184.

Simón, C., Piquette, G.N., Frances, A., Polan, M.L., 1993. Localization of interleukin-1 type I receptor and interleukin-1 beta in human endometrium throughout the menstrual cycle. The Journal of Clinical Endocrinology & Metabolism. 77, 549-555.

Skarzynski, D.J., Miyamoto, Y., Okuda, K., 2000. Production of prostaglandin F2 alpha by cultured bovine endometrial cells in response to tumor necrosis factor alpha: cell type specificity and intracellular mechanisms. Biology of Reproduction. 69, 1907-1913.

Skarzynski, D.J., Woclawek-Potocka, I., Korzekwa, A., Bah, M.M., Piotrowska, K., Barszczewska, B., Okuda, K., 2007. Infusion of exogenous tumor necrosis factor dose dependently alters the length of the luteal phase in cattle: differential responses to treatment with indomethacin and L-NAME, a nitric oxide synthase inhibitor. Biology of Reproduction. 76, 619-627.

Smith, K.A., Lachman, L.B., Oppenheim, J.J., Favata, M.F., 1980. The functional relationship of the interleukins. The Journal of experimental medicine. 151, 1551-1556.

Song, G., Bazer, F.W., Spencer, T.E., 2007. Pregnancy and interferon tau regulate RSAD2 and IFIH1 expression in the ovine uterus. Reproduction. 133, 285-295.

Song, G., Bazer, F.W., Wagner, G.F., Spencer, T.E., 2006. Stanniocalcin (STC) in the Endometrial Glands of the Ovine Uterus: Regulation by Progesterone and Placental Hormones. Biology of Reproduction. 74, 913-922.

Song, Y., Hu, Z., Long, H., Peng, Y., Zhang, X.A., Que, T., Zheng, S., Li, Z., Wang, G., Yi, L., 2014. A complex mechanism for HDGF-mediated cell growth, migration, invasion, and TMZ chemosensitivity in glioma. Journal of Neuro-Oncology. 119, 285-295.

Soto, P., Natzke, R.P., Hansen, P.J., 2003. Actions of Tumor Necrosis Factor-α on Oocyte Maturation and Embryonic Development in Cattle1. American Journal of Reproductive Immunology. 50, 380-388.

Spencer, T.E., Bazer, F.W., 1995. Temporal and spatial alterations in uterine estrogen receptor and progesterone receptor gene expression during the estrous cycle and early pregnancy in the ewe. Biology of Reproduction. 53, 1527-1543.

Spencer, T.E., Bazer, F.W., 2002. Biology of progesterone action during pregnancy recognition and maintenance of pregnancy. Frontiers in bioscience: a journal and virtual library. 7, 1879-1898.

Spencer, T.E., Bazer, F.W., 2004. Conceptus signals for establishment and maintenance of pregnancy. Reproductive Biology and Endocrinology. 2, 195-209.

Spencer, T.E., Forde, N., Dorniak, P., Hansen, T.R., Romero, J.J., Lonergan, P., 2013. Conceptus-derived prostaglandins regulate gene expression in the endometrium prior to pregnancy recognition in ruminants. Reproduction. 146, 377-387.

Spencer, T.E., Johnson, G.A., Bazer, F.W., Burghardt, R.C., 2004a. Implantation mechanisms: insights from the sheep. Reproduction. 128, 657-668.

Spencer, T.E., Johnson, G.A., Burghardt, R.C., Bazer, F.W., 2004b. Progesterone and placental hormone actions on the uterus: insights from domestic animals. Biology of Reproduction. 71, 2-10.

Spencer, T.E., Sandra, O., Wolf, E., 2008. Genes involved in conceptus-endometrial interactions in ruminants: insights from reductionism and thoughts on holistic approaches. Reproduction. 135, 165-179.

Stephens, A.N., Hannan, N.J., Rainczuk, A., Meehan, K.L., Chen, J., Nicholls, P.K., Rombauts, L.J., Stanton, P.G., Robertson, D.M., Salamonsen, L.A., 2010. Post-translational modifications and protein-specific isoforms in endometriosis revealed by 2D DIGE. Journal of Proteome Research. 9, 2438-2449.

Stojkovic, M., Büttner, M., Zakhartchenko, V., Riedl, J., Reichenbach, H.-D., Wenigerkind, H., Brem, G., Wolf, E., 1999. Secretion of interferon-tau by bovine embryos in long-term culture: comparison of in vivo derived, in vitro produced, nuclear transfer and demi-embryos. Animal Reproduction Science. 55, 151-162.

Sturmey, R., Bermejo-Álvarez, P., Gutierrez-Adan, A., Rizos, D., Leese, H., Lonergan, P., 2010. Amino acid metabolism of bovine blastocysts: a biomarker of sex and viability. Molecular Reproduction and Development. 77, 285-296.

Suarez, S.S., 2006. Interactions of spermatozoa with the female reproductive tract: inspiration for assisted reproduction. Reproduction, Fertility and Development. 19, 103-110.

Sugawara, K., Kizaki, K., Herath, C.B., Hasegawa, Y., Hashizume, K., 2010. Transforming growth factor beta family expression at the bovine feto-maternal interface. Reproductive Biology and Endocrinology. 8, 120-132.

Tabibzadeh, S., Zupi, E., Babaknia, A., Liu, R., Marconi, D., Romanini, C., 1995. Physiology: Site and menstrual cycle-dependent expression of proteins of the tumour necrosis factor (TNF) receptor family, and BCL-2 oncoprotein and phase-specific production of TNFalpha in human endometrium. Human Reproduction. 10, 277-286.

Takacs, P., Kauma, S., 1996. The expression of interleukin-1α, interleukin-1β, and interleukin-1 receptor type I mRNA during preimplantation mouse development. Journal of Reproductive Immunology. 32, 27-35.

Tang, P., Hung, M.C., Klostergaard, J., 1996. Human pro-tumor necrosis factor is a homotrimer. Biochemistry. 35, 8216-8225.

Tanikawa, M., Acosta, T.J., Fukui, T., Murakami, S., Korzekwa, A., Skarzynski, D.J., Piotrowska, K.K., Park, C.K., Okuda, K., 2005. Regulation of prostaglandin synthesis by interleukin-1α in bovine endometrium during the estrous cycle. Prostaglandins and Other Lipid Mediators. 78, 279-290.

Tanikawa, M., Kim, T.S., Okuda, K., Ryoo, Z.Y., Park, S.B., Shin, J.H., Park, C.K., Lee, D.S., 2009. Cell-type specificity of interleukins 1α and 1β on prostaglandin and plasminogen activator production in bovine endometrial cells. Animal Reproduction Science. 114, 32-42.

Tao, F., Ye, M.F., Sun, A.J., Lv, J.Q., Xu, G.G., Jing, Y.M., Wang, W., 2014. Prognostic significance of nuclear hepatoma-derived growth factor expression in gallbladder cancer. World journal of gastroenterology. 20, 9564-9569.

Taskin, E.A., Baltacı, V., Çağıran, G., Aytaç, R., 2012. Detection of IL-1β in culture media supernatants of pre-implantation human embryos; its relation with embryo grades and development. Gynecological Endocrinology. 28, 296-298.

Thakar, K., Kröcher, T., Savant, S., Gollnast, D., Kelm, S., Dietz, F., 2010. Secretion of hepatoma-derived growth factor is regulated by N-terminal processing. Biological Chemistry. 391, 1401-1410.

Thirant, C., Galan-Moya, E.M., Dubois, L.G., Pinte, S., Chafey, P., Broussard, C., Varlet, P., Devaux, B., Soncin, F., Gavard, J., 2012. Differential proteomic analysis of human glioblastoma and neural stem cells reveals HDGF as a novel angiogenic secreted factor. Stem Cells. 30, 845-853.

Thornberry, N.A., Bull, H.G., Calaycay, J.R., Chapman, K.T., Howard, A.D., Kostura, M.J., Miller, D.K., Molineaux, S.M., Weidner, J.R., Aunins, J., 1992. A novel heterodimeric cysteine protease is required for interleukin-1β processing in monocytes. Nature. 356, 768-774.

Tiffin, G., Rieger, D., Betteridge, K., Yadav, B., King, W., 1991. Glucose and glutamine metabolism in pre-attachment cattle embryos in relation to sex and stage of development. Journal of Reproduction and Fertility. 93, 125-132.

Tuo, W., Harney, J.P., Bazer, F.W., 1996. Developmentally regulated expression of interleukin-1β by peri-implantation conceptuses in swine. Journal of Reproductive Immunology. 31, 185-198.

Ulbrich, S.E., Groebner, A.E., Bauersachs, S., 2013. Transcriptional profiling to address molecular determinants of endometrial receptivity-lessons from studies in livestock species. Methods. 59, 108-115.

Upadhyay, R.D., Balasinor, N., Kumar, A.V., Sachdeva, G., Parte, P., Dumasia, K., 2013. Proteomics in reproductive biology: beacon for unraveling the molecular complexities. Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics. 1834, 8-15.

Urrego, R., Rodriguez-Osorio, N., Niemann, H., 2014. Epigenetic disorders and altered gene expression after use of Assisted Reproductive Technologies in domestic cattle. Epigenetics. 9, 803-815.

Uzumcu, M., Braileanu, G.T., Carnahan, K.G., Ludwig, T.E., Mirando, M.A., 1998. Oxytocin-stimulated phosphoinositide hydrolysis and prostaglandin F secretion by luminal epithelial, glandular epithelial, and stromal cells from pig endometrium. I. Response of cyclic pigs on day 16 postestrus. Biology of Reproduction. 59, 1259-1265.

Van Soom, A., Boerjan, M.L., Bols, P.E., Vanroose, G., Lein, A., Coryn, M., De Kruif, A., 1997. Timing of compaction and inner cell allocation in bovine embryos produced in vivo after superovulation. Biology of Reproduction. 57, 1041-1049.

Vigers, G.P.A., Anderson, L.J., Caffes, P., Brandhuber, B.J., 1997. Crystal structure of the type-I interleukin-1 receptor complexed with interleukin-1[beta]. Nature. 386, 190-194.

Von Wolff, M., Classen-Linke, I., Heid, D., Krusche, C.A., Beier-Hellwig, K., Karl, C., Beier, H.M., 1999. Tumour necrosis factor-α (TNF- α) in human endometrium and uterine secretion: an evaluation by immunohistochemistry, ELISA and semiquantitative RT-PCR. Molecular Human Reproduction. 5, 146-152.

Walker, C., Meier, S., Littlejohn, M., Lehnert, K., Roche, J., Mitchell, M., 2010. Modulation of the maternal immune system by the pre-implantation embryo. BMC Genomics. 11, 474-487.

Walters, K.W., Corbin, C.J., Anderson, G.B., Roser, J.F., Conley, A.J., 2000. Tissue-specific localization of cytochrome P450 aromatase in the equine embryo by in situ hybridization and immunocytochemistry. Biology of Reproduction. 62, 1141-1145.

Wallach, D., Engelmann, H., Nophar, Y., Aderka, D., Kemper, O., Hornik, V., Holtmann, H., Brakebusch, C., 1990. Soluble and cell surface receptors for tumor necrosis factor. Agents and Actions. Supplements. 35, 51-57.

Wang, C., Davamani, F., Sue, S., Lee, S., Wu, P., Tang, F., Shih, C., Huang, T., Wu, W., 2011. Cell surface heparan sulfates mediate internalization of the PWWP/HATH domain of HDGF via macropinocytosis to fine-tune cell signalling processes involved in fibroblast cell migration. Biochem. J. 433, 127-138.

Wang, L., Jiang, Q., Hua, S., Zhao, M., Wu, Q., Fu, Q., Fang, W., Guo, S., 2014. High nuclear expression of HDGF correlates with disease progression and poor prognosis in human endometrial carcinoma. Disease Markers. 2014, article ID 298795.

Wang, X.L., Wang, K., Han, G.C., Zeng, S.M., 2013. A potential autocrine role for interferon tau in ovine trophectoderm. Reproduction in Domestic Animals. 48, 819-825.

Watson, A.J., 1992. The cell biology of blastocyst development. Molecular Reproduction and Development. 33, 492-504.

Watts, A.D., Hunt, N.H., Wanigasekara, Y., Bloomfield, G., Wallach, D., Roufogalis, B.D., Chaudhri, G., 1999. A casein kinase I motif present in the cytoplasmic domain of members of the tumour necrosis factor ligand family is implicated in 'reverse signalling'. The EMBO journal. 18, 2119-2126.

Weber, A., Wasiliew, P., Kracht, M., 2010. Interleukin-1 (IL-1) pathway. Science signaling. 3, cm1.

Wesche H, K.C., Kracht M, Falk W, Resch K, Martin Mu, 1997. The Interleukin-1 receptor accessory protein (IL1RacP) is essential for IL1-induced activation of Interleukin-1 receptor-associated kinase (IRAK) and stress-activated protein kinases (SAP kinases). Journal of Biological Chemistry. 272, 7727-7731.

Wheeler, M.B., Rutledge, J.J., Fischer-Brown, A., Vanetten, T., Malusky, S., Beebe, D.J., 2006. Application of sexed semen technology to in vitro embryo production in cattle. Theriogenology. 65, 219-227.

Wiley, S.R., Goodwin, R.G., Smith, C.A., 1996. Reverse signaling via CD30 ligand. The Journal of Immunology. 157, 3635-3639.

Witkin, S.S., Liu, H.-C., Davis, O.K., Rosenwaks, Z., 1991. Tumor necrosis factor is present in maternal sera and embryo culture fluids during in vitro fertilization. Journal of Reproductive Immunology. 19, 85-93.

Wolf, E., Arnold, G., Bauersachs, S., Beier, H., Blum, H., Einspanier, R., Frohlich, T., Herrler, A., Hiendleder, S., Kolle, S., 2003. Embryo-maternal communication in bovine - strategies for deciphering a complex cross-talk. Reproduction in Domestic Animals. 38, 276 - 289.

Wooding, F., 1992. The synepitheliochorial placenta of ruminants: binucleate cell fusions and hormone production. Placenta. 13, 101-113.

Wu, B., Zan, L., 2012. Enhance Beef Cattle Improvement by Embryo Biotechnologies. Reproduction in Domestic Animals. 47, 865-871.

Yelavarthi, K., Hunt, J., 1993. Analysis of p60 and p80 tumor necrosis factor-alpha receptor messenger RNA and protein in human placentas. The American journal of pathology. 143, 1131-1141.

Yelavarthi, K.K., Chen, H., Yang, Y., Cowley, B., Fishback, J., Hunt, J., 1991. Tumor necrosis factor-alpha mRNA and protein in rat uterine and placental cells. The Journal of immunology. 146, 3840-3848.

Young, B., Heath, J.W., 2000. Wheather's Histología funcional, texto y atlas en color.

Zhou, Z., Yamamoto, Y., Sugai, F., Yoshida, K., Kishima, Y., Sumi, H., Nakamura, H., Sakoda, S., 2004. Hepatoma-derived growth factor is a neurotrophic factor harbored in the nucleus. Journal of Biological Chemistry. 279, 27320-27326.

CAPÍTULO 2 EARLY EMBRYONIC AND ENDOMETRIAL REGULATION OF TNF AND TNFR2 IN THE CATTLE UTERUS

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RESUMEN EN CASTELLANO

El factor de necrosis tumoral alfa (TNF) podría intervenir en la comunicación materno-embrionaria en mamíferos. En bovino, hemos identificado previamente que el fluido uterino (FU) de novillas portadoras de embriones tempranos muestra una regulación a la baja de TNF y del sistema NFKB. En este trabajo hemos analizado la expresión de TNF y su receptor TNFR2 en el endometrio y embrión bovinos durante el desarrollo del blastocisto. Además, para investigar la respuesta inmune del endometrio frente a los embriones tempranos, evaluamos el número de leucocitos CD45 en el endometrio bovino. A partir de animales sujetos a transferencia embrionaria en Día 5, se recogieron muestras de endometrio y blastocistos en Día 8. Tanto el endometrio como los blastocistos mostraron trancripción de *TNF* y *TNFR2,* y colocalización de las correspondientes proteínas. En el endometrio, la presencia de embriones incrementó las proteínas TNF y TNFR2 y redujo el número de leucocitos. Los blastocistos expuestos al tracto uterino mostraron niveles por debajo del límite de detección de *TNF* y menor transcripción de *TNFR2*. Estos resultados sugieren que el endometrio podría disminuir la concentración de TNF en el blastocisto mediante (1) la regulación de la secreción de TNF en el fluido uterino y (2) la reducción de la actividad trancripcional de *TNF* y *TNFR2* en el embrión. Por tanto, es probable que TNF y TNFR2 participen en la comunicación materno-embrionaria temprana.

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Capítulo 2

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Early embryonic and endometrial regulation of tumor necrosis factor and tumor necrosis factor receptor 2 in the cattle uterus

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ABSTRACT

Tumor necrosis factor (TNF) alpha likely mediates embryomaternal communication in mammals. In bovine, we have previously found that the uterine fluid of heifers that carried early embryos shows downregulation in the TNF and nuclear factor κB system. In this work, we assessed the expression of TNF and its receptor TNFR2 in the bovine endometrium and embryos during blastocyst development. Moreover, to explore the endometrial immune response to early embryos, we analyzed the number of CD45 leukocytes in the bovine endometrium. Day 8 endometrium and blastocyst recovered from animals after transfer of Day 5 embryos showed TNF and TNFR2 mRNA transcription and protein colocalization. The presence of embryos increased endometrial TNF and TNFR2 protein, whereas endometrial leukocytes decreased. Blastocysts exposed to the uterine tract had undetectable levels of *TNF* and lower levels of *TNFR2* mRNA. These results suggest that the endometrium might lower the TNF concentration in the blastocyst by (1) regulating TNF secretion into the uterine fluid and (2) inducing decreased *TNF* and *TNFR2* mRNA transcription in the embryo. Thus, TNF and TNFR2 might participate in early embryomaternal communication.

1. Introduction

Embryomaternal communication is essential for embryo implantation and successful pregnancy to term [1]. Failure to replicate the maternal environment surrounding the embryo hampers the *in vitro* reproduction procedures [2], making research in this field a priority for reproductive biology. In natural conditions, communication is governed by growth factors produced and secreted by the embryo and the endometrium [3]. One of such factors might be tumor necrosis factor (TNF), a proinflammatory cytokine that mediates cell differentiation, survival, renewal, and tissue homeostasis [4]. Tumor necrosis factor activates several intracellular pathways through its binding to two distinct receptors, TNFR1 and TNFR2 [5]. Receptor TNFR1 associates with apoptosis and TNFR2 is more versatile leading to apoptotic or proliferation processes depending on the stimulus.

Some evidence supports the participation of TNF and TNFR2 in normal reproduction. Thus, TNF and TNFR2 endometrial expression vary throughout the estrous cycle in humans and domestic animals [6–9], and both seem to exert a role during pregnancy in humans, mice, and dogs [8,10–12]. In bovine, TNF and TNFR2 mRNA and protein have been detected in the cyclic and pregnant endometrium [7,13]. Expression of

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the *TNF* and *TNFR2* genes is not regulated by pregnancy [13], although their mRNA levels change throughout the estrous cycle, being regulated by TNF itself, other cytokines (interleukin 1alpha), and sexual hormones such as oxytocin [7]. Similarly, TNF and TNFR2 protein and mRNA synthesis occurs at specific developmental stages in embryos from humans, rats, and mice [14], and also in cattle [15,16]. Uterine fluid (UF) contains TNF in cyclic cows, humans, and mice [16–19] as well as in cows and monkeys that have carried embryos [16,20]. Tumor necrosis factor release has been observed from human endometrial cells cultured *in vitro* [6] and embryos [21]. Collectively, the previously mentioned data indicate that the cytokine is secreted by the endometrium and or embryos. In the embryo-carrying bovine endometrium, we have detected the 78 kDa TNF isoform downregulated in the UF [16]. Most of the previously mentioned studies provided significant knowledge on the TNF system in the embryo and maternal tissues, particularly during implantation [8,10,11,13]. However, little is known about the endometrial expression of TNF system during earlier stages. Furthermore, in pregnant bovine endometrium, only mRNA transcriptions of *TNF* and its receptor, but not protein expression, have been studied [13]. Similarly, in the bovine embryo only the mRNA for *TNFR2* has been found [15].

During blastocyst formation, ungulate embryos downregulate uterine nuclear factor κB at the protein and gene expression levels [16,22], probably depressing the immune response in favor of embryo tolerance. Other studies have described changes in the immune endometrial cells around implantation in response to embryos (Days 12, 15, 16, and 18) [23,24]. However, to our knowledge, the protein expression of immune cells in the bovine uterus in response to very early embryos has not been studied yet.

The aim of this study was to assess the regulation of TNF system and CD45 leukocytes by the embryo and the maternal tract as likely contributors to early embryomaternal communication in cattle. We tested the hypothesis that the embryo and the endometrium may reciprocally regulate TNF, TNFR2, and CD45 leukocytes at the time of blastocyst formation. Specifically, we examined whether (1) TNF and TNFR2 mRNA and protein are present in the bovine endometrium and embryos during the blastocyst stage; (2) their expression is reciprocally regulated; and (3) the presence of embryos affects the expression of endometrial CD45 leukocytes.

2. Materials and methods

Reagents were purchased from Sigma-Aldrich (Madrid, Spain) unless otherwise stated. Experiments involving animals complied with the Directive 2010/63/EU (Spanish RD 53/2013) and were sanctioned by the Servicio Regional de Investigación y Desarrollo Agroalimentario Animal Research Ethics Committee. Animal management, estrus synchronization, and embryo production and transfer procedures are previously described [16].

2.1. Embryo production

In vitro–produced (IVP) embryos were obtained as described [25], with minor modifications. Ovaries were obtained from a local abattoir, and from them antral follicles (from 3 to 8 mm) were aspirated. The recovered cumulus-oocyte complexes (COCs) were rinsed three times in a holding medium (HM) consisting of Medium 199 (Invitrogen, Barcelona, Spain), 25 mM HEPES, and BSA 0.4 g/L. Only oocytes enclosed in a compact cumulus with an evenly granulated cytoplasm were selected for maturation. Groups of approximately 50 COCs were placed in 500 μL maturation medium consisting of TCM199 $NaHCO_3$ (2.2 g/L), fetal calf serum (10% v:v), porcine FSH-LH (1:5 μg/mL, Stimufol; ULg FMV, Liège, Belgium, France), and 17 β-estradiol (1 μg/mL). Cumulus-oocyte complexes were matured for 24 hours at 38.7 °C in 5% CO_2 and saturated humidity.

For IVF (Day 0), sperm was prepared by the swim-up procedure [26]. Briefly, semen from one frozen straw corresponding to one bull was thawed in a water bath and added to a polystyrene tube containing 1 mL of pre-equilibrated Sperm-TALP (Tyrode's albumin lactate pyruvate). After 1 hour of incubation, the upper layer of supernatant containing motile sperm was recovered. The sperm were centrifuged for 7 minutes at 200× *g* and the supernatant aspirated to leave a pellet containing the sperm in which the concentration was determined with a hemocytometer. Meanwhile, COCs were washed twice in HM and placed in four-well culture dishes containing pre-equilibrated fertilization medium (Fert-TALP) with heparin (10 μg/mL; Calbiochem, La Jolla, CA, USA). Spermatozoa were added at a concentration of 2×10^6 cells/mL in 500 μL of medium per well containing a maximum of 100 COCs. The IVF was accomplished by incubating oocytes and sperm cells together for 18 to 20 hours at 39 °C in an atmosphere of 5% CO_2 with saturated humidity.

For IVC, cumulus cells were detached using a vortex, and presumptive zygotes were cultured in synthetic oviduct fluid modified with amino acids (BME Amino Acids Solution (SIGMA, Madrid, Spain), 45 μL/mL and MEM Non-essential Aminoacid Solution (SIGMA, Madrid, Spain), 5 μL/mL), citrate, myo-inositol, and BSA (6 g/L), as previously described [27]. Droplets of synthetic oviduct fluid modified (1–2 μL per embryo) were layered under mineral oil and embryos cultured in groups of 35 to 45. *In vitro* culture was carried out at 39 °C, 5% CO_2, 5% O_2, and saturated humidity. Culture medium was renewed on Days 3 and 6 by transferring the embryos to fresh droplets. Cleavage (Day 3) and development (from Days 6 to 8) rates were recorded following reported criteria [28].

2.2. Embryo transfer

Embryos were *in vitro* cultured until Day 8 or were nonsurgically transferred on Day 5 to the cranial third of the CL ipsilateral uterine horn of synchronized animals (50 morulae per embryo transferred [ET] cow). The transfer of multiple embryos to the uterus has been previously validated as a model to study early embryomaternal interactions in cattle [16,29]. A control group of animals was sham transferred (ST) with same volume (45 mL) of embryo HM (Instruments de Médecine Vétérinaire, Humeco, Huesca, Spain). Age-matched heifers and uniparous cows (n = 3 heifers + 2 cows for ET; 3 heifers + 3 cows for ST) were sacrificed in a nearby slaughterhouse (Matadero de Pravia, Asturias) on Day 8.

2.3. Collection of endometrial samples

Endometrial samples were taken from the uterine horn ipsilateral to the CL. Caruncular and intercaruncular regions of middle and cranial uterine thirds were sampled as previously described [29]. Endometrial tissues for immunohistochemistry (IHC) were fixed in 4% paraformaldehyde for 24 hours at 4 °C and embedded in paraffin (Panreac, Barcelona, Spain). Endometrial samples for Western blotting (WB) were snap frozen in Eppendorf LoBind Tubes (VWR, Hamburg, Germany) and kept at −150 °C until further use. Endometrial samples for real-time quantitative polymerase chain reaction (RT-qPCR) were incubated in RNA later (Ambion, Huntingdon, UK) overnight at 4 °C; afterward, excess RNA later was removed, and samples were snap frozen and stored at −150 °C until use.

2.4. Embryo processing

Embryos were recovered from the uterus by flushing five times the ipsilateral uterine horn with 45 mL of D-PBS (GIBCO 14190–094) + 1 mg/mL of polyvinylpyrrolidone, as described [29]. Only expanded blastocysts (32.2 ± 8.5 as a proportion of the recovered embryos) were further processed for RT-qPCR and IHC. Embryos for IHC were fixed in 4% paraformaldehyde for 20 minutes at room temperature and kept in 0.1 M of PBS + 0.2 mg/mL of polyvinyl alcohol (pH = 7.4, 4 °C) until further use. Embryos for RT-qPCR were snap frozen and kept at −150 °C until further use. As each individual embryo provided an insufficient quantity of mRNA, we used five pools of n = 10 expanded blastocysts per each experimental group (entirely *in vitro* produced vs. after passage through the uterine tract).

2.5. Immunohistochemistry

Immunostaining procedures on endometrium and embryos have been described elsewhere [30] and modified by Gómez et al. [29]. The antibodies used were as follows: TNF (LS-C43037; LSBio, Vizcaya, Spain; dilution 5 μg/mL) and TNFR2 (ab15563; Abcam, Cambridge, UK; dilution 4 μg/mL). Within endometrial sections for TNFR2, antigen retrieval was performed by further 10-minute incubation with 0.01 M citrate buffer (pH 6, 90 °C). Incubation times with antibodies were 72 hours (endometrium) and 24 hours (embryos). Afterward, samples were incubated with the secondary antibody Alexa 488 goat antirabbit IgG (A11034; Invitrogen, Oregon, USA; dilution 2.5 μg/mL) and mounted in Vectashield with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Inc., Burlingame, USA). A double TNF and TNFR2 immunostaining was also performed using the protocols described previously for endometrium and embryos with the following modifications: (1) the incubation times were 24 hours; (2) gentle washing and a second blocking step between both incubations; (3) secondary antibodies were linked to Alexa 488 (for TNF; dilution 3.3 μg/mL) and Alexa 555 (A21429; Invitrogen) (for TNFR2; dilution 2 μg/mL). Positive immunostaining was evaluated using a confocal microscope (Leica TCS-SP2-AOBS; Leica Microsystems) through serial Z-axis optical sections.

In case of endometrial samples, the staining intensity of TNF and TNFR2 was assessed after performing IHC with the VECTASTAIN ABC kit (following manufacturer instructions; Vector Laboratories, Peterborough, UK). The bound complex was made visible by reaction with 3,3′-diaminobenzidine (DAB). Finally, sections were counterstained in Harris hematoxylin, dehydrated, and mounted with DePeX Eukitt. Images were recorded using an Olympus BX51 fitted with an Olympus DP70 digital camera. Positive DAB immunostaining was scored by two independent observers blind to the nature of the tissue (0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining). An average area of 18.7 mm^2 for each tissue section was evaluated; within this area, the glandular epithelium, luminal epithelium, and stroma were differentiated. Three tissue sections or replicates for each endometrial region and animal were used.

Finally, the number of CD45-positive cells in the endometrial samples was evaluated using an antibody against this protein (CACTB51A; VMRD, Madrid, Spain; dilution 15 μg/mL) and following the protocol described previously (11 minutes and 45 seconds of incubation for the DAB reaction). The number of CD45-positive cells was determined as reported [24]. The Computer Assisted Stereological Toolbox software package (Olympus) was used to randomly move the counting frame (area, 5712 μm^2) throughout each tissue section (average area, 18.7 mm^2). At least three counting frames were counted per cellular structure within each tissue section by differentiating between glandular epithelia and stroma in the subepithelial, functional, and basal zones. The number of CD45-positive cells was evaluated using three cows per experimental group (embryo transfer or sham transfer).

Negative controls using normal goat serum instead of the primary antibody were included in all the protocols.

2.6. Western blotting

Endometrial tissue was homogenized with a high-intensity ultrasonic processor (Sonics & Materials, Newtown, USA) using 10 mL of tissue lysis reagent (CelLyticTM MT C3228) and 50 μL of protease inhibitor cocktail (P8340) per gram. After centrifugation at 3026× *g* for 10 minutes at 4 °C, the tissue supernatant fraction was collected and stored at −150 °C. Protein concentration in total lysate was determined using the Bradford assay (Bio-Rad Laboratories, Hercules, USA). Equal amounts of protein (10 μg) were separated on 12% (v:v) SDS-PAGE for 1.5 hours, then transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, USA) and probed with the antibodies for TNF (5 μg/mL) and TNFR2 (2 μg/mL), 24 hours at 4 °C. An enhanced chemiluminescence kit (Thermo Scientific, Rockford, USA) was used to visualize the blots. Signal intensity was quantified using the software Confocal Uniovi Image-J (University of Oviedo, Spain; http://www.sct.uniovi.es/index.php?option=content&task=view&id=224).

2.7. Extraction and reverse transcription of RNA

Total RNA was isolated from frozen tissue using TRIzol Reagent (Invitrogen, Cergy-Pontoise, France) (endometrial samples) or Picopure RNA isolation kit (Arcturus) (embryos).

All RNA samples were purified on Qiagen columns following the manufacturer's protocol (RNeasy Mini kit; Qiagen, Courtaboeuf, France) and subjected to a DNaseI treatment (Roche Diagnostics, Meylan, France).

Total purified RNA (1 μg of endometrial samples and 10 equivalent embryos) was reverse transcribed using the Superscript III enzyme (Invitrogen) and random primer hexamers (Roche). The PCR programs were as follows: (1) for the endometrium: 65 °C for 10 minutes, 42 °C for 50 minutes, and 70 °C for 15 minutes; and (2) for the embryo: 25 °C for 5 minutes, 50 °C for 60 minutes, and 70 °C for 15 minutes.

2.8. Quantitative real-time PCR

Primer sets (Table 1) were developed using known bovine sequences and designed using the Primer Express software (v3.0; Applied Biosystems, San Francisco, CA, USA), except for TNF, which were designed with Beacon Designer (Premier Biosoft, Palo Alto CA, USA). Such TNF primers did work well with endometrial samples, but they did not work well with embryonic samples. Therefore, TNF primers specific for embryos were designed according to the study by Yang et al. [31]. The RT-qPCRs were performed using StepOnePlus Real-Time PCR System and Master Mix SYBR Green (Applied Biosystems, Cergy-Pontoise, France). Assays were performed in duplicate for endometrium and triplicate for embryonic samples. The reaction mixture for amplification consisted of the following: (1) endometrium: 5 μL cDNA diluted 1:200 in a final reaction volume of 15 μL and (2) embryos: 10 μL cDNA containing 0.2 equivalent embryos in a final reaction volume of 25 μL. Nontemplate controls were run for each gene. The PCR program was as follows: two initial stages at 50 °C for 2 seconds and 95 °C for 10 minutes, followed by 45 (endometrium) or 40 (embryos) cycles at 95 °C for 15 seconds and a final annealing and extension step at 60 °C for 1 minute.

To confirm product specificity, melting-curve analyses were performed immediately after amplification following denaturation at 95 °C for 15 seconds, annealing at 60 °C for 20 seconds, and 1 cycle with 0.8 °C (endometrium) or 1 °C (embryos) increment. Primer annealing temperature was 60 °C. The standard curve was produced using cDNA retrotranscribed from an RNA pool of all endometrial samples and serially diluted. The amplification efficiency and correlation coefficients were higher than 80% and 0.98, respectively. Amplified endometrial PCR fragments were verified using DNA sequencing (Beckman Coulter Genomics, Takeley, UK) and analysis by the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For embryonic samples, fragment size was confirmed in a 1% agarose gel stained with ethidium bromide.

2.9. Data analysis of qPCR

The reference genes were chosen for the following: (1) for endometrium Solute Carrier Family 30 (Zinc Transporter), Member 6 (SLC30A6), C20RF29, and ribosomal protein L19 (RPL19) using the geNorm applet as detailed in the study by Vandesompele et al. [32]; and (2) for embryos Glyceraldehyde-3-Phosphate Dehydrogenase (GAPD), Succinate dehydrogenase complex, subunit A (SDHA), and Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (YWHAZ) according to the study by Goossens et al. [33]. All the Ct values were transformed into calibrated normalized relative quantities using the qBase plus software (Biogazelle, Gent, Belgium) [34].

2.10. Statistical analysis

Data were analyzed using the Proc generalized linear model module of the statistical analysis system/STAT software (Version 9.2; SAS Institute, Cary, USA). The models for endometrial samples included the following fixed effects:

Table 1
Primers used for the real-time quantitative polymerase chain reaction on bovine endometrium (*TNF*[a], *TNFRSF1B*, *SLC30A6*, *C20RF29*, and *RPL19*) and embryo (*TNF*[b], *TNFRSF1B*, *GAPD*, *SDHA*, and *YWHAZ*).

Name	Accession number	Primer sequence (concentration, μM)	Amplicon size (bp)
TNF[a]	NM_173966	F: TGTTCCTCACCCACACCAT (0.3) R: CTCTTGATGGCAGACAGGATG (0.3)	76
TNF[b]	NM_173966	F: CTTCTGCCTGCTGCACTTCG (0.3) R: GAGTTGATGTCGGCTACAACG (0.3)	156
TNFRSF1B	NM_001040490.2	F: ACCGCATGCTTTAGCTGTAACTC (0.3) R: GCAGATGCGATTCTGTTTCG (0.3)	84
SLC30A6	NM_001075766.1	F: TGATGAGGAAACCTAGCCCTGCC (0.3) R: TCGGGCTGCTCCAAAAAGCGT (0.3)	142
C20RF29	XM_582695.5	F: CCTTCAAGAGCCCCCTGT (0.3) R: GGGTCCTTTTCCAACTCTCC (0.3)	64
RPL19	NM_001040516	F: CCCCAATGAGACCAATGAAATC (0.3) R: CAGCCCATCTTTGATCAGCTT (0.3)	73
GAPD	XM_618013	F: TTCAACGGCACAGTCAAGG (0.2) R: ACATACTCAGCACCAGCATCAC (0.2)	119
SDHA	NM_174178	F: GCAGAACCTGATGCTTTGTG (0.3) R: CGTAGGAGAGCGTGTGCTT (0.3)	185
YWHAZ	BM446307	F: GCATCCCACAGACTATTTCC (0.2) R: GCAAAGACAATGACAGACCA (0.2)	120

Direction of all sequences is 5′ to 3′.
Abbreviations: F, forward; R, reverse; TNF, tumor necrosis factor.

uterine third, uterine region, presence of embryos, replicate, and animal (where appropriate). Models for embryos included replicate (pool) and origin (totally produced *in vitro* vs. exposed to the uterus). Least squares means and their errors (±Standard errors of the means) were estimated for each level of fixed effects with a significant F value. The Ryan-Einot-Gabriel-Welsch test was used to compare raw means.

3. Results

3.1. Tumor necrosis factor and TNFR2 in the endometrium

In both, the ET and ST endometrium, TNF and TNFR2 colocalized in most cells of the luminal and glandular epithelium, the stroma, and the endothelium. Tumor necrosis factor localized only in cytoplasm, whereas TNFR2 was present in both nucleus and cytoplasm (Fig. 1E, F). Staining was more intense in the apical side of the glandular epithelium for both proteins, and sometimes, staining appeared in the glandular lumen (Fig. 1F). Tumor necrosis factor expression in the stroma of the caruncles was higher than that in intercaruncles (2.5 ± 0.2 vs. 1.4 ± 0.2; $P < 0.01$) (Fig. 1A, B). In the presence of embryos, the expression of TNF in the basal glandular epithelia tended to increase ($P = 0.05$; Fig. 2A). Similarly, embryos increased the expression of TNFR2 protein in the basal glandular epithelia of ET versus ST endometrium (2.3 ± 0.2 vs. 1.7 ± 0.2; $P = 0.03$) (Figs. 1C, D and 2B). The number of animals analyzed was five ET and six ST, and the number of biological replicates was 20 ET and 24 ST.

Several TNF isoforms were detected using WB (Fig. 3B). These isoforms comprised a likely soluble 17 kDa protein and some membrane-bound isoforms (nonglycosylated 26 kDa and glycosylated 34, 37, 50, and 78 kDa). The presence of embryos significantly increased the 78-kDa endometrial isoform and tended to increase the following isoforms: 17, 26, and 37 kDa (Fig. 3A). There were no significant differences regarding the region or third analyzed, although there was a tendency for the 26 kDa isoform to be more abundant in the middle versus the cranial uterine third (25.9 ± 5.8 vs. 11.2 ± 5.8; $P = 0.09$). A unique band for TNFR2 was detected by WB at the predicted molecular weight of 55 kDa, which was not affected by the presence and absence of embryos in the uterus (Fig. 3A). However, TNFR2 protein expression was more abundant in caruncles versus intercaruncles (30.6 ± 2.2 vs. 24.6 ± 1.2; $P = 0.04$) and in cranial versus middle third (35.0 ± 3.6 vs. 20.2 ± 2.9; $P = 0.02$). The number of animals analyzed was five ET and six ST, and the number of biological replicates was 20 ET and 24 ST.

Expression of *TNF* and *TNFR2* transcripts was not significantly affected by the presence of embryos (Fig. 4). However, *TNF* abundance was higher in the middle versus the cranial area (3.01 ± 0.1 vs. 2.6 ± 0.1; $P = 0.03$). The expression of *TNFR2* did not change significantly between endometrial areas and regions. The number of animals analyzed was five ET and six ST, and the number of biological replicates was 12 ET and 16 ST.

3.2. Tumor necrosis factor and TNFR2 in embryos

Both IVP embryos and embryos exposed to the uterus contained mRNA and protein for TNF and TNFR2; these proteins were highly colocalized, mainly in the

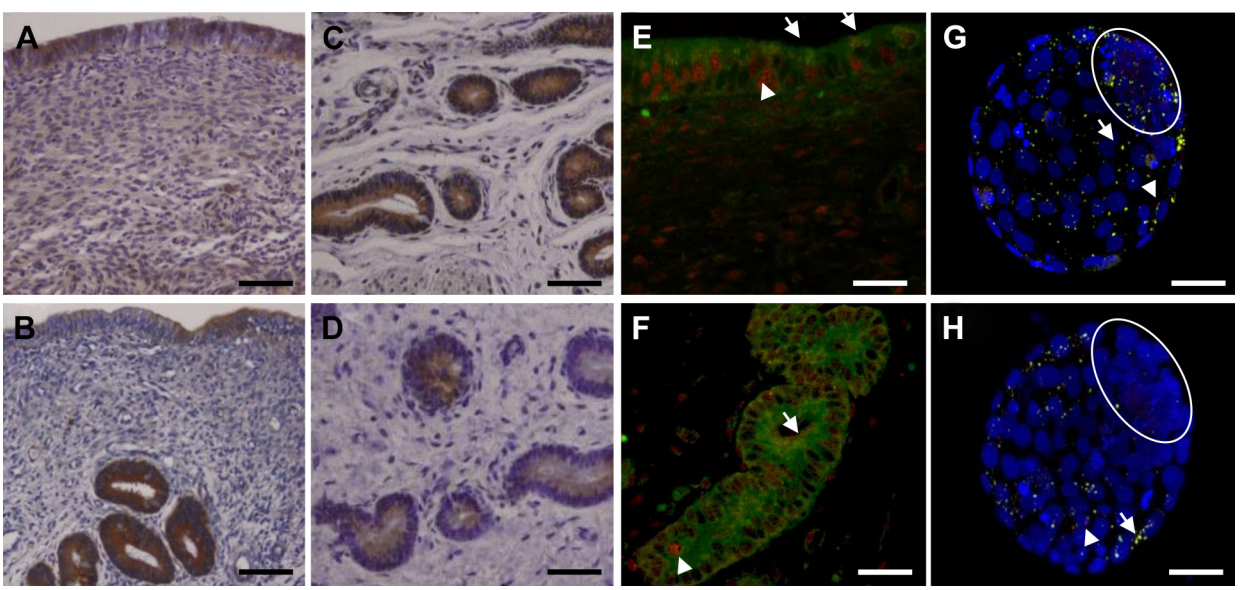

Fig. 1. Representative images of bovine Day 8 endometrium immunostained for the semiquantification of (A, B) TNF and (C, D) TNFR2. The staining for TNF and TNFR2 was more intense in the caruncles (A) than in the intercaruncles (B) and in the basal glandular epithelium of ET (C) compared with ST animals (D), respectively. Colocalization of TNF and TNFR2 in the endometrial (E) luminal and (F) glandular epithelium and in the blastocyst, (G) *in vitro* produced, and (H) after uterine passage. Images are maximal projections. Arrows show colocalized spots, and arrowheads show TNF (green) or TNFR2 (red) single staining. The inner cell mass area is highlighted within an ellipse. Scale bars (A–H: 200 μm; E–H: 65 μm). Data from n = 5 ET and n = 6 ST cows. ET, embryo transferred; ST, sham transferred; TNF, tumor necrosis factor. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

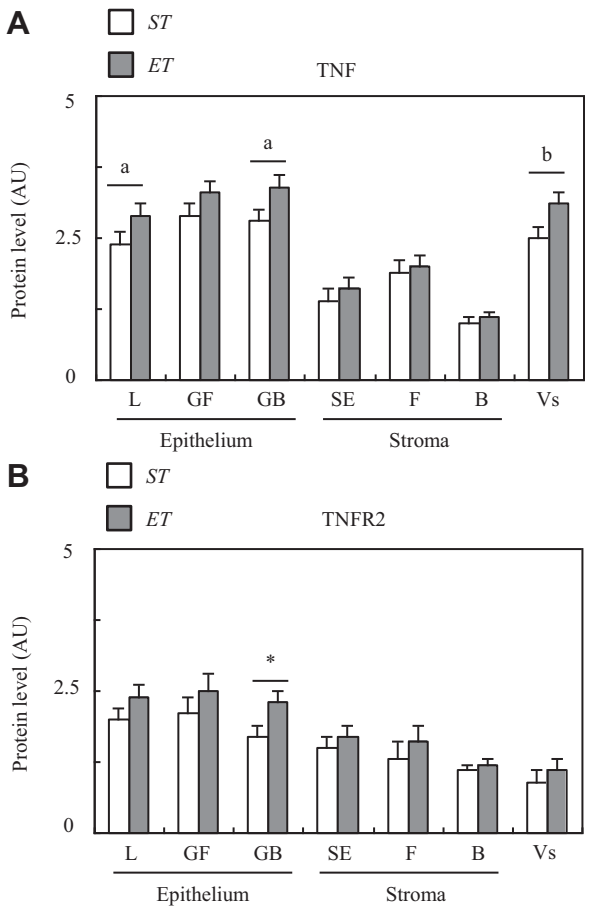

Fig. 2. Protein levels of (A) TNF and (B) TNFR2 in Day 8 uterus that was ET or ST on Day 5, as determined by semiquantitative analysis of immunostaining. The different endometrial cells, as well as the epithelium and stroma from the functional and basal zones, were differentiated. Data presented are the mean ± standard error of the mean; asterisk represents significant differences ($P = 0.03$). Lowercase letters represent a tendency ($^{a}P = 0.05$ and $^{b}P = 0.06$). Data from n = 5 ET and n = 6 ST cows. B, basal; ET, embryo transferred; F, functional; GB, glandular basal; GF, glandular functional; L, luminal; SE, subepithelial; ST, sham transferred; TNF, tumor necrosis factor; Vs, endothelium of the blood vessels.

trophectoderm (Fig. 1G, H). Tumor necrosis factor and TNFR2 proteins were found only in the cytoplasm, not in the nucleus.

Tumor necrosis factor was undetectable in embryos after passage through the uterine tract, whereas IVP embryos were found to have measurable values (Fig. 5). However, TNFR2 was detected in both types of embryos, and its concentrations were lower ($P = 0.006$) in IVP embryos (Fig. 5). For each treatment, five replicates were used, each replicate consisting of RNA extracted from 10 embryos.

3.3. Cell counts of CD45 cells

The presence of embryos led to decreased numbers of endometrial leukocytes in the functional glandular epithelium and the subepithelial stroma ($P = 0.01$ and $P = 0.0001$, respectively). Furthermore, the caruncles contained fewer leukocytes than intercaruncles in the glandular basal epithelium ($P = 0.004$) and in the functional stroma ($P = 0.001$) and basal stroma ($P = 0.03$). The cranial third tended to contain less leukocytes than the middle in the basal stroma ($P = 0.06$) (Table 2).

4. Discussion

4.1. Tumor necrosis factor and TNFR2 expression

Our study confirms for the first time the expression of mRNA and protein for TNF and its receptor TNFR2 in cattle endometrium and embryos during the very early development. Our endometrial pattern agrees with previous descriptions in pregnant and cyclic humans, rats, and mice [14,17], and cyclic dogs, horses, and cows [7–9,35]. The receptor TNFR2 might indeed mediate TNF binding in the endometrium and blastocyst, as both proteins colocalize in most cells from all the studied tissues. Previous studies revealed that TNFR2 differentially regulated in the endometrium during pregnancy and estrous cycle [7,11], and our study is the first to show TNFR2 protein present in the bovine blastocyst.

Endometrial protein expression of TNF is higher in caruncular than in intercaruncular region and tended to increase in the middle uterine third. Similarly, *TNF* mRNA is more abundant in the middle region, although it did not change within the endometrial region. We detected more TNF protein in caruncles, structures in charge of hosting embryo implantation [36]. Within the endometrial tissues, TNF and TNFR2 proteins localize to the epithelium, endothelium, and stroma, although expression is stronger in the glandular epithelium, more specifically in the glandular apical size. Their localization supports a secretory role of TNF and TNFR2 [9,37] and suggests that both proteins can be secreted into the uterine lumen. This is consistent with the presence of TNF in the UF of several species including cattle [16–20] and also with the existence of soluble forms of TNFR2 [38]. On the other hand, the nuclear localization of TNFR2 may be explained as protein withdrawal from the cytoplasm to downregulate cell responsiveness to TNF [39].

4.2. Endometrial responses to embryos

The passage of embryos through the uterus upregulates endometrial TNF protein expression without affecting *TNF* mRNA. Previous studies reported endometrial transcriptome changes induced by embryos on Day 13 but not earlier [40–42]. Similarly, the use of nanoscale liquid chromatography coupled to tandem mass spectrometry (nano-LC MS/MS) revealed proteomic changes in the UF of pregnant heifers from Days 10 to 19 [43]. Overall, the communication between mother and embryo seems to start earlier than classically understood, and the study of the proteome seems more promising than transcriptome to this respect. Given that mammalian embryos move in loops through the uterine horn axis [44], embryo-induced local gene expression might be dispensable to the embryo, as the embryo would be away from the induction site at the time a response is provided. Therefore, faster posttranscriptional responses affecting protein processing and/or protein or

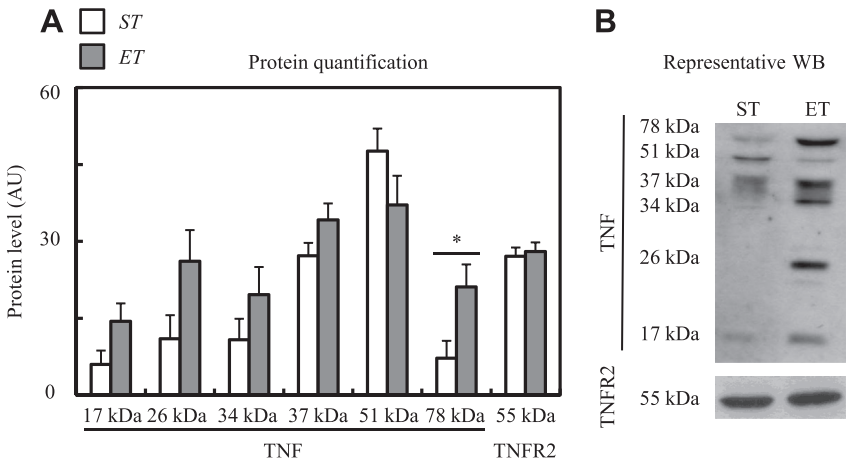

Fig. 3. (A) Protein levels of TNF and TNFR2 in Day 8 uterus that was ET or ST on Day 5, as determined using WB. Data expressed are the mean ± standard error of the mean; asterisk represents significant differences (P = 0.05). (B) Representative WBs. Data from n = 5 ET and n = 6 ST cows. ET, embryo transferred; ST, sham transferred; TNF, tumor necrosis factor; WB, Western blot.

metabolite secretion are more likely players in the very early embryomaternal communication. Recruitment of TNF from the UF could be also taking place, as the 78 kDa isoform that we reported to be upregulated in the endometrium is downregulated in the UF when embryos are present [16]. At the same time, inhibition of TNF endometrial secretion would be consistent with its accumulation into macrophages [45]. As a result of the previous processes, TNF concentration would become diminished in the UF. Provided that the presence of exogenous TNF in culture inhibits early embryo development *in vitro* in the mouse [12] and cattle [46], endometrial responses leading to TNF removal from UF could be intended to prevent deleterious effects on the embryo. The apparent discordance of TNF protein abundance between immunocytochemistry and WB analysis suggests that changes in response to embryos occur in specific endometrial cell structures and may not be detectable in a WB assay in whole tissue blocks.

Embryos in uterus led to reduced numbers of CD45-positive leukocytes in endometrial cell structures. Similarly, Groebner et al. [24] reported fewer CD45-positive

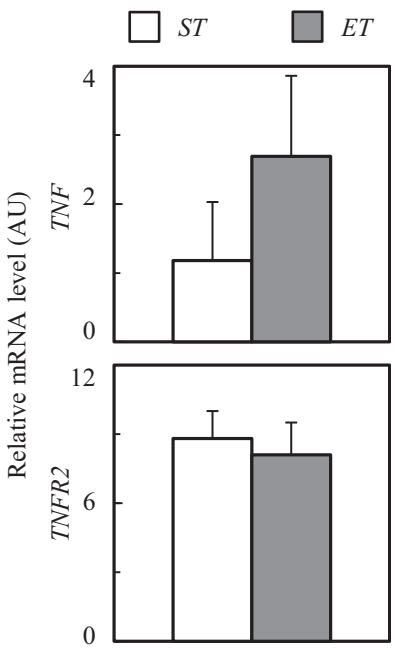

Fig. 4. Transcript levels of *TNF* and *TNFR2* genes in Day 8 uterus that was ET or ST on Day 5, as determined using real-time quantitative polymerase chain reaction. Data expressed are the mean ± standard error of the mean. Data from n = 5 ET and n = 6 ST cows. ET, embryo transferred; ST, sham transferred; TNF, tumor necrosis factor.

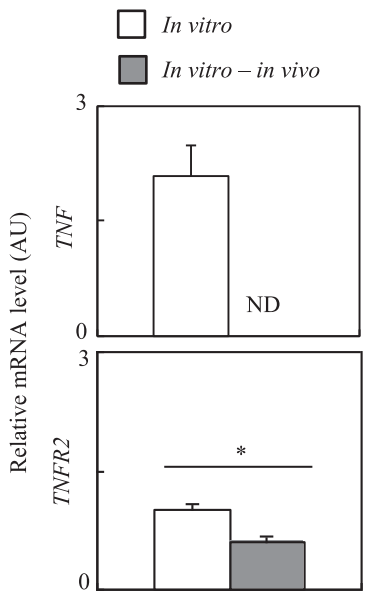

Fig. 5. Transcript levels of *TNF* and *TNFR2* genes in Day 8 bovine blastocyst, entirely *in vitro* produced (*in vitro*) or after uterine passage (*in vitro-in vivo*). Data expressed are the mean ± standard error of the mean. Asterisk represents significant differences (P = 0.006). Data from n = 5 pools *in vitro* and n = 5 pools *in vitro-in vivo* embryos. ND, not detectable; TNF, tumor necrosis factor.

Table 2
Mean (±standard error of the mean) CD45-positive leukocytes in Day 8 uterus that was embryo (+) or sham (−) transferred on Day 5, as determined by immunohistochemistry. The luminal and the glandular epithelia (cells per millimeter) and the stroma (cells per square millimeter) from the subepithelial, functional, and basal zones were differentiated.

Variable	Level	N	R	Luminal epithelium	Glandular epithelium		Stroma		
					Functional	Basal	Subepithelial	Functional	Basal
Embryos	+	3	36	4.0 ± 1.4	2.1 ± 1.2*	5.6 ± 2.1	117 ± 44**	55 ± 21	24 ± 20
	−	3	36	4.6 ± 1.6	4.5 ± 1.2	8.0 ± 2.3	256 ± 48	60 ± 23	46 ± 22
Region	C	6	36	3.7 ± 1.6		3.9 ± 2.4**	157 ± 51	26 ± 25**	15 ± 23*
	IC	6	36	4.9 ± 1.3		9.7 ± 2.0	215 ± 42	90 ± 20	56 ± 19
Third	Cr	6	36	4.0 ± 1.6	2.7 ± 1.4	7.0 ± 2.5	170 ± 50	53 ± 24	20 ± 23***
	M	6	36	4.0 ± 1.4	3.4 ± 1.1	6.7 ± 2.1	203 ± 43	63 ± 21	50 ± 20

Within variables, superscripts express significant differences: *P < 0.05; **P < 0.005; ***P = 0.06.
Abbreviations: C, caruncular; Cr, cranial; IC, intercaruncular; M, middle; N, number of cows; R, number of biological replicates.

leukocytes in the zona basalis of pregnant animals during peri-implantation. In the light of our findings, the embryo might induce downregulation of the maternal immune response as early as during the blastocyst stage, which could help establish the immune privilege for the embryo. Our results support the work of Almiñana et al. [22] in pigs, which showed downregulation of immune-related genes in endometrium in response to blastocysts. In addition, we also reported the regulation of nuclear factor κB system in the bovine UF on Day 8 [16]. Reduction in endometrial CD45 leukocytes observed in the present study was more pronounced in the caruncles. This could have a biological significance, provided that caruncles will support placentation.

In the present work, we have transferred several embryos to the bovine uterus. Previous studies have shown that multiple embryo transfer is consistent with artificial insemination in terms of blood progesterone concentration, quantity of protein recovered from UF, expression level of several abundant proteins in UF (purine nucleoside phosphorylase, heat shock 70kDa protein 5, and albumin) [16,29]. Nevertheless, more studies are needed to confirm the endometrial pattern expression of TNF and TNFR2 in cows carrying one or two embryos.

4.3. Embryo response to the uterus

Embryos that were exposed to the uterine tract express almost undetectable levels of *TNF* and lower levels of *TNFR2* mRNA than those produced entirely *in vitro*. This response has functional relevance, as increased expression of embryonic *TNF* mRNA in cows negatively correlates with pregnancy success [47]. Other studies in bovine and other species have shown that TNF addition to culture medium has a deleterious effect on embryo development *in vitro* [12,20,46,48–50]. Nonetheless, the effect of TNF on embryo viability *in vitro* seems to depend on TNF concentration and embryo developmental stage. Indeed, TNF concentration in the UF is far lower than that used in those *in vitro* studies [49]. Furthermore, TNF has been localized in placental embryonic cells [8,51], and it seems to be associated with trophoblast invasion [4,52]. At the very early stage analyzed in our work, embryos must not attach yet to the endometrium, as ruminant implantation is delayed [36]. Moreover, ruminants have a noninvasive placentation [53]. Therefore, it is conceivable that TNF levels differ between Day 8 and implantation in UF, and therefore, any excess of TNF must be counteracted or removed on Day 8.

4.4. Implications for early embryomaternal communication

This study demonstrates that TNF through its binding to TNFR2 may mediate embryomaternal communication at the time of blastocyst development. We have found that TNF and TNFR2 proteins are highly colocalized, and their expression is reciprocally regulated at this stage in bovine endometrium and embryos. Endometrial protein levels of TNF and TNFR2 were not consistent with their mRNA abundance, suggesting that the bovine blastocyst depends on posttranscriptional responses on the maternal side; so far, the regulation of TNF and TNFR2 is involved.

We have previously shown that TNF protein is downregulated in the bovine UF [16]. In the present study, we showed that *TNFR2* and likely *TNF* mRNA are downregulated in the bovine blastocyst at the same time that TNF and TNFR2 proteins are upregulated in the bovine endometrium. Thus, by regulating TNF secretion in the UF and/or possibly by recruitment of this cytokine from the UF through TNFR2 binding, the endometrium may keep low TNF concentrations available for the blastocyst.

Ultimately, we have counted less endometrial CD45 immune cells in response to embryos, which might indicate a downregulation of the maternal immune response at very early stages. Such a decrease was more prominent in caruncular versus intercaruncular regions and might be mediated by embryos, as significant differences were found between caruncles with embryos and intercaruncles with or without embryos for the basal glandular epithelium and the functional and basal stroma.

Collectively, our results suggest a role for TNF and TNFR2 in the very early embryomaternal communication in cattle.

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References

[1] Ulbrich SE, Wolf E, Bauersachs S. Hosting the preimplantation embryo: potentials and limitations of different approaches for analysing embryo-endometrium interactions in cattle. Reprod Fertil Dev 2012;25:62–70.
[2] Farin PW, Piedrahita JA, Farin CE. Errors in development of fetuses and placentas from in vitro-produced bovine embryos. Theriogenology 2006;65:178–91.
[3] Guzeloglu-Kayisli O, Kayisli UA, Taylor HS. The role of growth factors and cytokines during implantation: endocrine and paracrine interactions. Semin Reprod Med 2009;27:62–79.
[4] Haider S, Knöfler M. Human tumour necrosis factor: physiological and pathological roles in placenta and endometrium. Placenta 2009; 30:111.
[5] Aggarwal BB, Gupta SC, Kim JH. Historical perspectives on tumor necrosis factor and its superfamily: 25 years later, a golden journey. Blood 2012;119:651–65.
[6] Tabibzadeh S, Zupi E, Babaknia A, Liu R, Marconi D, Romanini C. Physiology: site and menstrual cycle-dependent expression of proteins of the tumour necrosis factor (TNF) receptor family, and BCL-2 oncoprotein and phase-specific production of TNF alpha in human endometrium. Hum Reprod 1995;10:277–86.
[7] Okuda K, Sakumoto R, Okamoto N, Acosta TJ, Abe H, Okada H, et al. Cellular localization of genes and proteins for tumor necrosis factor-a (TNF), TNF receptor types I and II in bovine endometrium. Mol Cell Endocrinol 2010;330:41–8.
[8] Payan-Carreira R, Pires MA, Ström Holst B, Rodriguez-Martinez H. Tumour necrosis factor in the canine endometrium: an immunohistochemical study. Reprod Domest Anim 2011;46:410.
[9] Galvão A, Valente L, Skarzynski DJ, Szóstek A, Piotrowska-Tomala K, Rebordão MR, et al. Effect of cytokines and ovarian steroids on equine endometrial function: an in vitro study. Reprod Fertil Dev 2013;25:985–97.
[10] Chen H-L, Yang Y, Hu XL, Yelavarthi KK, Fishback JL, Hunt JS. Tumor necrosis factor alpha mRNA and protein are present in human placental and uterine cells at early and late stages of gestation. Am J Pathol 1991;139:8.
[11] Roby K, Laham N, Kröning H, Terranova P, Hunt J. Expression and localization of messenger RNA for tumor necrosis factor receptor (TNF-R) I and TNF-RII in pregnant mouse uterus and placenta. Endocrine 1995;3:557–62.
[12] Kawamura K, Kawamura N, Kumagai J, Fukuda J, Tanaka T. Tumor necrosis factor regulation of apoptosis in mouse preimplantation embryos and its antagonism by transforming growth factor alpha/phosphatidylionsitol 3-kinase signaling system. Biol Reprod 2007; 76:611–8.
[13] Groebner AE, Schulke K, Unterseer S, Reichenbach HD, Reichenbach M, Büttner M, et al. Enhanced proapoptotic gene expression of XAF1, CASP8 and TNFSF10 in the bovine endometrium during early pregnancy is not correlated with augmented apoptosis. Placenta 2010;31:168–77.
[14] Hunt JS, Chen HL, Miller L. Tumor necrosis factors: pivotal components of pregnancy? Biol Reprod 1996;54:554–62.
[15] Mamo S, Mehta JP, Forde N, McGettigan P, Lonergan P. Conceptus-endometrium crosstalk during maternal recognition of pregnancy in cattle. Biol Reprod 2012;87:1–9.
[16] Muñoz M, Corrales FJ, Caamaño JN, Díez C, Trigal B, Mora MI, et al. Proteome of the early embryo-maternal dialogue in the cattle uterus. J Proteome Res 2012;11:751–66.
[17] von Wolff M, Classen-Linke I, Heid D, Krusche CA, Beier-Hellwig K, Karl C, et al. Tumour necrosis factor-a (TNF-a) in human endometrium and uterine secretion: an evaluation by immunohistochemistry, ELISA and semiquantitative RT-PCR. Mol Hum Reprod 1999;5: 146–52.
[18] Orsi NM, Ekbote UV, Walker JJ, Gopichandran N. Uterine and serum cytokine arrays in the mouse during estrus. Anim Reprod Sci 2007; 100:301–10.
[19] Boomsma CM, Kavelaars A, Eijkemans MJC, Lentjes EG, Fauser BCJM, Heijnen CJ, et al. Endometrial secretion analysis identifies a cytokine profile predictive of pregnancy in IVF. Hum Reprod 2009;24: 1427–35.
[20] Lalitkumar P, Sengupta J, Ghosh D. Effect of tumour necrosis factor-alpha (TNF-alpha) on protein synthesis by mouse preimplantation stage embryos in vitro. Indian J Physiol Pharmacol 2005;49:139–47.
[21] Lachapelle MH, Miron P, Hemmings R, Falcone T, Granger L, Bourque J, et al. Embryonic resistance to tumour necrosis factor-alpha mediated cytotoxicity: novel mechanism underlying maternal immunological tolerance to the fetal allograft. Hum Reprod 1993;8:1032–8.
[22] Almiñana C, Heath PR, Wilkinson S, Sanchez-Osorio J, Cuello C, Parrilla I, et al. Early developing pig embryos mediate their own environment in the maternal tract. PLoS One 2012;7:e33625.
[23] Leung S, Derecka K, Mann G, Flint A, Wathes D. Uterine lymphocyte distribution and interleukin expression during early pregnancy in cows. J Reprod Fertil 2000;119:25–33.
[24] Groebner AE, Schulke K, Schefold JC, Fusch G, Sinowatz F, Reichenbach HD, et al. Immunological mechanisms to establish embryo tolerance in early bovine pregnancy. Reprod Fertil Dev 2011;23:619–32.
[25] Trigal B, Gómez E, Caamaño JN, Muñoz M, Moreno J, Carrocera S, et al. In vitro and in vivo quality of bovine embryos in vitro produced with sex-sorted sperm. Theriogenology 2012;78:1465–75.
[26] Parrish J, Susko-Parrish J, Leibfried-Rutledge M, Critser E, Eyestone W, First N. Bovine in vitro fertilization with frozen-thawed semen. Theriogenology 1986;25:591–600.
[27] Holm P, Booth P, Schmidt M, Greve T, Callesen H. High bovine blastocyst development in a static in vitro production system using SOFaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. Theriogenology 1999;52:683–700.
[28] Lindner GM, Wright Jr RW. Bovine embryo morphology and evaluation. Theriogenology 1983;20:407–16.
[29] Gómez E, Caamaño JN, Corrales FJ, Díez C, Correia-Álvarez E, Martín D, et al. Embryonic sex induces differential expression of proteins in bovine uterine fluid. J Proteome Res 2013;12:1199–210.
[30] Muñoz M, Rodríguez A, De Frutos C, Caamaño JN, Díez C, Facal N, et al. Conventional pluripotency markers are unspecific for bovine embryonic-derived cell-lines. Theriogenology 2008;69:1159–64.
[31] Yang W, Zerbe H, Petzl W, Brunner RM, Günther J, Draing C, et al. Bovine TLR2 and TLR4 properly transduce signals from Staphylococcus aureus and E. coli, but S. aureus fails to both activate NF-κB in mammary epithelial cells and to quickly induce TNFα and interleukin-8 (CXCL8) expression in the udder. Mol Immunol 2008;45:1385–97.
[32] Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002;3:1–12. Research0034.
[33] Goossens K, Van Poucke M, Van Soom A, Vandesompele J, Van Zeveren A, Peelman L. Selection of reference genes for quantitative real-time PCR in bovine preimplantation embryos. BMC Dev Biol 2005;5:27.
[34] Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol 2007;8:R19.
[35] Woodward EM, Christoffersen M, Campos J, Betancourt A, Horohov D, Scoggin KE, et al. Endometrial inflammatory markers of the early immune response in mares susceptible or resistant to persistent breeding-induced endometritis. Reproduction 2013;145:289–96.
[36] Bazer FW, Spencer TE, Johnson GA, Burghardt RC, Wu G. Comparative aspects of implantation. Reproduction 2009;138:195–209.
[37] Miyamoto Y, Skarzynski DJ, Okuda K. Is tumor necrosis factor alpha trigger for the initiation of endometrial prostaglandin F(2alpha) release at luteolysis in cattle? Biol Reprod 2000;62:1109–15.
[38] Aggarwal BB. Signalling pathways of the TNF superfamily: a double-edged sword. Nat Rev Immunol 2003;3:745–56.
[39] Fischer R, Maier O, Naumer M, Krippner-Heidenreich A, Scheurich P, Pfizenmaier K. Ligand-induced internalization of TNF receptor 2 mediated by a di-leucin motif is dispensable for activation of the NFκB pathway. Cell Signal 2011;23:161.
[40] Forde N, Carter F, Spencer TE, Bazer FW, Sandra O, Mansouri-Attia N, et al. Conceptus-induced changes in the endometrial transcriptome: how soon does the cow know she is pregnant? Biol Reprod 2011; 85:144–56.
[41] Forde N, Duffy GB, McGettigan PA, Browne JA, Mehta JP, Kelly AK, et al. Evidence for an early endometrial response to pregnancy in cattle: both dependent upon and independent of interferon tau. Physiol Genomics 2012;44:799–810.

[42] Spencer TE, Forde N, Dorniak P, Hansen TR, Romero JJ, Lonergan P. Conceptus-derived prostaglandins regulate gene expression in the endometrium prior to pregnancy recognition in ruminants. Reproduction 2013;146:377–87.
[43] Forde N, McGettigan PA, Mehta JP, O'Hara L, Mamo S, Bazer FW, et al. Proteomic analysis of uterine fluid during the pre-implantation period of pregnancy in cattle. Reproduction 2014;147:575–87.
[44] Chen Q, Zhang Y, Elad D, Jaffa AJ, Cao Y, Ye X, et al. Navigating the site for embryo implantation: biomechanical and molecular regulation of intrauterine embryo distribution. Mol Aspects Med 2013;34:1024–42.
[45] Olszewski MB, Groot AJ, Dastych J, Knol EF. TNF trafficking to human mast cell granules: mature chain-dependent endocytosis. J Immunol 2007;178:5701–9.
[46] Jackson L, Farin C, Whisnant S. Tumor necrosis factor alpha inhibits in vitro bovine embryo development through a prostaglandin mediated mechanism. J Anim Sci Biotechnol 2012;3:7.
[47] El-Sayed A, Hoelker M, Rings F, Salilew D, Jennen D, Tholen E, et al. Large-scale transcriptional analysis of bovine embryo biopsies in relation to pregnancy success after transfer to recipients. Physiol Genomics 2007;28:84–96.
[48] Pampfer S, Moulaert B, Vanderheyden I, Wuu YD, De Hertogh R. Effect of tumour necrosis factor a on rat blastocyst growth and glucose metabolism. J Reprod Fertil 1994;101:199–206.
[49] Inagaki N, Stern C, McBain J, Lopata A, Kornman L, Wilkinson D. Analysis of intra-uterine cytokine concentration and matrix-metalloproteinase activity in women with recurrent failed embryo transfer. Hum Reprod 2003;18:608–15.
[50] Soto P, Natzke RP, Hansen PJ. Actions of tumor necrosis factor-α on oocyte maturation and embryonic development in cattle. Am J Reprod Immunol 2003;50:380–8.
[51] Ben-Yair E, Less A, Lev S, Ben-Yehoshua L, Tartakovsky B. Tumour necrosis factor alpha binding to human and mouse trophoblast. Cytokine 1997;9:830–6.
[52] Jerzak MG, Bischof P. Apoptosis in the first trimester human placenta: the role in maintaining immune privilege at the maternal-foetal interface and in the trophoblast remodelling. Eur J Obstet Gynecol Reprod Biol 2002;100:138–42.
[53] Spencer TE, Sandra O, Wolf E. Genes involved in conceptus-endometrial interactions in ruminants: insights from reductionism and thoughts on holistic approaches. Reproduction 2008;135:165–79.

CAPÍTULO 3 EXPRESSION AND LOCALIZATION OF INTERLEUKIN 1 AND INTERLEUKIN 1 RECEPTOR (TYPE I) IN THE BOVINE ENDOMETRIUM AND EMBRYO

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RESUMEN EN CASTELLANO

El sitema interleuquina-1 (IL1) podría participar en la comunicación materno-embrionaria en mamíferos. En bovino, hemos demostrado recientemente que el fluido uterino de novillas portadoras de embriones tempranos presenta concentraciones de IL1 beta (IL1B) más bajas, lo cual podría conducir a una disminución en la expresión de NFKB y a una represión de la respuesta inmune innata materna. En este trabajo, hemos evaluado la expresión de IL1B y su receptor, el receptor de IL1 tipo I (IL1R1) en el endometrio y embrión bovinos mediante RT-PCR, inmunohistoquímica y western blot durante el desarrollo del blastocisto. A partir de animales sujetos a transferencia embrionaria en Día 5, se recogieron muestras de endometrio y blastocistos en Día 8. Tanto el endometrio como los blastocistos mostraron trancripción de *IL1B* e *IL1R1* y colocalización de las proteínas correspondientes. El epitelio glandular, el epitelio luminal y el estroma mostraron tinción positiva a IL1B e IL1R1. En algunas regiones del endometrio, la expresión de IL1B se vio incrementada debido a la presencia de embriones, mientras que la expresión proteíca de IL1R1 o la expresión génica de *IL1B* e *IL1R1* no se vieron afectadas por la presencia de embriones. Estos resultados sugieren que, durante la preimplantación, la presencia de embriones tempranos en el tracto reproductivo regula el sistema inmune materno mediante la regulación del sistema IL1 a nivel local. A través de este mecanismo, el embrión podría actuar durante los estadios tempranos de la preñez para desencadenar respuestas locales necesarias para el desarrollo de un endometrio receptivo.

ABSTRACT

The interleukin-1 (IL1) system likely mediates embryo-maternal communication in mammals. In bovine, we have recently reported that the uterine fluid of heifers that carried early embryos shows down regulation of IL1 beta (IL1B) which potentially could lead to a reduction in NFKB expression and dampening of maternal innate immune responses. In this work, we assessed the expression of IL1B and its receptor, interleukin 1 receptor type I (IL1R1) in the bovine endometrium and embryos by RT-PCR, immunohistochemistry and western blotting at the time of blastocyst development. Day 8 endometrium and blastocyst recovered from animals after transfer of Day 5 embryos showed *IL1B* and *IL1R1* mRNA transcription and protein co localization. IL1B and IL1R1 immunostaining was observed in epithelial cells of the

surface and the glandular epithelium and in stromal cells. The presence of embryos increased endometrial IL1B protein locally, while no differences for IL1R1 protein and *IL1B* and *IL1R1* mRNA were detected. These results suggest that the presence of the early preimplantation bovine embryo in the maternal tract regulates the maternal immune system through local modulation of the IL1 system. Through such a mechanism, the embryo could act during early stages of pregnancy to elicit local responses which are required for the development of a receptive endometrium.

INTRODUCTION

Cytokines, initially identified as peptides and protein secretory products of immune cells, play important roles in endometrial physiology and maternal regulation of embryonic development (Robertson et al. 1992, Singh et al. 2011). In mammals, deregulated expression of cytokines can lead to total or partial failure of implantation and abnormal placental formation (Guzeloglu-Kayisli et al. 2009).

Interleukin-1 (IL1), a major pro-inflammatory cytokine, locally regulates many endometrial functions at the materno-fetal interface in mammalian and non-mammalian vertebrates (Paulesu et al. 2005). The IL1 system is composed of two agonists (IL1alpha, IL1beta), an antagonist (IL1 receptor antagonist, IL1RN), and a receptor family. The IL1 receptor family consists of the type I (IL1R1) and type II (IL1R2)IL1 receptors, and the IL1R accessory protein (IL1RAP) (Dinarello 1994). Both IL1alpha (IL1A) and IL1beta (IL1B) bind to IL1R1 and to IL1R2 (Dower et al. 1985), whereas IL1RAP does not recognize the ligand but increases the receptor affinity for interleukins (Colotta et al. 1993, Wesche H 1997). Only the IL1R1 transduces a signal in response to IL1, while the IL1R2 receptor suppresses IL1 activity by competing for IL1 binding (Sims et al. 1994).

The IL1 system has been identified in mammalian embryos and in the reproductive tract. Thus, IL1B has been detected in human, mouse and bovine endometrium (McMaster et al. 1992, Paula-Lopes et al. 1999, Simón et al. 1993b, Tabibzadeh and Babaknia 1995); in human, mouse, porcine and bovine embryos (De los Santos et al. 1996, Muñoz et al. 2012, Tuo et al. 1996); and in human, porcine and bovine uterine fluid (Davidson et al. 1995, Muñoz et al. 2012, Paula-Lopes et al. 1999, Ross et al. 2003, Simón et al. 1996). Similarly, the endometrium of humans, mice, pigs, and rabbits contains IL1A (McMaster et al. 1992, Tabibzadeh and Babaknia 1995, Tuo et al. 1996, Yang et al. 1995). Among the receptors, IL1R1 has been detected in human, murine, porcine and bovine endometrium (Ross et al. 2003, Simón et al. 1994, Simón et al. 1993a, Simón et al. 1993b, Tanikawa et al. 2005) and in preimplantation human and mouse embryos (De los Santos et al. 1996, Kruessel et al. 1997). In addition IL1RAP has been found in human and porcine endometrium (Ross et al. 2003, Simón et al. 1996, Tabibzadeh and Babaknia 1995), and in preimplantation human and mouse embryos (De los Santos et al. 1996, Kruessel et al. 1997).

Preimplantation embryos from various species produce and respond to IL1 (Austgulen et al. 1995, Pampfer et al. 1991, Sheth et al. 1991, Simón et al. 1994, Stewart and Cullinan 1997, Tuo et al. 1996, Zolti et al. 1991). Moreover, in humans, preimplantation embryos release IL1B into culture medium in an amount proportional to blastomere number (Barañao et al. 1997, Taşkın et al. 2012), suggesting that IL1B secretion might predict embryo viability to establish pregnancy. In cattle, addition of IL1B at 8-10 h after insemination increases the proportion of oocytes that develop to the blastocyst stage (Paula-Lopes et al. 1998). Furthermore, in endometrial cell cultures, IL1B inhibits growth of stromal cells but not epithelial cells (Davidson et al. 1995) whereas IL1B increases secretion of prostaglandins by epithelial and stromal endometrial cells (Betts and Hansen 1992, Davidson et al. 1995).

We have recently reported a decrease in IL1B in bovine uterine fluid during early pregnancy which potentially could lead to a reduction in NFkB expression and dampening of maternal innate immune responses (Muñoz et al. 2012). Such an observation, coupled with evidence of IL1B participating in early embryonic development and endometrial function in the cow (Betts and Hansen 1992, Davidson et al. 1995, Paula-Lopes et al. 1998), suggests that the IL1 system may play an important role during early embryonic development in cattle.

The main hypothesis of this study is that the presence of the early preimplantation bovine embryo in the maternal tract regulates the maternal immune system through local modulation of the IL1 system. Through such a mechanism, the embryo could act during early stages of pregnancy to elicit local responses which are required for the development of a receptive endometrium. To test that hypothesis, changes in gene and protein expression of IL1B and IL1R1 during transient in uterus development of embryos produced *in vitro* were evaluated in the bovine endometrium and embryos during the blastocyst stage. Caruncular and intercaruncular endometrial and embryonic expression of IL1B and IL1R1was examined by RT-qPCR, immunohistochemistry and western blotting at the time of blastocyst development.

MATERIALS AND METHODS

All experimental procedures involving animals were performed according to the European Community Directive 2010/63/EU (Spanish Real Decreto 53/2013), and were sanctioned by the Animal Research Ethics Committee of SERIDA.

All reagents were purchased from Sigma (Madrid, Spain) unless otherwise stated.

Animals and embryo transfer

Procedures involving animal feeding and management, estrus synchronization, embryo transfer (ET) and recovery, and progesterone (P4) blood sampling and analysis have been described elsewhere (Gómez et al. 2008, Hidalgo et al. 2004, Muñoz et al. 2012).

Embryos were produced *in vitro* (IVP) using oocytes collected from slaughterhouse ovaries as previously reported (Gómez et al. 2008). *In vitro* fertilization (IVF: Day 0) was performed with frozen/thawed sperm by using a swim-up procedure. The resultant zygotes were either cultured until Day 8 post-IVF or were cultured until Day 5 and then transferred to the cranial third of the uterine horn ipsilateral to the formerly detected preovulatory follicle at Day 5 after estrus. A total of 50 morulae were transferred into each female. Sham transfers (ST) were also performed by infusing an equivalent volume of the medium used to transfer embryos (Instruments de Médecine Vétérinaire, Humeco, Huesca, Spain; embryo holding medium). Age-matched heifers and primiparous cows (n = 3 heifers and 2 cows for ET; 3 heifers and 3 cows for ST) were sacrificed in a nearby slaughterhouse (Matadero dePravia, Asturias) on Day 8 after estrus.

Collection of endometrial tissues

Within 1 h after slaughter, uteri were trimmed free of surrounding tissue, and embryos were recovered from the uterus by flushing the ipsilateral uterine horn with 45 mL of Dulbecco's phosphate buffered saline (D-PBS) (Gibco-Life technologies, Alcobendas, Spain) + 1 mg/mL polyvinylpyrrolidone (PVP), as described by Gómez et al. (2013). The uterus was flushed five times. The presence of embryos was verified by careful examination of uterine flushings under a stereomicroscope. Subsequently, only reproductive tracts containing embryos at the expected stage of development were processed (n=5 cows and n=6 heifers). Endometrial samples were collected from caruncular and intercaruncular regions from the ipsilateral uterine horn.

Caruncular regions were first carefully cut out; then the intercaruncular endometrium was sampled (Mansouri-Attia et al. 2009). Tissue samples were immediately collected and stored. Samples for real-time quantitative PCR (RT-qPCR) were maintained in RNAlater (Ambion, Huntingdon, Cambridgeshire, UK) for 24 h at 4 ºC and subsequently at -80 ºC. Samples for western blotting (WB) were immediately transferred into Eppendorf Protein LoBind tubes (Eppendorf, Hamburg, Germany), frozen in liquid nitrogen and stored at -145 °C until processing. Samples for immunohistochemistry (IHC) were fixed with 4 % (w/v) paraformaldehyde in phosphate-buffered saline (PBS, 8.1 mM sodium phosphate dibasic, 137.9 mM sodium chloride, 2.07 mM potassium chloride, 1.5 mM potassium phosphate monobasic; pH 7.4) for 24 h at 4 ºC and subsequently dehydrated and embedded in paraffin (Muñoz et al. 2005).

Embryo processing

Flushed embryos were washed 3 times in PBS+ 1 mg/mL PVP (PBS-PVP) and grouped according to their development stage (degenerated, morula, blastocyst, expanded blastocyst and hatched blastocyst). Only expanded blastocysts were further processed for RT-qPCR and IHC.

Flushed embryos and Day 8 IVP blastocysts for IHC were fixed for 20 min at room temperature in 4% (w/v) paraformaldehyde in PBS. Fixed embryos were stored in PBS at 4°C until use. Flushed embryos and Day 8 IVP blastocysts for RT-qPCR were snap-frozen in Eppendorf DNA LoBind tubes (Eppendorf, Hamburg, Germany) and stored at -145 °C until use.

Quantitative real-time PCR

RNA Extraction and Reverse Transcription

Total RNA was isolated from frozen tissue using TRIzol Reagent (Invitrogen, Cergy-Pontoise, France) for endometrial samples or Picopure RNA isolation Kit (Arcturus Ltd. United Kingdom) for embryos. Endometrial RNA samples were purified on Qiagen columns following the manufacturer's protocol (RNeasy Mini kit; Qiagen, Courtaboeuf, France). All RNA samples were subjected to a DNaseI treatment (Roche Diagnostics, Meylan, France for endometrium and Qiagen for embryos).

Total purified RNA (1 µg for endometrial samples or 10 equivalent embryos) was reverse transcribed using Superscript III enzyme (Invitrogen, Cergy-Pontoise, France) and random primer hexamers (Roche, Meylan, France). The reverse transcrition conditions for endometrium were as follows: 65 ºC for 10 min, 42 ºC for 50 min, and 70 ºC for 15 min. Conditions for embryos wereas follows: 25 ºC for 5 min, 50 ºC for 60 min, and 70 ºC for 15 min.

Quantitative real-time PCR

Primer sets (Table 3.1) were designed using published bovine sequences and Primer Express software (v3.0, Applied Biosystems, USA). The RT-qPCR was performed using StepOnePlus Real Time PCR System and Master Mix SYBR Green (Applied Biosystems, Cergy-Pontoise, France). Assays were performed in duplicate for endometrium and triplicate for embryos. The reaction mixture for amplification in a final reaction volume of 25 µl contained 5 µl cDNA diluted 1:200 in a final reaction volume of 15 µl (endometrium) or 10 µl cDNA containing 0.2 equivalent embryos. Non-template controls were run for each gene. The PCR program involved two initial stages at 50 ºC for 2 s and 95 ºC for 10 min, followed by 45 (endometrium) or 40 (embryos) cycles at 95 ºC for 15 s and a final annealing and extension step at 60 ºC for 1 min.

To confirm product specificity, melting-curve analyses were performed immediately after amplification following denaturation at 95 ºC for 15 s, annealing at 60 ºC for 20 s and 1 cycle with 0.8 ºC (endometrium) / 1 ºC (embryos) increment. Primer annealing temperature was 60 ºC. The standard curve was produced using cDNA retrotranscribed from a RNA pool of all endometrial samples and serially diluted. The amplification efficiency and correlation coefficients were higher than 80 % and 0.98, respectively. Amplified endometrial PCR fragments were verified by DNA sequencing (Beckman Coulter Genomics, Takeley, UK) and alignment to known sequences using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For embryonic samples, fragment size was confirmed in a 1% agarose gel stained with Ethidium Bromide.

Data analysis of qPCR

The reference genes for endometrium were *SLC30A6*, *C20RF29*, *RPL19* using the geNorm applet as detailed by Vandesompele et al. (2002). Reference genes for embryos

were *GAPDH*, *SDHA* and *YWHAZ*, based on results of Goossens et al. (2005). All Ct values were transformed into normalized relative quantities using qBase plus software (Biogazelle, Zwijnaarde, Belgium) (Hellemans et al. 2007).

Western blotting

The protein concentration of endometrial samples was determined using Bradford's assay (Bio-Rad, Hercules, CA). Endometrial samples consisting of 5 µg total protein, were boiled in sodium dodecyl sulfate (SDS) sample buffer for 5 min and electrophoresed on 10% (w/v) SDS polyacrylamide gels for 120 min at 80 V. Separated proteins were then electrically (0.150 A, 120 min) transferred to a PVDF membrane. Non-specific binding was blocked with 5% (w/v) fat-free milk in PBS-0.05% (v/v) Tween-20 (PBS-T) for 60 min and incubated with primary antibodies in PBS-T + 5% (w/v) fat-free milk overnight at room temperature. Antisera specific to IL1B(Abbiotec, San Diego, USA; 250716) and IL1R1 (Abcam, Cambridge, UK; Ab106278) were used at 1:750 and 1:1500 dilution respectively. After washing, the membrane was incubated with the required secondary antibody (goat anti-rabbit/*horseradish peroxidase* (*HRP*) conjugate; Amersham, Piscataway, NJ, USA) at 1:2000 dilution, and detected with the Pierce ECL or ECL plus detection kits (Thermo Scientific, Waltham, MA). Protein concentration was quantified by scanning densitometry using the software Confocal Uniovi Image-J (University of Oviedo, Spain; http://www.sct.uniovi.es/index.php?option=content&task=view&id=224) in 3 replicates. Differences in mean protein abundance between samples from ET and ST animals were analyzed and expressed as fold change [least squares means (LSM ± SEM)]. Equal loading of total proteins was assessed by Coomassie staining of SDS-PAGE gels.

Statistical analysis

Data were analyzed by analysis of variance using the Proc GLM module of the SAS/STAT software (Version 9.2; SAS Institute, Cary, USA). The models for endometrial samples included the following fixed effects: uterine region, presence of embryos, animal (where appropriate), and replicate (technical and biological). Models for embryos included replicate and origin (IVP or in utero). Least squares means (LSMs) and their errors (±SEM) were estimated for each level of fixed effects with a significant F-value. The Ryan-Einot-Gabriel-Welsch was used to compare raw means.

Single immunofluorescence analysis

Embryos

Protein localization was investigated using whole-mount immunofluorescence methods for both IL1B (Abbiotec, San Diego, USA; 250716) and IL1R1 (Abcam, Cambridge, UK; Ab106278) in blastocysts recovered by uterine flushings and also in blastocysts entirely produced *in vitro*.

Samples were washed in PBS-PVP and permeabilized in 0.1% (v/v) Triton X-100 in PBS-PVP. Blocking was performed by 30 min incubation with Image-iT FX Signal Enhancer (Molecular Probes-Life technologies, Alcobendas, Spain) and in PBS + 5% (v/v) normal goat serum (NGS). Primary antibodies to IL1Band IL1R1 were diluted 1:100 in PBS+ 10% (v/v) NGS and incubated overnight at 4 ºC. After a wash, primary antibodies were detected with Alexa 488 goat anti-rabbit IgG (Invitrogen-;A11034) diluted 1:600 in PBS + 5 % (v/v) NGS for 2h. Nuclei were stained with Hoescht 33342 (2.3 mg/mL) for 15 min. Subsequently, samples were examined using a confocal microscope (Leica Ultra-Espectral Confocal Microscope TCS-SP2-AOBS). Negative controls using only secondary antibody were performed in parallel.

Endometrial samples

Paraffin-embedded samples were sectioned at 4 µm and mounted on glass slides. After paraffin removal and rehydration, endometrial sections were submitted to antigen retrieval with sodium citrate buffer (10 mM sodium citrate, 0.05% (v/v) Tween 20, pH 6.0) for 20 min at 95 °C. Subsequently, non-specific binding was blocked with Image-iT FX Signal Enhancer (Invitrogen- Life technologies, Alcobendas, Spain) and PBS + 5% (v/v) NGS. Rabbit anti-IL1B or anti-IL1R1were diluted 1:300 and 1:200, respectively, in 10% (v/v) NGS in PBS and incubated overnight at 4 °C. After washes in PBS, sections were incubated with Alexa 488 goat anti-rabbit IgG (Invitrogen- Life technologies, Alcobendas, Spain; A11034) diluted 1:600 in PBS + 10 % (v/v) NGS for 2 h. Following further washes in PBS, sections were incubated in Hoescht 33342 reagent (2.3 mg/mL) for 15 min. Sections were washed, mounted using Vectashield Mounting medium (Vector Labs, Burlingame,USA), and examined using a confocal microscope (Leica Ultra-Espectral Confocal Microscope TCS-SP2-AOBS).Negative controls using only secondary antibody were performed in parallel.

Two-colour immunofluorescence

A summary of procedures are presented in Table 3.2; all detections were performed sequentially. After blocking, samples were washed with phosphate-buffered saline three times, 10 min each, between incubations.

Confocal microscopy

Samples were examined using a confocal microscope (Leica Ultra-Espectral Confocal Microscope TCS-SP2-AOBS). Non-specific signals during image acquisition were subtracted from other images. Similarly, settings were adjusted so that no part of the image was saturated, in order to maximize detection of differences in labelling intensity. For each confocal microscopy session, settings were maintained for all samples.

Serial z-axis optical 1 µm sections were acquired to accurately characterize the pattern of expression of IL1B and IL1RI.

RESULTS

Expression of mRNA and protein for IL1B and IL1R1 in bovine endometrium

The relative abundance of *IL1B* and *IL1R1* mRNA was examined in endometrial caruncular and intercaruncular regions of ET and ST females by RT-qPCR. Both transcripts were present in all samples. Expression of neither *IL1B* nor *IL1R1* mRNAs were affected by pregnancy status or region analyzed (i.e. caruncle *versus*. intercaruncle) (Figures 3.1A and 3.1B respectively; $p > 0.05$). However, *IL1B* mRNA tended to be more abundant in caruncular than in intercaruncular areas ($p < 0.06$).

Western blot analysis of IL1B revealed an immunoreactive 35 kDa band whose apparent molecular weight corresponded to the pro-form of IL1B and a 17 kDa band with the molecular weight predicted for the mature form of IL1B. Densitometric analysis of IL1B showed significant ($p < 0.04$) increase in the mature form of IL1B in ET caruncles as compared to caruncles from sham-treated animals. However no differences for the mature form of IL1B were found in the intercaruncular samples from ET and ST animals (Figure 3.2A). In contrast, IL1 pro-form expression was not affected by the presence of embryos in the uterus in any of the regions analyzed (Figure 3.2B; $p > 0.05$).

Immunoblot analysis for IL1R1 revealed an immunoreactive 65 kDa band consistent with the reported form of IL1R1. There was no effect of ET on IL1R1 in caruncular or intercaruncular regions ($p > 0.05$). In ST animals, abundance of IL1R1 was higher in the endometrial caruncular than intercaruncular regions (Figure 3.3; $p < 0.04$). This effect of region was not apparent for ET recipients.

Distribution of immunoreactive IL1B and IL1R1 in bovine endometrium

Immunoreactive IL1B was detected in the luminal epithelium of both caruncular and intercaruncular tissues and in the glandular epithelium of intercaruncular tissues, in all groups. The most intense staining for IL1B was localized in the apical sites of luminal and glandular epithelial cells (Figures 3.4 A-E wide arrows) suggesting that IL1B is secreted by these cells. There was also additional diffuse staining throughout the stroma (Figures 3.4 A-E). IL1R1 showed a similar pattern of localization to IL1B in both caruncular and intercaruncular tissues (Figures 3.4 F-J). However, unlike IL1B, IL1R1

expression was also observed in the walls of blood vessels (Figure 3.4G, thin arrow) and in the myometrium (Figure 3.4H, arrowhead).

Two-colour immunofluorescence was performed to assess IL1B and IL1R1 co-localization. Combined images revealed that the majority of cells that co-expressed IL1B and IL1R1 were localized in the luminal and glandular epithelia (Figures 3.4 K-O, wide arrows). There were no differences in staining pattern for IL1B or IL1R1 between intercaruncular and caruncular regions or due to the presence of embryos. The control samples in which the primary antibodies had been omitted did not show positive signals (Supplementary Figures 3.1 A-C).

Expression of IL1Band IL1R1 mRNA in day 8 bovine blastocysts by RT-qPCR

IL1R1 mRNA was detected in all samples analysed. Expression pattern did not differ between embryos developed in the uterus for three Days and those that developed *in vitro* until Day 8 (Figure 3.5, $p<0.6$). *IL1B* transcript was also detected in 5 out of 6 samples and 3 out of 6 samples of IVP and uterus-exposed embryos, respectively. However, reliable quantification could not be performed due to the very low level of mRNA expression in some samples.

Distribution of immunoreactive IL1Band IL1R1 in bovine blastocysts

Immunofluorescence analysis was used to establish IL1B and IL1R1 localization in the blastocyst. When primary antibody was omitted, control embryos did not exhibit any detectable signal (Supplementary Figures 3.1 D-E). There was expression of IL1B and IL1R1 for both embryos exposed to the uterine environment (Figure 3.6A) and *in vitro* cultured until Day 8 (Figure 3.6B). Evaluation of serial optical sections by laser confocal microscopy revealed that immunoreactive IL1B and IL1R1 were mainly distributed over the trophectoderm (Figures3.6A-B) and were predominantly localized in the cytoplasm (Figure 3.6 inserts). There was no difference in signal intensity between blastocysts exposed to the uterus exposed and those that were *in vitro* cultured until Day 8.

DISCUSSION

In previous work we found decreased levels of the pro-inflammatory cytokines IL1B and TNF alpha (TNF) in uterine fluid during early pregnancy (Muñoz et al. 2012). Such findings prompted us to investigate if the reported change in IL1B could be the result of local regulation of the IL1 system by the presence of the embryo. If so, the IL1 system could be involved in the formation of a receptive phenotype in the maternal tract regulated by developing embryos. The improvement of the embryotrophic properties in the uterine environment in response to the presence of embryos has been described not only in the cow (Muñoz et al. 2012), but also in pigs (Almiñana et al. 2012).

Our main findings in the present work are the following: (1) IL1R1 is expressed in the bovine caruncular and intercaruncular endometrium at the mRNA and protein level; (2) expression is not affected by the presence of developing embryos; (3) the transient development of embryos in the uterus for 3 Days up-regulates endometrial IL1B protein without affecting *IL1B* mRNA and (4) IL1B and IL1R1 are detectable at protein and mRNA levels in embryos produced *in vitro* and in embryos exposed to the uterine environment.

The IL1 system during early preimplantation development in the bovine endometrium

The results of the present study provide first evidence of local expression of IL1R1 protein and mRNA in the bovine endometrium during early preimplantation development. The most intense labelling for IL1R1 was localized to the apical portion of luminal and glandular epithelial cells. The reported pattern of expression is in agreement with previous descriptions for IL1R1 protein in human endometrium throughout the menstrual cycle (Simón et al. 1993b). Similar to our results, *IL1R1* transcription has also been detected in porcine uterine luminal and glandular epithelium during the estrous cycle and early pregnancy (Ross et al. 2003, Seo et al. 2012) and in bovine intercaruncular endometrium during the estrous cycle (Tanikawa et al. 2005).

The presence of early bovine embryos in the uterus did not alter *IL1R1* mRNA or protein expression. In mice, *IL1R1* mRNA and protein levels significantly increase during the short window of uterine receptivity and embryo implantation (Days 1 and 2 of gestation as well as Day 4) (Bourdiec et al. 2014). The discordant results reported between murine and bovine species for IL1R1 expression during early pregnancy might

be due to differences in implantation. Thus, bovine embryos show delayed superficial implantation, while murine and human blastocysts invade the endometrium layer after hatching (Bazer et al. 2011, Bazer et al. 2012, Bazer et al. 2009, Roberts et al. 2008).

Our study also showed that IL1B protein is strongly expressed in the luminal and glandular epithelium and lightly in stromal cells in both pregnant and cyclic animals. In agreement, Paula-Lopes and co-workers (1999) identified immunoreactive IL1B in the luminal and glandular epithelium and stroma of the endometrium during the estrous cycle.

The presence of growth factor receptors on cell surfaces is compelling evidence that growth factor exposure regulates cellular function in the endometrium. The co-expression of IL1B and its functional receptor at mRNA and protein level in the luminal and glandular epithelium suggests that IL1B may act in a paracrine/autocrine manner in the bovine endometrium. In porcine, IL1B secreted by the conceptus has been suggested to exert both autocrine and paracrine effects (Geisert et al. 2012).

Recent studies in pigs show that the presence of early embryos (Day 6 blastocysts) in the uterus alters the expression of genes that promote a maternal receptive environment that favours conceptus growth and development (Almiñana et al. 2012). In our work, the presence of developing embryos did not affect *IL1B* and *IL1R1* mRNA expression in the endometrium; however the presence of embryos led to increased levels of IL1B protein within endometrial caruncles, structures in charge of hosting embryo implantation (Bazer et al. 2009).

The confined expression of members of the IL1 system in certain endometrial regions might be part of a complex mechanism controlling IL1 activity. Complex mechanisms operating locally that target cytokines have been described in cultures of both endometrial epithelial cell lines and primary human endometrial epithelial cells. Such mechanisms seem to tightly regulate IL1 biological effects to ensure normal reproductive functions (Bellehumeur et al. 2009). The binding of IL1B by the IL1R1 in the luminal epithelium may help minimize the pro-inflammatory responses that this cytokine triggers in the endometrium during early development.

Our results concerning site-specific IL1B expression are also consistent with recent proteomic analyses of caruncular and intercaruncular endometrium in sheep (Al-Gubory et al. 2014, Wang et al. 2013). The protein pattern profiles in pregnant ewes at

preattachment (Day 12), implantation (Day 16) and early postimplantation (Day 20) markedly differed between caruncle and intercaruncle (Al-Gubory et al. 2014). In addition, in cattle, distinct patterns of gene expression between caruncular and intercaruncular regions at Day 17 and 20 of pregnancy have been previously reported (Mansouri-Attia et al. 2009, Walker et al. 2010). These differences are likely to underlie important functional differences between the referred endometrial regions.

Similarly to IL1B, we have recently reported that endometrial protein expression of TNF is higher in the caruncular than in the intercaruncular region (Correia-Álvarez et al. 2015). This work shows that the passage of embryos through the uterus increases accumulation of endometrial TNF without affecting *TNF* mRNA. As the mammalian embryo moves in loops through the uterine horn (Chen et al. 2013), it is unlikely that it will be in a transcription induction site at the time a response is provided. Thus, it seems that very early embryo-maternal communication does not rely on gene regulated mediated responses but on faster posttranscriptional responses. Extensive posttranslational modifications and thus not correlated with data from gene array, exist in human endometrial tissue (Stephens et al. 2010). These data and our own studies suggest that posttranslational modifications might be a key factor in the endometrial physiology and embryo-maternal signalling.

IL1 system during early embryo-maternal communication

Growth and development of the preimplantation embryo takes place in a cytokine and growth factor rich fluid secreted by the conceptus, epithelial cells lining the oviduct and the uterus, endometrial glands and by immune-resident cells (Sargent et al. 1998, Sjöblom et al. 1999). Some of these molecules are directly involved in embryonic development, whereas others are endocrine and immune signals needed for the establishment of pregnancy (Bazer et al. 2010, Geisert and Yelich 1996, Spencer and Bazer 2004).

To the best of our knowledge, our report is first demonstrating the presence of *IL1R1* mRNA and protein and the co-localization of IL1B and its cognate receptor IL1R1 in both IVP embryos and embryos exposed to the uterus. These results suggest that bovine blastocysts may secrete and respond to IL1, one of the earliest signals released by embryonic cells (Bourdiec et al. 2012, Geisert and Yelich 1996, Krüssel et al. 2003, Paulesu et al. 2008, Simón et al. 1994, Spencer and Bazer 2004). In humans and pigs,

IL1B is produced by preimplantation blastocysts (Sheth et al. 1991) and IL1 concentration in embryo culture-conditioned media has been positively correlated with successful implantation after *in vitro* fertilization and embryo transfer (Barañao et al. 1997, Sheth et al. 1991). In addition, mRNA and protein of IL1R1 has been detected in preimplantation embryos in several species (De los Santos et al. 1998, Huang et al. 1997, Kruessel et al. 1997, Krüssel et al. 1998, Ross et al. 2003, Takacs and Kauma 1996).

Conclusions

The multiple aspects and mechanisms that have evolved for the control of the IL1 system highlight the potency of IL1 and the need of appropriate management of inflammation during early pregnancy. Cytokine production in cells is regulated by cell signalling and includes mRNA and protein synthesis. In addition, conventional and novel secretory pathways have recently been implicated in dictating the release and activity of cytokines (Stow and Murray 2013). Thus, a full understanding of the physiology of the IL1 system during very early pregnancy requires an interdisciplinary approach combining genomic, transcriptomic and proteomic profiling.

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Table 3.1 Primers used for the qRT-PCR on bovine endometrium (*IL1B*[a], *IL1R1*[a], *SLC30A6, C20RF29,* and *RPL19*) and embryo (*IL1B*[b], *IL1R1*[b], *GAPD, SDHA, and YWHAZ*). Direction of all sequences is 5´ to 3´.

Name	Accesion number	Primer sequence (concentration, µM)	Amplicon size (bp)
IL1B[a]	NM_174093.1	F: CTACGAATCTCCGACCAC (0.1) R: AACCAGCATCTTCCTCAG (0.1)	96
IL1B[b]	NM_174093.1	F: GCATGAGCTTTGTGCAAGGA (0.1) R: CTTGGGGTAGACTTTGGGGTC (0.1)	149
IL1R1[a]	NM_001206735.1	F: ACCGCATGCTTTAGCTGTAACTC (0.3) R: GCAGATGCGATTCTGTTTCG (0.3)	118
IL1R1[b]	NM_001206735.1	F:TTGGTGACTCCTGTCTTCGG (0.3) R:CCTGCTCCCTTAATCCGGTC (0.3)	87
SLC30A6	NM_001075766.1	F: TGATGAGGAAACCTAGCCCTGCC (0.3) R: TCGGGCTGCTCCAAAAAGCGT (0.3)	142
C20RF29	XM_582695.5	F: CCTTCAAGAGCCCCCTGT (0.3) R: GGGTCCTTTTCCAACTCTCC (0.3)	64
RPL19	NM_001040516	F: CCCCAATGAGACCAATGAAATC (0.3) R: CAGCCCATCTTTGATCAGCTT (0.3)	73
GAPD	XM_618013	F: TTCAACGGCACAGTCAAGG (0.2) R: ACATACTCAGCACCAGCATCAC (0.2)	119
SDHA	NM_174178	F: GCAGAACCTGATGCTTTGTG (0.3 R: CGTAGGAGAGCGTGTGCTT (0.3)	185
YWHAZ	BM446307	F: GCATCCCACAGACTATTTCC (0.2) R: GCAAAGACAATGACAGACCA (0.2)	120

Table 3.2 Summary of procedures used for IL1B / IL1R1 colocalization.

Endometrial samples	Blastocysts
Citrate retrieval	
Image-iT Block	Image-iT Block
NGS block	NGS block
IL1R1: 1:100; 72 h	IL1B: 1:100; 24 h
Alexa 488 goat anti Rabbit IgG: 1:1000	Alexa 488 goat anti Rabbit IgG: 1:600
NGS block	NGS block
IL1B : 1:100; 24 h	IL1R1: 1:100; 72 h
Alexa 555 goat anti Rabbit IgG: 1:800	Alexa 555 goat anti Rabbit IgG: 1:1000
Hoescht 33342 reagent (2.3 mg/ml)	Hoescht 33342 reagent (2.3 mg/ml)

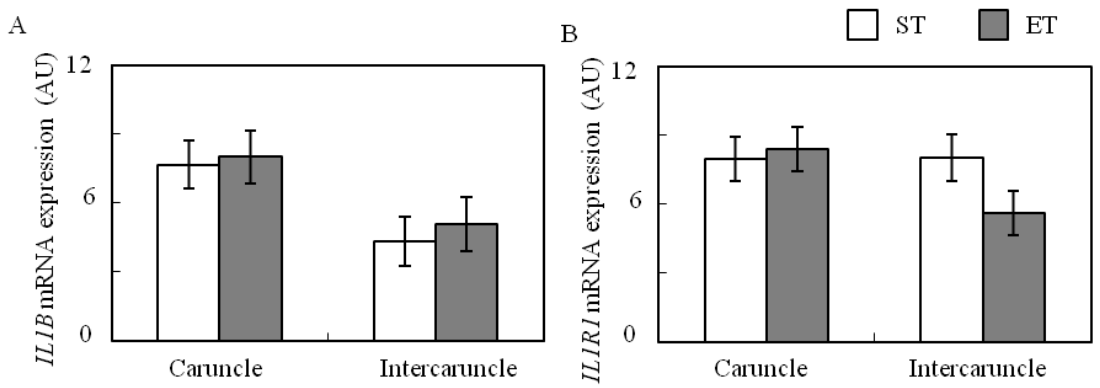


Figure 3.1 Real-time quantitative RT-PCR of *IL1B* mRNA (a) and *IL1R1* mRNA (b) from day 8 bovine endometrium collected from animals that received embryos (ET) and sham-treated control animals (ST). Expression of *IL1B* and *IL1R1* was not affected by pregnancy status. However, *IL1B* mRNA was significantly more abundant in caruncular than in intercaruncular regions in both groups of animals. Relative expression values are plotted as the LSM ± SEM. Asterisk represents significant differences: $p < 0.06$.

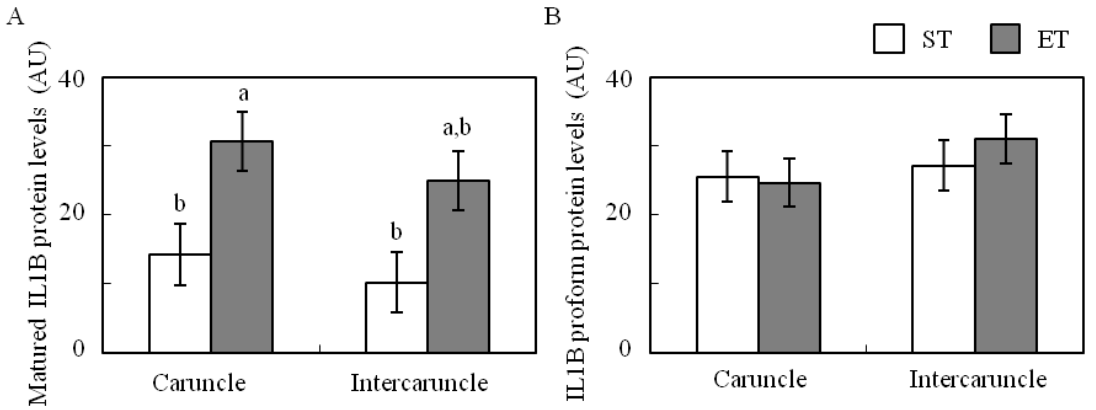


Figure 3.2 Amounts of IL1B mature form (A) and IL1B proform (B) as determined using Western blotting in day 8 bovine endometrium from cows that received embryos (ET) or were sham-transferred (ST) on day 5. Immunoreactive mature form of IL1B was higher in ET than ST within caruncles ($P < 0.04$). Data are LSM ± SEM.

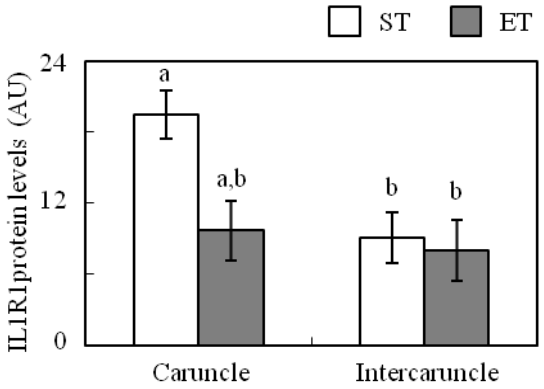


Figure 3.3 Immunoreactive IL1R1 in day 8 endometrium as determined by Western blotting of samples from cows that received embryos (ET) or were sham-transferred (ST) on day 5. IL1R1 was higher in caruncular than intercaruncular samples of ST animals ($p < 0.04$). Data are LSM ± SEM.

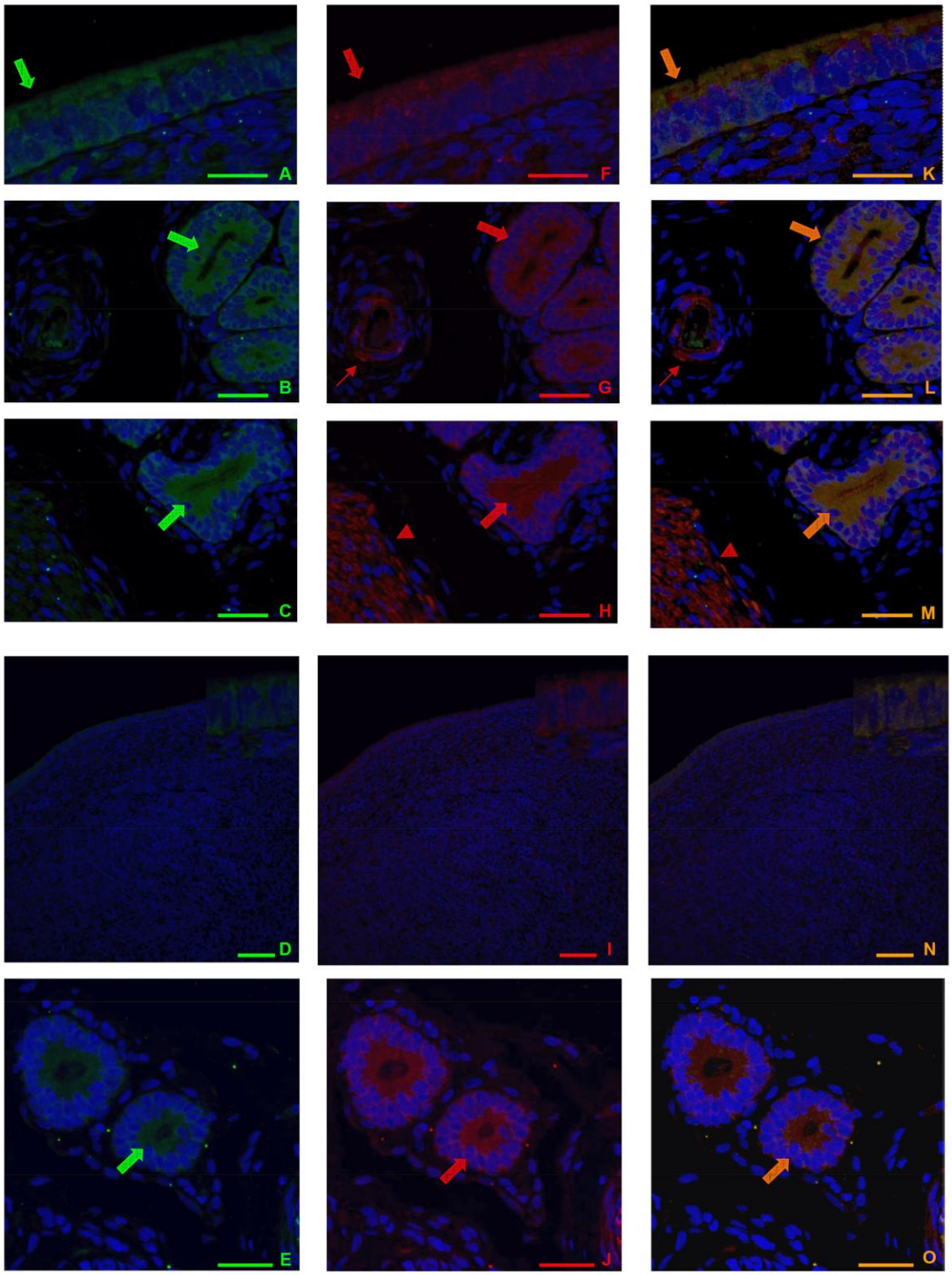


Figure 3.4 Representative images of localization of immunoreactive IL1B (green, A-E) and IL1R1 (red, F-J) in bovine endometrium. Colocalization of both proteins (orange) was found in luminal (K) and glandular epithelial cells (L, M). No differences in immunolabeling pattern or intensity was found between the intercaruncular (A-C: IL1B; F-H: IL1R1) and caruncular regions or between females that received embryos or were sham-transferred (D, E: IL1B; I, J: IL1R1). Scale bars, 25 µm except figures 3.4 D, I and N, where scale bars = 50 µm. Wide arrows indicate the apical sites of luminal and glandular epithelium. Thin arrow shows the walls of blood vessels. Arrowhead indicates the myometrium.

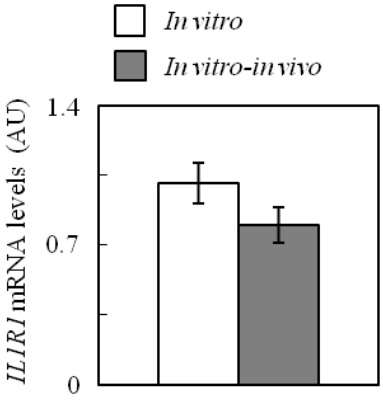


Figure 3.5. Transcript abundance for *IL1R1* in day 8 bovine blastocysst entirely *in vitro* produced (*in vitro*) or after uterine passage (*in vitro-in vivo*). Data are LSM ± SEM.

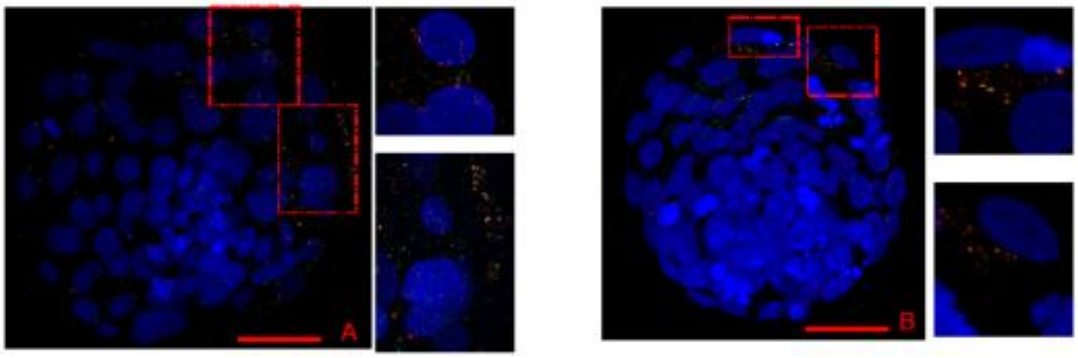
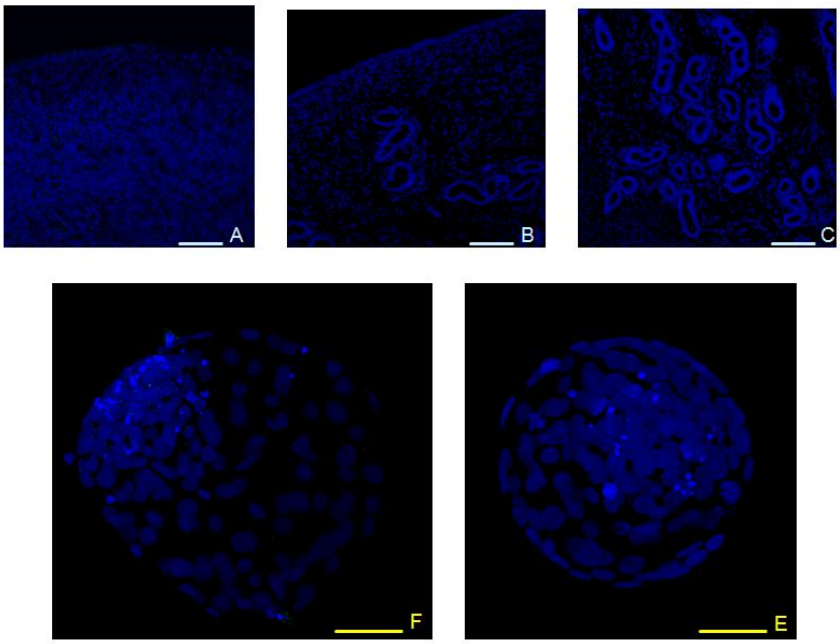


Figure 3.6 Representative images of immunolocalization of IL1B and IL1R1 in bovine embryos. Both proteins were detected in blastocysts after uterine passage (6A) and in blastocysts that developed entirely *in vitro* (6B). Detailed inspection of single z-axis optical sections from different focal planes confirmed that IL1B and IL1R1 were expressed exclusively in trophectoderm cells and not in cells of the inner cell mass. Embryonic nuclei were counter-stained with DAPI (blue). Scale bar, 50 µm.



Supplementary Figure 3.1 Immunoreactive IL1B and IL1R1 were not detected in bovine endometrium (A-C) or blastocysts, regardless of whether embryos experienced uterine passage (D) or developed entirely *in vitro* (E). Negative control samples were not treated with primary antibody. White scale bar: 200 µm, Yellow scale bar: 50µm.

REFERENCES

Al-Gubory, K.H., Arianmanesh, M., Garrel, C., Bhattacharya, S., Cash, P., Fowler, P.A., 2014. Proteomic analysis of the sheep caruncular and intercaruncular endometrium reveals changes. Reproduction. 147, 599-614.

Almiñana, C., Heath, P.R., Wilkinson, S., Sanchez-Osorio, J., Cuello, C., Parrilla, I., et al., 2012. Early developing pig embryos mediate their own environment in the maternal tract. PLoS One. 7, e33625.

Austgulen, R., Arntzen, K., Vatten, L., Kahn, J., Sunde, A., 1995. Detection of cytokines (interleukin-1, interleukin-6, transforming growth factor-β) and soluble tumour necrosis factor receptors in embryo culture fluids during in-vitro fertilization. Hum. Reprod. 10, 171-176.

Barañao, R., Piazza, A., Rumi, L., Fried, E., 1997. Determination of IL-1 and IL-6 Levels in Human Embryo Culture-Conditioned Media. Am. J. Reprod. Immunol. 37, 191-194.

Bazer, F., Spencer, T., Johnson, G., Burghardt, R., 2011. Uterine receptivity to implantation of blastocysts in mammals. Frontiers in bioscience (Scholar edition). 3, 745-767.

Bazer, F.W., Song, G., Kim, J., Dunlap, K.A., Satterfield, M.C., Johnson, G.A., et al., 2012. Uterine biology in pigs and sheep. J Anim Sci Biotechnol. 3, 1-23.

Bazer, F.W., Spencer, T.E., Johnson, G.A., Burghardt, R.C., Wu, G., 2009. Comparative aspects of implantation. Reproduction. 138, 195-209.

Bazer, F.W., Wu, G., Spencer, T.E., Johnson, G.A., Burghardt, R.C., Bayless, K., 2010. Novel pathways for implantation and establishment and maintenance of pregnancy in mammals. Mol. Human. Reprod. 16, 135-152.

Bellehumeur, C., Blanchet, J., Fontaine, J.Y., Bourcier, N., Akoum, A., 2009. Interleukin 1 regulates its own receptors in human endometrial cells via distinct mechanisms. Hum. Reprod. 24, 2193-2204.

Betts, J., Hansen, P., 1992. Regulation of prostaglandin secretion from epithelial and stromal cells of the bovine endometrium by interleukin-1 beta, interleukin-2, granulocyte-macrophage colony stimulating factor and tumor necrosis factor-alpha. Life Sci. 51, 1171-1176.

Bourdiec, A., Martel, V., Akoum, A., 2014. Synchronous regulation of the determinants of endometrial receptivity to interleukin 1 at key stages of early embryo implantation in vivo. Fertil. Steril. 101, 1183-1193.

Bourdiec, A., Shao, R., Rao, C., Akoum, A., 2012. Human chorionic gonadotropin triggers angiogenesis via the modulation of endometrial stromal cell responsiveness to interleukin 1: a new possible mechanism underlying embryo implantation. Biol. Reprod. 87, 66.

Colotta, F., Re, F., Muzio, M., Bertini, R., Polentarutti, N., Sironi, M., et al., 1993. Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4. Science. 261, 472-475.

Correia-Álvarez, E., Gómez, E., Martin, D., Carrocera, S., Pérez, S., Peynot, N., et al., 2015. Early embryonic and endometrial regulation of TNF and TNFR2 in the cattle

uterus. Theriogenology, in press, doi: http://dx.doi.org/10.1016/j.theriogenology.2014.12.007.

Chen, Q., Zhang, Y., Elad, D., Jaffa, A.J., Cao, Y., Ye, X., et al., 2013. Navigating the site for embryo implantation: biomechanical and molecular regulation of intrauterine embryo distribution. Mol. Aspects Med. 34, 1024-1042.

Davidson, J., Tiemann, U., Betts, J., Hansen, P., 1995. DNA synthesis and prostaglandin secretion by bovine endometrial cells as regulated by interleukin-1. Reprod. Fertil. Dev. 7, 1037-1043.

De los Santos, M.J., Anderson, D.J., Racowsky, C., Simón, C., Hill, J.A., 1998. Expression of interleukin-1 system genes in human gametes. Biol. Reprod. 59, 1419-1424.

De los Santos, M.J., Mercader, A., Francés, A., Portoles, E., Remohí, J., Pellicer, A., et al., 1996. Role of endometrial factors in regulating secretion of components of the immunoreactive human embryonic interleukin-1 system during embryonic development. Biol. Reprod. 54, 563-574.

Dinarello, C.A., 1994. The interleukin-1 family: 10 years of discovery. The FASEB Journal. 8, 1314-1325.

Dower, S., Kronheim, S.R., March, C.J., Conlon, P.J., Hopp, T.P., Gillis, S., et al., 1985. Detection and characterization of high affinity plasma membrane receptors for human interleukin 1. The Journal of experimental medicine. 162, 501-515.

Geisert, R., Fazleabas, A., Lucy, M., Mathew, D., 2012. Interaction of the conceptus and endometrium to establish pregnancy in mammals: role of interleukin 1β. Cell Tissue Res. 349, 825-838.

Geisert, R., Yelich, J., 1996. Regulation of conceptus development and attachment in pigs. J. Reprod. Fertil. Suppl. 52, 133-149.

Gómez, E., Caamaño, J.N., Corrales, F.J., Díez, C., Correia-Álvarez, E., Martín, D., et al., 2013. Embryonic sex induces differential expression of proteins in bovine uterine fluid. J. Proteome Res. 12, 1199-1210.

Gómez, E., Rodríguez, A., Muñoz, M., Caamaño, J.N., Carrocera, S., Martín, D., et al., 2008. Development and quality of bovine morulae cultured in serum-free medium with specific retinoid receptor agonists. Reprod. Fertil. Dev. 20, 884-891.

Goossens, K., Van Poucke, M., Van Soom, A., Vandesompele, J., Van Zeveren, A., Peelman, L., 2005. Selection of reference genes for quantitative real-time PCR in bovine preimplantation embryos. BMC Dev. Biol. 5, 27.

Guzeloglu-Kayisli, O., Kayisli, U.A., Taylor, H.S., 2009. The role of growth factors and cytokines during implantation: endocrine and paracrine interactions. Semin. Reprod. Med. 27, 062-079.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., Vandesompele, J., 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. 8, R19.

Hidalgo, C.O., Gómez, E., Prieto, L., Duque, P., Goyache, F., Fernández, L., et al., 2004. Pregnancy rates and metabolic profiles in cattle treated with propylene glycol prior to embryo transfer. Theriogenology. 62, 664-676.

Huang, H.-Y., Krüssel, J.S., Wen, Y., Polan, M.L., 1997. Use of reverse transcription-polymerase chain reaction to detect embryonic interleukin-1 system messenger RNA in individual preimplantation mouse embryos co-cultured with Vero cells. Hum. Reprod. 12, 1537-1544.

Kruessel, J., Huang, H.-Y., Wen, Y., Kloodt, A., Bielfeld, P., Polan, M., 1997. Different pattern of interleukin-1β- (IL-1β), interleukin-1 receptor antagonist- (IL-1ra) and interleukin-1 receptor type I- (IL-1R tI) mRNA-expression in single preimplantation mouse embryos at various developmental stages. J. Reprod. Immunol. 34, 103-120.

Krüssel, J.S., Bielfeld, P., Polan, M.L., Simón, C., 2003. Regulation of embryonic implantation. European Journal of Obstetrics & Gynecology and Reproductive Biology. 110, S2-S9.

Krüssel, J.S., Simón, C., Rubio, M.C., Pape, A.R., Wen, Y., Huang, H.Y., et al., 1998. Expression of interleukin-1 system mRNA in single blastomeres from human preimplantation embryos. Hum. Reprod. 13, 2206-2211.

Mansouri-Attia, N.r., Aubert, J., Reinaud, P., Giraud-Delville, C., Taghouti, G.r., Galio, L., et al., 2009. Gene expression profiles of bovine caruncular and intercaruncular endometrium at implantation. Physiol. Genomics. 39, 14-27.

McMaster, M., Newton, R., Dey, S., Andrews, G., 1992. Activation and distribution of inflammatory cells in the mouse uterus during the preimplantation period. The Journal of Immunology. 148, 1699-1705.

Muñoz, M., Corrales, F.J., Caamaño, J.N., Díez, C., Trigal, B., Mora, M.I., et al., 2012. Proteome of the Early Embryo-Maternal Dialogue in the Cattle Uterus. J. Proteome Res. 11, 751-766.

Muñoz, M., Peirson, S.N., Hankins, M.W., Foster, R.G., 2005. Long-term constant light induces constitutive elevated expression of mPER2 protein in the murine SCN: a molecular basis for Aschoff's rule? J. Biol. Rhythms. 20, 3-14.

Pampfer, S., Arceci, R.J., Pollard, J.W., 1991. Role of colony stimulating factor-1 (CSF-1) and other lympho-hematopoietic growth factors in mouse pre-implantation development. Bioessays. 13, 535-540.

Paula-Lopes, F.F., de Moraes, A.A.S., Edwards, J.L., Justice, J.E., Hansen, P.J., 1998. Regulation of preimplantation development of bovine embryos by interleukin-1β. Biol. Reprod. 59, 1406-1412.

Paula-Lopes, F.F., Moraes, A.A.D., Hansen, P., 1999. The presence of interleukin-1beta in the bovine reproductive tract. J. Interferon Cytokine Res. 19, 279-285.

Paulesu, L., Jantra, S., Ietta, F., Brizzi, R., Bigliardi, E., 2008. Interleukin-1 in reproductive strategies. Evolution & development. 10, 778-788.

Paulesu, L., Romagnoli, R., Bigliardi, E., 2005. Materno–fetal immunotolerance: is interleukin-1 a fundamental mediator in placental viviparity? Dev. Comp. Immunol. 29, 409-415.

Roberts, R.M., Chen, Y., Ezashi, T., Walker, A.M., 2008. Interferons and the maternal–conceptus dialog in mammals. Semin. Cell Dev. Biol. 19, 170-177.

Robertson, S.A., Brännström, M., Seamark, R.F., 1992. Cytokines in rodent reproduction and the cytokine-endocrine interaction. Curr. Opin. Immunol. 4, 585-590.

Ross, J.W., Malayer, J.R., Ritchey, J.W., Geisert, R.D., 2003. Characterization of the interleukin-1β system during porcine trophoblastic elongation and early placental attachment. Biol. Reprod. 69, 1251-1259.

Sargent, I., Martin, K., Barlow, D., 1998. The use of recombinant growth factors to promote human embryo development in serum-free medium. Hum. Reprod. 13, 239-248.

Seo, H., Choi, Y., Shim, J., Choi, Y., Ka, H., 2012. Regulatory mechanism for expression of IL1B receptors in the uterine endometrium and effects of IL1B on prostaglandin synthetic enzymes during the implantation period in pigs. Biol. Reprod. 87, 31.

Sheth, K., Roca, G., Al-Sedairy, S., Parhar, R., Hamilton, C., al-Abdul, J.F., 1991. Prediction of successful embryo implantation by measuring interleukin-1-alpha and immunosuppressive factor (s) in preimplantation embryo culture fluid. Fertil. Steril. 55, 952-957.

Simón, C., Frances, A., Piquette, G., El Danasouri, I., Zurawski, G., Dang, W., et al., 1994. Embryonic implantation in mice is blocked by interleukin-1 receptor antagonist. Endocrinology. 134, 521-528.

Simón, C., Mercader, A., Frances, A., Gimeno, M.J., Polan, M.L., Remohí, J., et al., 1996. Hormonal regulation of serum and endometrial IL-1α, IL-1β and IL-1ra: IL-1 endometrial microenvironment of the human embryo at the apposition phase under physiological and supraphysiological steroid level conditions. J. Reprod. Immunol. 31, 165-184.

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

Simón, C., Piquette, G.N., Frances, A., Polan, M.L., 1993b. Localization of interleukin-1 type I receptor and interleukin-1 beta in human endometrium throughout the menstrual cycle. The Journal of Clinical Endocrinology & Metabolism. 77, 549-555.

Sims, J.E., Giri, J.G., Dower, S.K., 1994. The two interleukin-1 receptors play different roles in IL-1 actions. Clin. Immunol. Immnopathol. 72, 9-14.

Singh, M., Chaudhry, P., Asselin, E., 2011. Bridging endometrial receptivity and implantation: network of hormones, cytokines, and growth factors. J. Endocrinol. 210, 5-14.

Sjöblom, C., Wikland, M., Robertson, S.A., 1999. Granulocyte–macrophage colony-stimulating factor promotes human blastocyst development in vitro. Hum. Reprod. 14, 3069-3076.

Spencer, T.E., Bazer, F.W., 2004. Conceptus signals for establishment and maintenance of pregnancy. Reprod. Biol. Endocrinol. 2, 195-209.

Stephens, A.N., Hannan, N.J., Rainczuk, A., Meehan, K.L., Chen, J., Nicholls, P.K., et al., 2010. Post-translational modifications and protein-specific isoforms in endometriosis revealed by 2D DIGE. J. Proteome Res. 9, 2438-2449.

Stewart, C.L., Cullinan, E.B., 1997. Preimplantation development of the mammalian embryo and its regulation by growth factors. Dev. Genet. 21, 91-101.

Stow, J.L., Murray, R.Z., 2013. Intracellular trafficking and secretion of inflammatory cytokines. Cytokine Growth Factor Rev. 24, 227-239.

Tabibzadeh, S., Babaknia, A., 1995. The signals and molecular pathways involved in implantation, a symbiotic interaction between blastocyst and endometrium involving adhesion and tissue invasion. Mol. Human Reprod. 1, 179-202.

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

Tanikawa, M., Acosta, T.J., Fukui, T., Murakami, S., Korzekwa, A., Skarzynski, D.J., et al., 2005. Regulation of prostaglandin synthesis by interleukin-1α in bovine endometrium during the estrous cycle. Prostaglandins Other Lipid Mediat. 78, 279-290.

Taskin, E.A., Baltaci, V., Cagiran, G., Aytac, R., 2012. Detection of IL-1β in culture media supernatants of pre-implantation human embryos; its relation with embryo grades and development. Gynecol. Endocrinol. 28, 296-298.

Tuo, W., Harney, J.P., Bazer, F.W., 1996. Developmentally regulated expression of interleukin-1β by peri-implantation conceptuses in swine. J. Reprod. Immunol. 31, 185-198.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., et al., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3, research0034.0031 - research0034.0011.

Walker, C., Meier, S., Littlejohn, M., Lehnert, K., Roche, J., Mitchell, M., 2010. Modulation of the maternal immune system by the pre-implantation embryo. BMC Genomics. 11, 474.

Wang, Y., Wang, C., Hou, Z., Miao, K., Zhao, H., Wang, R., et al., 2013. Comparative analysis of proteomic profiles between endometrial caruncular and intercaruncular areas in ewes during the peri-implantation period. J Anim Sci Biotechnol. 4, 39.

Wesche H, K.C., Kracht M, Falk W, Resch K, Martin MU, 1997. The interleukin-1 receptor accessory protein (IL1RacP) is essential for IL1-induced activation of interleukin-1 receptor-associated kinase (IRAK) and stress-activated protein kinases (SAP kinases). J. Biol. Chem. 272, 7727–7731.

Yang, Z., Chen, D., Le, S., Harper, M., 1995. Interleukin-1 alpha in the rabbit uterus during early pregnancy. Early pregnancy: biology and medicine: the official journal of the Society for the Investigation of Early Pregnancy. 1, 201-205.

Zolti, M., Ben-Rafael, Z., Meirom, R., Shemesh, M., Bider, D., Mashiach, S., et al., 1991. Cytokine involvement in oocytes and early embryos. Fertil. Steril. 56, 265-272.

CAPÍTULO 4 HEPATOMA-DERIVED GROWTH FACTOR: FROM THE BOVINE UTERUS TO THE IN VITRO EMBRYO CULTURE

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RESUMEN EN CASTELLANO

Durante el desarrollo tembrano bovino, el fluido uterino contiene concentraciones detectables del factor de crecimiento derivado del hepatoma (HDGF). Se desconoce tanto el origen de HDGF en los tejidos maternos como su efecto en el desarrollo embrionario. En el presente trabajo analizamos la expresión de HDGF en el endometrio de Día 8 expuesto a embriones, así como los efectos de HDGF recombinante (rHDGF) en el desarrollo embrionario. En el endometrio, los niveles de expresión proteíca o génica de *HDGF* no se vieron afectados por la exposición a embriones. La proteína de HDGF se localizó en los núcleos del epitelio luminal y de las glándulas superficiales, y en el citoplasma apical de las glándulas profundas. En los embriones, los niveles de expresión del mRNA de *HDGF* disminuyeron como consecuencia del pasaje uterino y la expresión proteíca de HDGF se detectó solamente en el trofectodermo. La adición de rHDGF al medio de cultivo de fibroblastos fetales promovió la proliferación celular. La adición de rHDGF al medio de cultivo con polivinil alcohol y libre de proteína durante el cultivo de mórulas tempranas (Día 5) en grupo, inhibió el desarrollo de blastocistos y no afectó a los contajes celulares. Sin embargo, la adición de rHDGF durante el cultivo de mórulas compactas (Día 6), tanto en grupo como individuales, incrementó los índices de desarrollo de blastocistos y el número de células. Nuestros experimentos con rHDGF indican que este factor de crecimiento estimula el desarrollo embrionario y la proliferación celular. El HDGF es sintetizado tanto por el endometrio como por el embrión, y podría ejercer efectos embriotróficos mediante mecanismos autocrinos o paracrinos.

RESEARCH

Hepatoma-derived growth factor: from the bovine uterus to the *in vitro* embryo culture

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Abstract

Early in cow embryo development, hepatoma-derived growth factor (HDGF) is detectable in uterine fluid. The origin of HDGF in maternal tissues is unknown, as is the effect of the induction on developing embryos. Herein, we analyze HDGF expression in day 8 endometrium exposed to embryos, as well as the effects of recombinant HDGF (rHDGF) on embryo growth. Exposure to embryos did not alter endometrial levels of *HDGF* mRNA or protein. HDGF protein localized to cell nuclei in the luminal epithelium and superficial glands and to the apical cytoplasm in deep glands. After uterine passage, levels of embryonic *HDGF* mRNA decreased and HDGF protein was detected only in the trophectoderm. In fetal fibroblast cultures, addition of rHDGF promoted cell proliferation. In experiments with group cultures of morulae in protein-free medium containing polyvinyl alcohol, adding rHDGF inhibited blastocyst development and did not affect cell counts when the morulae were early (day 5), whereas it enhanced blastocyst development and increased cell counts when the morulae were compact (day 6). In cultures of individual day 6 morulae, adding rHDGF promoted blastocyst development and increased cell counts. Our experiments with rHDGF indicate that the growth factor stimulates embryonic development and cell proliferation. HDGF is synthesized similarly by the endometrium and embryo, and it may exert embryotropic effects by autocrine and/or paracrine mechanisms.

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Introduction

The acidic, heparin-binding protein hepatoma-derived growth factor (HDGF) was originally isolated from a conditioned medium of human hepatoma cells (Nakamura *et al*. 1994). HDGF is a multifunctional protein that participates in the regulation of many cellular events, including ribosome biogenesis, RNA processing, DNA damage repair, and transcription (Zhao *et al*. 2011). Exogenous HDGF promotes proliferation of numerous cell types (e.g. vascular smooth muscle and endothelial and fibroblast cells; Klagsbrun *et al*. 1986, Everett *et al*. 2001), including cancer cell lines (Bernard *et al*. 2003, Hu *et al*. 2003, Chang *et al*. 2007, Lee *et al*. 2010, Liao *et al*. 2010, Yang *et al*. 2013). In tumor cells, overexpressed HDGF increases proliferation, migration, and neoangiogenesis (Sasaki *et al*. 2011, Thirant *et al*. 2012, Tsai *et al*. 2013), and it inhibits apoptosis (Lee *et al*. 2010, Liao *et al*. 2010).

HDGF is involved in organ development and tissue differentiation (Oliver & Al-Awqati 1998, Everett 2001, Everett *et al*. 2001, Enomoto *et al*. 2002). The molecular details of how HDGF influences development remain unclear because membrane or nuclear receptors for HDGF have yet to be identified. Nevertheless, it does appear that this soluble factor promotes remodeling by activating the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway (Kung *et al*. 2012). Factors regulating *HDGF* gene expression and activity also remain unclear, though studies indicate that restoring p53 activity in cancer cells represses transcription of the *HDGF* gene (Sasaki *et al*. 2011).

In the bovine species, previous studies have already identified numerous growth factors produced by the reproductive tract, which improve *in vitro* embryo development. These include insulin-like growth factor 1 (IGF1), epidermal growth factor (EGF), activin, granulocyte-macrophage colony-stimulating factor (GMCSF or CSF2), hyaluronan, fibroblast growth factor 2 (FGF2), and platelet-activating factor (PAF) (Gopichandran & Leese 2006, Block *et al*. 2011, Bonilla *et al*. 2011, Fields *et al*. 2011, Trigal *et al*. 2011, Sakagami *et al*. 2012). CSF2 has also been shown to act during gastrulation development (Loureiro *et al*. 2009, 2011). Several of these growth factors have been shown to improve survival-to-term following embryo transfer, including EGF, CSF2, CSF3, IGF1, and hyaluronan (Lim *et al*. 2007,

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Loureiro *et al*. 2009, Block *et al*. 2011, Sakagami *et al*. 2012). In a previous study combining two-dimensional difference gel electrophoresis with liquid chromatography–electrospray ionization mass spectrometry, we identified HDGF among 23 proteins that were overexpressed in day 8 uterine fluid (UF) collected from pregnant cows (Muñoz *et al*. 2012). This suggests that this growth factor may be involved in the regulation of blastocyst development, prompting the need for i) a detailed analysis of *HDGF* gene expression and ii) first investigation of the biological actions this growth factor will exert at the endometrium–embryo interface during early pregnancy.

In this study, we examined the origin of lumenal HDGF by determining the endometrial and embryonic localization of the protein and gene expression during transient *in utero* development of embryos produced *in vitro*. We also analyzed the bioactivity of recombinant HDGF (rHDGF) on fetal fibroblast cell proliferation and its effects on the *in vitro* development of bovine embryos.

Materials and methods

All chemicals were purchased from Sigma, unless otherwise indicated.

Animals

All experimental procedures with animals were carried out according to the European Community Directive 2010/63/EU (Spanish Regulation R.D. 53/2013) and were approved by the Animal Research Ethics Committee of SERIDA.

The experimental design has been described previously (Muñoz *et al*. 2012). Briefly, crossbred, age-matched, mixed heifers (Asturiana de los Valles and Asturiana de la Montaña cross, $n=6$) and uniparous cows ($n=5$) were synchronized to estrus (day 0) by using an intravaginal progesterone device (PRID ALPHA, Ceva, Barcelona, Spain) for 10 days combined with a prostaglandin analog (Dynolitic, Pfizer) injected 48 h before progestagen removal. Animals were assigned to either a cyclic group ($n=6$) or an *in vitro*-produced (IVP) group ($n=5$). The IVP group received 50 IVP embryos on day 5 (Gómez *et al*. 2013), while the cyclic group underwent sham transfer. Animals were killed in a nearby slaughterhouse (Matadero de Pravia, Asturias, Spain) on day 8 of their cycle.

Uterine horns were flushed, and the presence of embryos at appropriate development stages was examined in washes from embryo-transferred females. Endometrial samples and embryos were processed as described below.

Endometrial sample collection

Genital apparatuses were transported to the laboratory at 4 °C, and middle and cranial thirds were sampled as described previously (Gómez *et al*. 2013). Briefly, endometrial cross-sections were taken from caruncular and intercaruncular regions from the horn ipsilateral to the corpus luteum. Samples were processed for immunohistochemistry (IHC), western blotting (WB), and mRNA expression analysis. For WB and RT-PCR analysis, the caruncular and intercaruncular endometria, including deep endometrial glands, were dissected from the underlying myometrium. To allow for histological evaluation of the endometrium, IHC samples were fixed overnight at 4 °C in 4% paraformaldehyde in 0.1 M PBS (8.1 mM sodium phosphate dibasic, 137.9 mM sodium chloride, 2.07 mM potassium chloride, and 1.5 mM potassium phosphate monobasic; pH 7.4), dehydrated, embedded in paraffin, sectioned to a thickness of 4 μm, and stained with hematoxylin and eosin (Panreac, Barcelona, Spain).

Samples collected for WB were snap frozen in liquid nitrogen and stored at −145 °C until homogenization. Endometrial tissue was homogenized using a high-intensity ultrasonic processor (Sonics & Materials, Newton, MA, USA) in 10 ml lysis buffer (CelLytic, Sigma, Madrid, Spain, MT C3228) and 50 μl protease inhibitors (P8340, Sigma) per gram of tissue. After centrifugation at 3026 ***g*** for 10 min at 4 °C, the supernatant was collected and stored at −145 °C. Total protein concentration in tissue lysates was analyzed using the Quick Start Bradford protein assay kit (Bio-Rad Laboratories).

Samples for mRNA expression analysis with quantitative real-time reverse transcriptase PCR (qRT-PCR) were incubated overnight at 4 °C in RNAlater (Ambion, Huntingdon, Cambridgeshire, UK). Subsequently, excess RNAlater was removed and samples were stored at −145 °C until use.

Analysis of HDGF protein levels in bovine endometrium by IHC

Cell-specific expression of HDGF was analyzed with a mouse polyclonal antibody against HDGF (43668, Abcam, Cambridge, UK; diluted 1:1000) in endometrial cross-sections following a classical immunostaining protocol (Muñoz *et al*. 2005). Immunostained sections were rinsed in tap water, counterstained using modified Harris hematoxylin solution (AHHSS16), cleared, and mounted. Images of representative fields were recorded under bright-field illumination using an Olympus BX51 microscope fitted with an Olympus DP70 digital camera. Positive immunostaining was scored by two independent observers blind to the nature of the tissue. Negative control sections were incubated with 5% normal blocking serum instead of the primary antibody. No significant positive staining was observed in negative control sections (data not shown).

Analysis of HDGF protein levels in bovine endometrium by WB

Immunoblotting was performed as described previously (Muñoz *et al*. 2012). Briefly, total endometrial protein (5 μg/sample) was electrophoresed on 12% SDS–PAGE gels. Proteins were then transferred electrically onto a PVDF membrane (Millipore, Billerica, MA, USA), which was incubated with a mouse polyclonal antibody against HDGF (43668, Abcam; diluted 1:1500) and a mouse MAB against β-actin (2228, Sigma; diluted 1:6600). After washing, the membrane was incubated with the required secondary antibody (goat anti-mouse/HRP conjugate; Amersham Pharmacia Biotech) at 1:4000 dilution and detected using the Pierce ECL or ECL

plus detection kits (Thermo Scientific, Waltham, MA, USA). Protein levels were quantified using scanning densitometry and the ImageJ Software (http://rsbweb.nih.gov/ij/), with three replicate lanes quantified per biological condition. Differences in mean protein abundance were analyzed and expressed as fold change (least square mean (LSM) ± S.E.M.). As levels of β-actin vary significantly across uterus regions and even between replicate samples, protein expression was normalized based on protein loading visualized by Coomassie staining.

Analysis of HDGF *mRNA levels in bovine endometrium by real-time RT-PCR*

Total RNA from frozen endometrial tissue was extracted and purified, and its quality was assessed as described previously (Mansouri-Attia *et al.* 2009). Total purified RNA (1 μg) was reverse transcribed in a final reaction volume of 20 μl using Superscript III enzyme (Invitrogen) and random primers (Roche). Reaction conditions were 65 °C for 10 min followed by 5 min cooling on ice to allow annealing, 42 °C for 50 min to allow synthesis, and finally 70 °C for 15 min to denature the enzyme.

This reverse-transcribed DNA was then subjected to real-time RT-PCR using primers targeting the *HDGF* gene or one of the following reference genes: β-actin (*ACTB* (*ACT*)); chromosome 11 open reading frame, human C2orf29 (*CNOT11* (*C2ORF29*)); glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*); ribosomal protein L19 (*RPL19*); solute carrier family 30 (zinc transporter), member 6 (*SLC30A6*); and succinate dehydrogenase flavoprotein subunit A (*SDHA*). Primers for all genes were designed using Primer Express version 3.0 (Applied Biosystems; Table 1). Real-time PCRs were carried out using Master Mix SYBR Green and the Step One Plus system (Applied Biosystems), in a total volume of 15 μl containing 5 μl cDNA (diluted 1:200) and 0.3 μM primers. Amplification conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, 45 cycles at 95 °C for 15 s (denaturation), and 60 °C for 1 min (primer annealing and extension). Melting curve analyses were performed immediately after amplification; all samples were analyzed on the same plate to minimize variation between runs. A calibration curve was produced using cDNA retro-transcribed from total RNA pooled from all endometrial samples (several replicates) and serially diluted as described previously (Mansouri-Attia *et al.* 2009).

All samples were reverse transcribed and amplified in duplicate. Negative control samples for each gene were run in parallel without template or without primers. None of the negative control reactions showed amplicons. Amplicon sequences for all target genes were verified by DNA sequencing (Beckman Coulter Genomics, Takeley, UK).

QRT-PCR data were analyzed using the qBase plus Software (Biogazelle, Ghent, Belgium). *C*q values were transformed into normalized relative quantities (NRQs) based on several reference genes and after correcting for differences in amplification efficiency, using the approach described by Hellemans *et al.* (2007). To identify the reference genes best suited for calculating the required normalization factors (NFs), we used qBase to calculate two quality measures for the six reference genes mentioned above: the coefficient of variation of the normalized reference gene expression levels and the geNorm stability *M*-value. Subsequently, the data for another, less stable reference gene were integrated to give NFs, following the procedure described by Vandesompele *et al.* (2002). Using this stepwise approach, we determined that *C*q data for *SLC30A6, CNOT11,* and *RPL19* were best suited for normalizing the HDGF data.

In vivo *embryo sample collection for protein and gene expression analysis*

Embryos were washed three times in PBS–polyvinylpyrrolidone and grouped according to their development stage (degenerated, morulae, blastocyst, expanded blastocyst, and hatched blastocyst). Only expanded blastocysts were further processed for qRT-PCR and IHC.

Embryos for IHC were fixed for 20 min at room temperature in 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Fixed embryos were stored in 0.1 M PBS (pH 7.4) at 4 °C until use.

Table 1 Primers used to amplify regions of the *HDGF* gene in bovine endometrium and embryos or to amplify regions of reference genes in endometrium (*SLC30A6*, *CNOT11*, and *RPL19*) and embryos (*GAPDH*, *SDHA*, and *YWHAZ*).

Symbols	Names	Accession nos	Primer sequences	Concentrations (μM)	Fragment length (bp)
HDGF	Hepatoma-derived growth factor	NM_175832.2	GACCCACGAGACGGCATT	0.3	97
			GCTGAACCCTTTCCTCTTGTTG	0.3	
SLC30A6	Solute carrier family 30 (zinc transporter), member 6	NM_001075766.1	TGATGAGGAAACCTAGCCCTGCC	0.3	142
			TCGGGCTGCTCCAAAAAGCGT	0.3	
CNOT11	Chromosome 11 open reading frame, human C2orf29	XM_582695.5	CCTTCAAGAGCCCCCTGT	0.3	64
			GGGTCCTTTTCCAACTCTCC	0.3	
RPL19	Ribosomal protein L19	NM_001040516	CCCCAATGAGACCAATGAAATC	0.3	73
			CAGCCCATCTTTGATCAGCTT	0.3	
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	XM_618013	TTCAACGGCACAGTCAAGG	0.2	119
			ACATACTCAGCACCAGCATCAC	0.2	
SDHA	Succinate dehydrogenase flavoprotein subunit A	NM_174178	GCAGAACCTGATGCTTTGTG	0.3	185
			CGTAGGAGAGCGTGTGCTT	0.3	
YWHAZ	Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, zeta polypeptide	BM446307	GCATCCCACAGACTATTTCC	0.2	120
			GCAAAGACAATGACAGACCA	0.2	

Embryos for qRT-PCR were snap frozen in Eppendorf DNA LoBind tubes (catalog no. 022431021, Eppendorf, Hamburg, Germany) and stored at −145 °C until use.

Analysis of HDGF protein expression in bovine embryos by IHC

HDGF protein expression was analyzed by IHC in day 8 blastocysts ($n=8$/condition) using a mouse polyclonal primary antibody against HDGF (43668, Abcam) as described previously (Muñoz *et al.* 2008). Serial optical sections were acquired by confocal laser scanning microscopy in order to characterize the pattern of HDGF expression. Negative controls using only secondary antibody were performed in parallel.

Quantification of HDGF mRNA levels in bovine embryos by qRT-PCR

RT-PCR was performed with embryos according to Khan *et al.* (2012), with some modifications. Briefly, RNAs from ten embryos were extracted using the Picopure RNA isolation Kit (Arcturus, Applied Biosystems) and incubated in extraction buffer containing a mixture of Luciferase RNA (Promega) and 16S–18S carrier RNA (Roche Diagnostics) at 42 °C for 30 min, according to the manufacturer's instructions. Rates of mRNA recovery were evaluated by optical density measurement at a wavelength of 260 nm (using Nanodrop, Thermo Fisher Scientific, Madrid, Spain). cDNA was synthesized starting from the same number of 'equivalent embryos' in each sample and using the following conditions: 25 °C for 5 min to allow annealing, 50 °C for 60 min for RT, and 70 °C for 15 min to denature the enzyme.

PCR amplification was performed using the same primers targeting *HDGF* as were used for RT-PCR of endometrial samples. Data were analyzed using the qBase plus Software (Biogazelle). In contrast to the reference genes amplified in endometrial samples, the following reference genes were amplified in embryo samples, based on Goossens *et al.* (2005) (Table 1): *GAPDH, SDHA*, and the gene encoding tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*). The qRT-PCRs were performed in a final volume of 25 µl containing 10 µl cDNA from 0.3 embryo equivalents. Cycling conditions were the same as for endometrial samples, except that 40 cycles of denaturation, annealing, and extension were performed. Amplified PCR fragments were verified by 1% agarose gel electrophoresis and ethidium bromide staining.

For amplification of each gene, all samples from a single experiment were analyzed on a single plate, and each PCR was performed in triplicate. Each experiment was conducted with five batches of embryos. The same calibration curve as for endometrial samples was used. Amplification efficiencies were >80% and correlation coefficients were >0.98. Negative control amplifications lacking template were run in parallel for each gene, and no false positives were observed.

*C*t values were transformed into NRQs using qBase plus (Biogazelle) as described for endometrial samples. Data were analyzed using the Wilcoxon–Mann–Whitney *U* test.

IVP of bovine embryos

IVP embryos were obtained as described previously (Trigal *et al.* 2012), with minor modifications. Briefly, ovarian follicles with diameters of 3–8 mm from ovaries collected in a slaughterhouse were aspirated and cumulus–oocyte complexes recovered. After *in vitro* maturation for 24 h, oocytes were subjected to IVF (day 0) using frozen/thawed semen from a single bull obtained through a swim-up procedure. Embryos were cultured in synthetic oviduct fluid (SOF) containing amino acids, citrate, and myo-inositol. SOF droplets were layered under mineral oil and incubated at 38.7 °C in an atmosphere of 5% CO_2 and 5% O_2 with saturated humidity. In order to produce morulae, SOF was supplemented with 6 mg/ml BSA (SOF–BSA) from days 1 to 5 or 6. Within experiments (i.e. from day 5 or 6 onwards), embryos were cultured in SOF with 0.5 mg/ml polyvinyl alcohol (PVA) (SOF–PVA), in groups or singly, as single culture allows discarding paracrine effects from neighboring embryos. Group cultures comprised 50 µl droplets with 25–35 embryos/drop. Single cultures were performed in 12 µl droplets (Muñoz *et al.* 2014*a*,*b*,*c*).

Differential cell counts and apoptosis in blastocysts

Cell counts and apoptosis levels were measured in day 8 expanded blastocysts. Embryonic cells in the inner cell mass (ICM) and trophectoderm (TE) were differentially counted using a propidium iodide/bisbenzimide method (Trigal *et al.* 2012) with minor modifications.

TUNEL staining was carried out as described previously (Rodriguez *et al.* 2006), and nuclei were classified as showing apoptotic or necrotic morphology using the criteria of Gjørret *et al.* (2003). The numbers of apoptotic cells and necrotic cells were expressed as percentages of total cells to give the apoptotic index (AI) and necrotic index respectively. AI was measured in the ICM and in the TE.

Tissues were stained for caspase 3 using a procedure described by Wydooghe *et al.* (2001) and modified in our laboratory (Gómez *et al.* 2013). Caspase protein was detected in the cytoplasm of target embryos, while negative controls showed no positive staining.

Synthesis of rHDGF

Abyntek Laboratories (Derio, Bizkaia, Spain) produced rHDGF using the following procedure. Briefly, *HDGF* cDNA from bovine species was chemically synthesized with codon optimization for mammalian cell expression. A Flag tag sequence was inserted at the 5′-end of the coding region, followed by a Kozak sequence. The resulting *Flag-HDGF* cDNA was cloned into the pcDNA3.1 (−) expression vector (Life Technologies), which was transiently transfected into HEK293 cells. At 72 h after transfection, cells were harvested and HDGF protein was purified by affinity chromatography against the Flag tag. Protein purification was monitored by SDS–PAGE and WB against the Flag tag.

Proliferation assay of bovine fetal fibroblasts cultured with rHDGF

Fibroblasts were obtained from a 3-month-old bovine fetus, passaged for multiplication, and stored in aliquots in a medium containing 90% DMSO as described previously (Muñoz *et al.* 2008). For rHDF proliferation assay, thawed fibroblasts were routinely maintained in a basal medium (DMEM supplemented with 10% FCS, 1% non-essential amino acids, and 1% penicillin–streptomycin–amphotericin mixture) in a humidified incubator at 38.5 °C in an atmosphere of 5% CO_2. Cells were treated with rHDGF in a medium with and without FCS at 24 h post-seeding and then cultured for 48 h in the presence of rHDGF. Real-time cell proliferation was measured using xCelligence System (Acea, Izasa, Barcelona, Spain). Serum-containing and serum-free conditions (as intended for *in vitro* embryo culture) were used to test rHDGF cell proliferation activity. A 96-well plate was filled with 100 µl basal media/well and equilibrated at room temperature for 30 min. The plate was placed into a station cradle (housed in a humidified incubator at 38.5 °C in an atmosphere of 5% CO_2) to establish background reading. Cells were enumerated by Trypan blue staining and plated at a density of 1×10^4 cells/well in five replicates. Cells were allowed to settle for 30 min outside the incubator before returning the plate to the work station. Cell growth was monitored by electrical impedance measurements expressed as a normalized cell proliferation index (CPI).

Experimental design

Experiment 1: levels of HDGF mRNA and protein in endometrium and embryos

This experiment aimed to explore the uterine and/or embryonic origins of *HDGF*. Endometrial tissue was analyzed by IHC to explore distribution and localization of HDGF protein, by WB to analyze HDGF levels and by qRT-PCR to measure *HDGF* mRNA levels. Analogously, three groups of seven expanded blastocysts (day 8) were analyzed by ICH and five pools of ten such blastocysts were analyzed by qRT-PCR. Blastocysts had been either entirely produced *in vitro* or transferred into cows on day 5.

Experiment 2: effect of rHDGF on bovine fibroblast cell proliferation assay

We analyzed rHDGF bioactivity using a real-time cell proliferation assay. Several rHDGF concentrations (0, 0.1, 1, 10, 100, and 250 ng/ml) were tested both in medium containing 10% FCS and in serum-free conditions (i.e. as intended to be used within embryo culture). Normalized CPI was calculated at 24 h (serum-containing conditions) and 48 h (serum-free conditions) as described for fetal fibroblasts.

Experiment 3: effect of rHDGF on in vitro embryo development

First, we analyzed group embryo cultures. On day 5, early morulae produced in SOF–BSA were selected and cultured to day 8 in SOF–PVA in the presence of rHDGF at 0, 0.1, 1, or 10 ng/ml. Embryo development was recorded and cells were differentially counted in day 8 blastocysts.

Secondly, we compared embryo development over a shorter culture period (days 6–8) in SOF–PVA in the presence of 0, 1, or 100 ng/ml rHDGF. Approximately, 95% of the embryos cultured were day 6 morulae, with the remainder early blastocysts. When the embryos had become day 8 blastocysts, their embryonic development was analyzed and differential cell counts were performed.

Finally, we tested the optimal culture conditions (i.e. 100 ng/ml rHDGF in SOF–PVA) in individual embryo culture from days 6 to 8. Individual culture allows discarding paracrine effects associated with embryo culture in groups. Data on embryo development were recorded daily, and day 8 blastocysts were differentially counted and subjected to TUNEL staining.

Statistical analysis

Sources of variation affecting fibroblast proliferation (CPI), and embryo development and recovery, i.e. cell counts, apoptosis (TUNEL staining), and expression of protein and mRNA (WB and qRT-PCR), were identified by fitting linear models using the Proc GLM module of SAS/STAT (version 9.2; SAS Institute, Cary, NC, USA). The models included the following fixed effects: experimental treatment, embryonic stage, animal (where appropriate), and replicate (technical and biological). LSMs and their errors (±S.E.M.) were estimated for each level of fixed effects with a significant *F* value. The Ryan–Einot–Gabriel–Welsch *Q*-test was used to compare the raw means of the levels from the fixed effects.

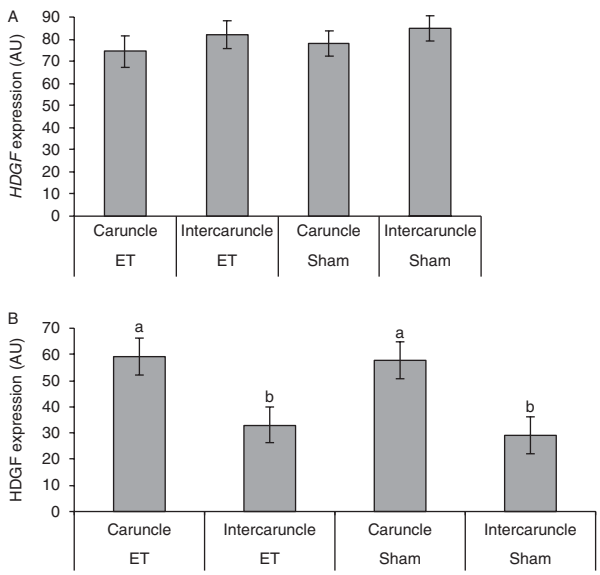

Figure 1 Real-time quantitative RT-PCR of *HDGF* mRNA (A) and western blot analysis of HDGF protein (B) from day 8 bovine endometrium collected from animals that received embryos (ET) and sham-treated control animals. Levels of mRNA and protein in caruncular and intercaruncular areas were similar between both groups of animals. HDGF protein was significantly more abundant in caruncular than in intercaruncular areas in both groups of animals. Results marked with different superscripts (a,b) are significantly different ($P<0.001$). Relative expression values are given in arbitrary units (AUs) and are plotted as the LSM±S.E.M.

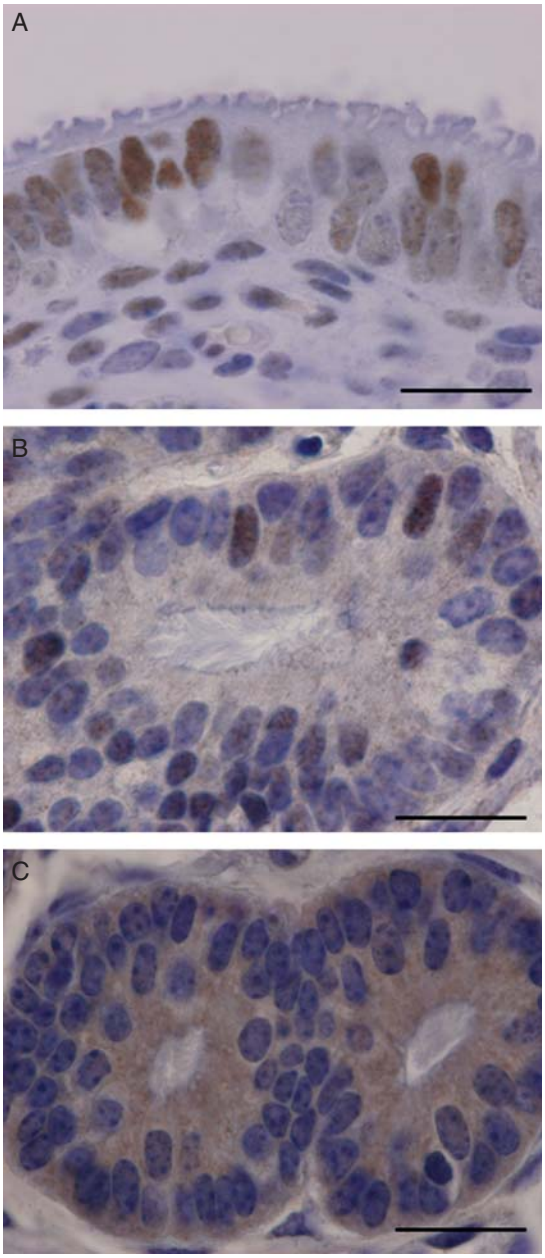

Figure 2 Representative immunohistochemical localization of HDGF protein in bovine endometrium. Positive labeling was found in the luminal (A) and glandular epithelial cells (B and C) and sub-epithelial stromal cells (A). In the luminal epithelium and superficial glands, immunostaining localized to the cell nuclei (A and B); in the deep glands, it localized to the apical cytoplasm adjacent to the glandular lumen (C). Scale bar, 20 μm.

Results

Experiment 1: levels of HDGF *mRNA and protein in endometrium and embryos*

The average embryo recovery rate, expressed as the percentage of total embryos transferred, was 36.8 ±4.0% (range 10–32, $n=5$ cows). The live embryo recovery rate was 54.1±4.0% (range 8–16).

Endometrial *HDGF* mRNA expression on day 8 was similar in caruncular and intercaruncular areas in the absence and presence of embryos ($P=0.66$; Fig. 1A), as were mRNA levels in cranial and middle horn regions ($P=0.70$; data not shown). HDGF protein levels were also similar in caruncular and intercaruncular areas in both groups of animals ($P>0.10$; Fig. 1B). However, in both pregnant and cyclic animals, HDGF protein levels were significantly higher in caruncular areas (56.2±4.3) than in intercaruncular ones (33.3 ±4.4, $P<0.001$).

Immunoreactive HDGF was detected in all endometrial samples. HDGF protein was localized to the nuclei of the luminal epithelium, sub-epithelial stroma, and superficial gland cells (Fig. 2A and B respectively). By contrast, localization of HDGF in deep uterine glands was observed in the cytoplasm (Fig. 2C). Interestingly, some sparse cells with strongly stained cytoplasm were detected in the caruncles (Supplementary Fig. 1, see section on supplementary data given at the end of this article). Negative control samples processed without any primary antibody showed no significant HDGF staining (Supplementary Fig. 2).

Relative abundance of *HDGF* mRNA, expressed in arbitrary units, was significantly lower in embryos recovered from the uterus (0.60±0.08) than in embryos produced entirely *in vitro* (1.0±0.08; $P=0.016$; Fig. 3). HDGF protein was detected in blastocysts after uterine passage (Fig. 4A) and in blastocysts produced entirely *in vitro* (Fig. 4D). In both cases, evaluation of serial optical sections analyzed by confocal laser scanning microscopy showed positive staining in the TE (Fig. 4C and F) but none in the ICM (Fig. 4B and E). Negative control samples processed without any primary antibody showed no significant HDGF staining (Supplementary Fig. 3, see section on supplementary data given at the end of this article).

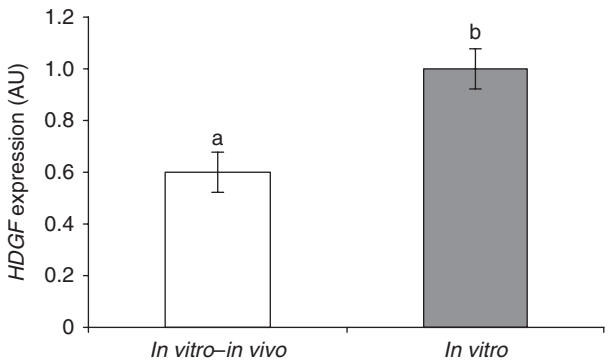

Figure 3 Real-time quantitative RT-PCR of *HDGF* mRNA in bovine embryos. Levels of mRNA were significantly lower in blastocysts after uterine passage (*in vitro–in vivo*) than in blastocysts that developed entirely *in vitro* (*in vitro*; $^{a,b}P=0.016$).

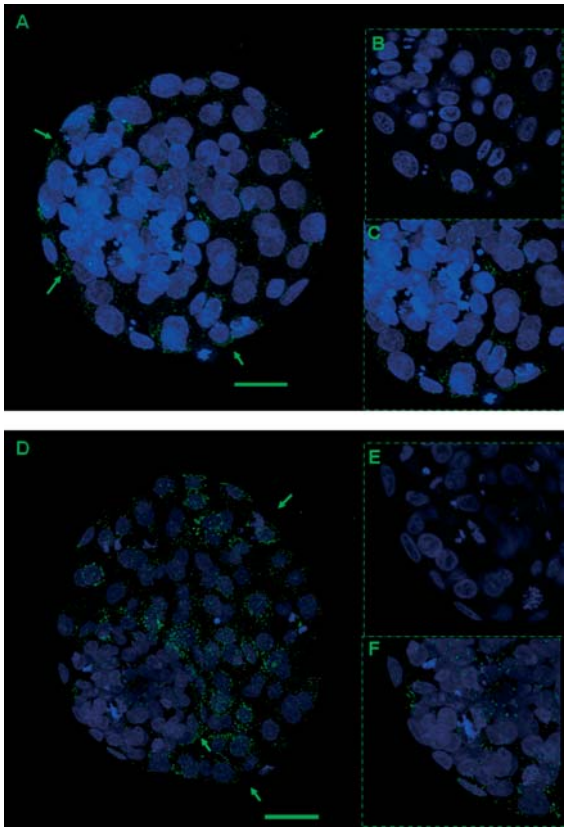

Figure 4 Immunofluorescence staining of HDGF protein in bovine embryos. HDGF was detected in blastocysts after uterine passage (A, B and C) and in blastocysts that developed entirely *in vitro* (D, E and F). HDGF localized to the trophectoderm (TE) cells (arrows, A and D; inserts, C and F), based on staining with a mouse polyclonal antibody. Detailed inspection of single *z*-axis optical sections from different focal planes confirmed that HDGF was expressed exclusively in TE cells (inserts, C and F) and not in cells of the inner cell mass (inserts, B and E). Embryonic nuclei were counterstained with DAPI (blue). Scale bar, 25 μm.

Experiment 2: effect of rHDGF on bovine fibroblast cell proliferation assay

In the presence of serum, rHDGF concentrations ranging between 1 and 250 μg/ml significantly increased the CPI ($P<0.02$; Fig. 5A). In the absence of serum, only 1 μg/ml rHDGF showed a highly significant effect ($P<0.001$; Fig. 5B) on CPI. Representative real-time fibroblast cell growth curves are shown in Supplementary Fig. 4A, see section on supplementary data given at the end of this article (serum-containing) and Supplementary Fig. 4B (serum-free). Therefore, we initially selected 1 μg/ml rHDGF and its closer concentrations (i.e. 0.1 and 10 μg/ml) to analyze within *in vitro* embryo culture experiments.

Experiment 3: effect of rHDGF on in vitro *embryo development*

We analyzed how adding rHDGF to the culture medium affects *in vitro* embryo development in SOF–PVA. First, we analyzed group cultures. On day 5, early morulae were selected and cultured up to day 8 in the presence of rHDGF at concentrations of 0, 0.1, 1, or 10 ng/ml (Table 2). All rHDGF concentrations significantly reduced blastocyst expansion rates on day 7 ($P<0.01$), but none of the rHDGF concentrations affected blastocyst development on day 8. Nevertheless, 0, 1, and 10 ng/ml rHDGF reduced day 8 hatching rates. Addition of rHDGF at any concentration did not significantly affect cell counts in the ICM or TE on day 8 (Table 3).

To gain more insight into whether the observed effects of rHDGF depend on embryo developmental stage, we repeated the experiments with day 6 morulae cultured to day 8 using rHDGF at a concentration of 1 or 100 ng/ml in SOF–PVA. We increased the upper concentration up to levels at which other uterine growth factors (i.e. EGF and IGF1) that activate the same downstream pathways as HDGF increased pregnancy rates using embryos cultured in similar conditions (Block & Hansen 2007, Sakagami *et al*. 2012). Adding 100 ng/ml rHDGF to the culture medium significantly increased the rate of formation of day 7 morulae and day 8 blastocyst and expanded blastocysts ($P<0.05$; Table 4). Counts in TE and total cell numbers increased with 100 ng/ml rHDGF (Table 5). The adverse effects on day 7 expansion rates observed when 1 ng/ml HDGF was added to the culture medium on day 5 were not observed when an equal

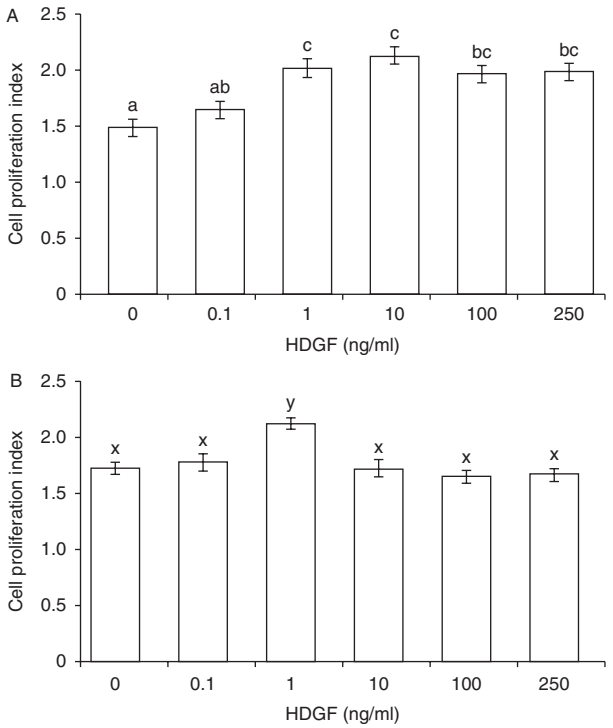

Figure 5 Cell proliferation index (CPI) of fetal fibroblasts growing in cultures with several rHDGF concentrations in the presence (A) and the absence (B) of 10% FCS. Cell growth was permanently monitored, and the CPI calculated at 48 h (A) and 24 h (B). Superscripts show significant differences: $^{a,b,c}P<0.02$; $^{x,y}P<0.001$.

Table 2 *In vitro* development of early bovine morulae cultured from days 5 to 8 in groups in synthetic oviduct fluid containing recombinant HDGF (rHDGF).

		Morulae (%)		**Day 7 blastocysts** (%)		**Day 8 blastocysts** (%)		
rHDGF (ng/ml)	***n***	Day 6	Day 7	Total	Expanded	Total	Expanded	Hatched
0	96	84.7±2.4	67.0±3.3	42.7±5.2	19.2±2.0*	52.1±3.8	33.2±4.9	3.3±0.7‡
0.1	96	86.8±2.4	60.7±3.3	26.0±5.2	5.8±2.0†	49.0±3.8	25.2±4.9	0.0§
1	97	85.6±2.5	75.2±3.3	38.0±5.2	8.1±2.0†	56.7±3.8	35.7±4.9	0.1±0.7
10	97	88.3±2.4	75.3±3.3	41.5±5.2	9.3±2.0†	59.1±3.8	34.5±4.9	0.0§

n, day 5 cultured early morulae. Data were collected from four replicates. Values in the same column marked with different symbols indicate significant differences: *,† $P<0.01$ and ‡,§ $P<0.05$.

concentration of growth factor was added on day 6. Although not tested in the same experimental replicates, cell counts in the ICM were numerically lower in expanded blastocysts cultured in groups from day 5 onwards (Table 3) than from day 6 onwards (Table 5).

Then, we analyzed the effects of rHDGF on the development of individual embryo cultures from days 6 to 8 in SOF–PVA. This technique allows for efficient, non-invasive sex detection (Muñoz *et al*. 2014*a*) and prediction of pregnancy viability based on spectroscopic analysis of embryo-conditioned culture medium (Muñoz *et al*. 2014*b*,*c*).

Adding rHDGF to the culture medium led to a significantly higher day 7 morulae rate ($P<0.03$) as well as a higher day 7 blastocyst rate ($P<0.01$) (Table 6). Blastocyst and expanded blastocyst development tended to be higher ($P<0.07$) in the presence of rHDGF. Addition of rHDGF to the culture medium (Table 7) significantly increased cell counts in the ICM and total cell counts ($P<0.03$), and the increases observed in the TE tended to be significant ($P<0.06$). Individual embryo cultures in our experiments showed similar levels of apoptosis based on TUNEL staining (data not shown).

Discussion

This study is the first detailed characterization of HDGF expression and function during early pregnancy in mammals. A previous work identified bovine HDGF in the UF (Muñoz *et al*. 2012), prompting us to ask whether the embryo and/or the mother express HDGF and how HDGF affects embryo development. The results of this study suggest that the presence of developing embryos does not significantly affect the levels of *HDGF* mRNA or protein in the endometrium. Nevertheless, *HDGF* mRNA and protein are detectable in embryos produced *in vitro* and in embryos exposed to the uterine environment. Exposing day 5 morulae to the uterus downregulates *HDGF* mRNA expression in recovered day 8 blastocysts to levels lower than those in embryos that developed entirely *in vitro*. Proliferation assay with bovine fetal fibroblasts demonstrated that rHDGF is a bioactive peptide. Experiments with embryos cultured individually or in groups in the presence of various concentrations of bioactive rHDGF indicate that this growth factor improves embryo development and cell counts *in vitro*. Our findings suggest that HDGF may act as a paracrine and/or autocrine factor to regulate embryo development *in vivo*.

Growing evidence points to the involvement of HDGF in controlling early embryo development, raising the question as to what molecule(s) triggers production of this growth factor in the first place. One candidate is hepatocyte growth factor (HGF), an angiogenic factor (Okada *et al*. 1999) that induces HDGF expression in cancer cells (Mao *et al*. 2008, Lee *et al*. 2010). In fact, the downstream effects of HGF may be mediated in part by HDGF induction of vascular endothelial growth factor (Mao *et al*. 2008, Lee *et al*. 2010) and angiogenesis via paracrine mechanisms (Sasaki *et al*. 2011, Thirant *et al*. 2012, Tsai *et al*. 2013).

To take into account the possibility that HDGF is regulated at transcriptional and/or post-transcriptional levels, we assayed levels of both mRNA and protein in our experiments. In this study, the presence of embryos did not alter endometrial *HDGF* mRNA expression in caruncular and intercaruncular areas, nor did it affect the localization of HDGF protein to cranial or middle horn regions. HDGF staining patterns only differed by structure: localization was primarily cytoplasmic with apparent secretion in deep uterine glands, while the protein localized to the nucleus in the luminal epithelium and superficial glands. In addition, some sparse positive cells were found in the caruncles. The lack of regulation of endometrial *HDGF* expression in the uterus by the embryo is intriguing, as P4 does not control *HGF* expression in the ruminant uterus either (Mitko *et al*. 2008, Satterfield *et al*. 2008). However,

Table 3 Differential cell counts in day 8 expanded blastocysts cultured in groups from days 5 to 8 in synthetic oviduct fluid containing PVA with recombinant HDGF (rHDGF).

rHDGF (ng/ml)	***n***	**ICM**	**TE**	**Total**
0	20	14.4±1.9	113.6±5.6	128.7±5.7
0.1	19	14.0±2.0	110.0±6.6	123.7±6.0
1	29	15.4±1.6	111.8±5.0	131.1±4.7
10	29	15.6±1.6	117.3±4.8	133.2±4.7

ICM, inner cell mass; *n*, number of embryos; TE, trophectoderm. Data were collected from four replicates. None of the values within each column indicated significant differences from one another ($P>0.10$).

Table 4 *In vitro* development of bovine morulae cultured from days 6 to 8 in synthetic oviduct fluid with PVA containing recombinant HDGF (rHDGF).

			Day 7 blastocysts (%)		**Day 8 blastocysts** (%)		
rHDGF (ng/ml)	***n***	**Day 7 morulae** (%)	Total	Expanded	Total	Expanded	Hatched
0	139	81.8±2.1*	42.2±4.0	13.8±2.6	54.2±3.0‡	39.0±2.9‡	4.7±1.5
1	99	78.9±2.4*	47.4±4.7	12.8±3.0	67.2±3.4	52.2±3.3	8.4±1.8
100	99	96.8±2.4†	57.8±4.7	18.2±3.0	70.9±3.4§	54.7±3.3§	8.3±1.8

n, day 6 cultured embryos (90% morulae+early blastocysts). Data were collected from five replicates. Values in the same column with different symbols indicate significant differences: *,†$P<0.01$ and ‡,§$P<0.05$.

luminal and glandular epithelial cells express *HGF* receptor and epithelial c-met proto-oncogene (*MET*), under P4 regulation (Mitko *et al*. 2008, Satterfield *et al*. 2008). On the embryonic side, passage through the uterine tract led to reduced levels of *HDGF* mRNA in recovered day 8 blastocysts in comparison to those produced entirely *in vitro*. Interestingly, no differences were found in HDGF expression or distribution in TE cells, between both types of embryonic samples analyzed. In line with our findings, expression of *MET* has been localized to the ovine TE (Chen *et al*. 2000*a*,*b*).

We postulate that embryonal and uterine HDGF may act in an autocrine and/or paracrine way with the possible participation of HGF to induce HDGF synthesis and secretion in deep uterine glands and regulate *HDGF* gene expression. This crosstalk may lead the embryo to downregulate its own *HDGF* transcription to compensate for the presence of maternal HDGF.

In contrast to the cytoplasmic and secretory localization of HDGF in deep glands, we found the protein to localize to the nucleus in superficial glands and epithelium. This nuclear localization suggests that the protein was not secreted from these regions. Consistent with our findings, HDGF localizes to the nucleus and cytoplasm in human fibroblasts (Abouzied *et al*. 2004).

In addition to providing first evidence of endometrial and embryonic localization and gene expression of HDGF, our study demonstrates that the growth factor is potentially involved in regulating *in vivo* and *in vitro* development. We performed proliferation assays with rHDGF in more usual conditions with serum, whereby HDGF bioactivity has been previously shown with fibroblasts not only in other species (Klagsbrun *et al*. 1986, Abouzied *et al*. 2005, Wang *et al*. 2011), but also in defined conditions, as intended for embryo culture. Serum may provide carriers and cofactors necessary for GFs to exert their physiological effects (Francis 2010). However, a chemically defined medium is interesting for the normalization of embryo culture conditions and absence of sanitary risks associated with the compounds of animal origin. The effects of HDGF on *in vitro* embryo development depended on the developmental stage. When HDGF was added to group cultures of early morulae from day 5 onwards, blastocyst expansion on day 7 was severely reduced and blastocyst cell counts were unchanged. By contrast, adding HDGF to group cultures of late morulae on day 6 promoted embryo development and increased TE cell counts. Such time-dependent effects of growth factors on *in vitro* embryo development have also been reported for FGF2, activin, CSF2, IGF1, TGFβ (TGFB), TGFα (TGFA), and PDGF (PDGFA; Larson *et al*. 1992*a*,*b*, Loureiro *et al*. 2009, Fields *et al*. 2011, Trigal *et al*. 2011, Dobbs *et al*. 2013). In fact, the developmental stage at which CSF2 and IGF1 are added to cultures strongly affects the yield and quality of the resulting blastocysts, as well as pregnancy and birth rates (Loureiro *et al*. 2009). A well-established explanation for such stage-specific effects relies on the timed expression of growth factor receptors in the embryo, which renders it responsive to uterine growth factors only during specific periods of development. There was also an apparent, side effect of the day and/or developmental stage of replacement of BSA by PVA on the ICM cells (day 5 vs day 6). Thus, in group culture, ICM cell numbers were lower with PVA added on day 5 than on day 6, suggesting that removal of protein from culture out of certain limits might compromise embryonic quality.

Our results suggest that HDGF helps to advance the day 6 morulae throughout blastulation. In bovine blastocysts, E-cadherin and vimentin are TE markers during the epithelial to mesenchymal transition (EMT; Yamakoshi *et al*. 2012), with each protein serving essential and specific roles at different stages of blastocyst development. Interestingly, HDGF down-regulates E-cadherin expression and upregulates vimentin expression to help drive EMT in both healthy and cancer cells (Chen *et al*. 2012, Tsai *et al*. 2013). Future research should explore stage-dependent effects of

Table 5 Differential cell counts in day 8 expanded blastocysts cultured in groups from days 6 to 8 in synthetic oviduct fluid containing PVA with recombinant HDGF (rHDGF).

rHDGF (ng/ml)	***n***	**ICM**	**TE**	**Total**
0	27	25.9±2.6	115.3±5.2‖	138.6±5.8‡
1	37	27.4±2.2	108.3±4.4*	132.9±5.2‡
100	38	23.2±2.7	132.2±5.4†,¶	155.4±4.9§

ICM, inner cell mass; *n*, number of embryos; TE, trophectoderm. Data were collected from four replicates. Values in the same column with different symbols indicate significant differences: *,†$P<0.01$ and ‡,§$P<0.05$ or a tendency, ‖,¶$P<0.07$.

Table 6 *In vitro* development of bovine morulae cultured individually from days 6 to 8 in droplets of synthetic oviduct fluid with PVA containing 100 ng/ml recombinant HDGF (rHDGF).

rHDGF (ng/ml)	*n*	Day 7 (%)	Day 7 blastocysts (%)			Day 8 blastocysts (%)			
		Morulae	Total	Blastocysts	Expanded	Total	Blastocysts	Expanded	Hatched
0	229	74.1‡	55.2	32.2*	15.3	62.5	52.1$^{‖}$	37.2$^{‖}$	6.4
100	230	91.0§	68.4	49.5†	20.6	76.7	67.7¶	50.9¶	5.7
LSM		±4.5	±5.1	±3.8	2.8	±6.6	5.6	±4.6	±1.6

LSMs, least square means. *n*, day 6 cultured early morulae. Data were collected from 11 replicates. Values in the same column with different symbols indicate significant differences: $^{*,†}P<0.01$ and $^{‡,§}P<0.05$; or tends, $^{‖,¶}P<0.07$.

HDGF in bovine embryo cultures related to the timed expression of E-cadherin and/or vimentin.

Embryotropic effects of rHDGF were observed with both group and single cultures. Our IHC studies showing the presence of substantial HDGF in TE but no localization in the ICM suggest that HDGF directly stimulates TE proliferation, while we speculate that proliferative effects on the ICM, clearly observed in individual culture, are likely to be mediated by a second factor in a paracrine way. Our apparent finding that HDGF does not affect apoptosis is surprising, given that HDGF overexpression inhibits caspase 3 activity in cancer cells (Liao *et al*. 2010). However, consistent with our observations, in healthy mouse fibroblasts, HDGF is dispensable for apoptotic signaling (Gallitzendoerfer *et al*. 2008). Alternatively, this unexpected result may be an artifact reflecting the fact that a certain percentage of cells showing unequivocal morphological signs of apoptosis fail to show TUNEL staining (Leidenfrost *et al*. 2011).

HDGF appears to activate the PI3K/Akt (Kung *et al*. 2012), and/or ERK (Mao *et al*. 2008, Lee *et al*. 2010) and p38 MAPK pathways (Wang *et al*. 2011). Early bovine IVP embryos show active ERK and p38 MAPK pathways (Madan *et al*. 2005), and the ERK pathway appears to be capable of activating p38 MAPK downstream kinases in the absence of p38 MAPK activity to maintain development, showing functional redundancy. Accordingly, numerous uterine signaling factors promote bovine embryo growth *in vitro* by acting through the above pathways. These factors include EGF, TGFβ, insulin, IGF1, FGF2, leukemia inhibitory factor (LIF), activin, and PDGF (Larson *et al*. 1992*a*,*b*, Loureiro *et al*. 2009, Bonilla *et al*. 2011, Fields *et al*. 2011, Trigal *et al*. 2011, Sakagami *et al*. 2012, Dobbs *et al*. 2013, Wydooghe *et al*. 2013, Cebrian-Serrano *et al*. 2014). Even small molecules in the uterus, such as retinoic acid, can also activate PI3K/Akt (Bastien *et al*. 2006) and ERK1/2 (Persaud *et al*. 2013). Interestingly, retinoic acid also improves bovine blastocyst development *in vitro* (Rodríguez *et al*. 2006, 2007*a*, Gómez *et al*. 2008). As shown with these factors, adding HDGF to culture medium can improve the performance of cultures of individual embryos, allowing studies not only for cow breeding *per se* but also for model studies of human embryo culture. These factors can also be used to treat cow donors that provide few viable oocytes by aspiration and hence require individual oocyte culturing (Hansen 2010, Wydooghe *et al*. 2013).

In our *in vitro* embryo culture study, we found that rHDGF showed embryotropic effects at a concentration of 100 ng/ml, which is similar or close to the concentration dynamic range at which other growth factors increase pregnancy rates using embryos cultured in defined and undefined conditions. Examples include bovine embryo culture in the presence of BSA and recombinant bovine CSF2 (10 ng/ml; Loureiro *et al*. 2009), as well as bovine embryo culture in the presence of PVA and human IGF1 (50 ng/ml) and mouse EGF (100 ng/ml; Sakagami *et al*. 2012) or in the presence of BSA and recombinant human IGF1 (100 ng/ml; Block & Hansen 2007). These growth factors were probably selected for inclusion in *in vitro* embryo production systems based on sequence homology with the bovine species. The pregnancy rates reported for these factors suggest functional redundancy even among factors from different species. Nevertheless, we recommend the use of homospecific growth factors based on the substantial differences we obtained in development and quality of bovine embryos *in vitro* depending on whether we used human or mouse LIF (Rodríguez *et al*. 2007*b*).

In this study, we have shown that HDGF is synthesized by the endometrium and embryo similarly and that it may act by both autocrine and paracrine mechanisms to promote early embryo development *in vitro* and probably *in vivo*. Adding rHDGF to IVP embryos increases blastocyst development and cell proliferation. These effects depend on the developmental stage at which the growth factor is added. HDGF seems to be an effective growth factor both in serum-containing

Table 7 Differential cell counts in day 8 expanded blastocysts cultured individually from days 6 to 8 in single drops of synthetic oviduct fluid supplemented with PVA in the presence of 100 ng/ml recombinant HDGF (rHDGF).

rHDGF (ng/ml)	*n*	ICM	TE	Total cells
0	23	18.0±2.3	99.3±7.1	117.0±6.9
100	19	25.1±2.4	118.0±7.4	139.7±7.8
P value		0.03	0.06	0.03

ICM, inner cell mass; *n*, number of blastocysts; TE, trophectoderm. Data were collected from four replicates.

medium and also in defined culture conditions, which is advantageous for media standardization and optimization and individual tracking of cultured embryos.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-14-0304.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

Abouzied MM, Baader SL, Dietz F, Kappler J, Gieselmann V & Franken S 2004 Expression patterns and different subcellular localization of the growth factors HDGF (hepatoma-derived growth factor) and HRP-3 (HDGF-related protein-3) suggest functions in addition to their mitogenic activity. *Biochemical Journal* **378** 169–176. (doi:10.1042/BJ20030916)

Abouzied MM, El-Tahir HM, Prenner L, Häberlein H, Gieselmann V & Franken S 2005 Hepatoma-derived growth factor. Significance of amino acid residues 81–100 in cell surface interaction and proliferative activity. *Journal of Biological Chemistry* **280** 10945–10954. (doi:10.1074/jbc.M414652200)

Bastien J, Plassat JL, Payrastre B & Rochette-Egly C 2006 The phosphoinositide 3-kinase/Akt pathway is essential for the retinoic acid-induced differentiation of F9 cells. *Oncogene* **25** 2040–2047. (doi:10.1038/sj.onc.1209241)

Bernard K, Litman E, Fitzpatrick JL, Shellman YG, Argast G, Polvinen K, Everett AD, Fukasawa K, Norris DA, Ahn NG *et al.* 2003 Functional proteomic analysis of melanoma progression. *Cancer Research* **63** 6716–6725.

Block J & Hansen PJ 2007 Interaction between season and culture with insulin-like growth factor-1 on survival of *in vitro* produced embryos following transfer to lactating dairy cows. *Theriogenology* **67** 1518–1529. (doi:10.1016/j.theriogenology.2007.03.012)

Block J, Hansen PJ, Loureiro B & Bonilla L 2011 Improving post-transfer survival of bovine embryos produced *in vitro*: actions of insulin-like growth factor-1, colony stimulating factor-2 and hyaluronan. *Theriogenology* **76** 1602–1609. (doi:10.1016/j.theriogenology.2011.07.025)

Bonilla AQ, Ozawa M & Hansen PJ 2011 Timing and dependence upon mitogen-activated protein kinase signaling for pro-developmental actions of insulin-like growth factor 1 on the preimplantation bovine embryo. *Growth Hormone & IGF Research* **21** 107–111. (doi:10.1016/j.ghir.2011.03.003)

Cebrian-Serrano A, Salvador I & Silvestre MA 2014 Beneficial effect of two culture systems with small groups of embryos on the development and quality of *in vitro*-produced bovine embryos. *Anatomia, Histologia, Embryologia* **43** 22–30. (doi:10.1111/ahe.12043)

Chang KC, Tai MH, Lin JW, Wang CC, Huang CC, Hung CH, Chen CH, Lu SN, Lee CM, Changchien CS *et al.* 2007 Hepatoma-derived growth factor is a novel prognostic factor for gastrointestinal stromal tumors. *International Journal of Cancer* **121** 1059–1065. (doi:10.1002/ijc.22803)

Chen C, Spencer TE & Bazer FW 2000*a* Expression of hepatocyte growth factor and its receptor c-met in the ovine uterus. *Biology of Reproduction* **62** 1844–1850. (doi:10.1095/biolreprod62.6.1844)

Chen C, Spencer TE & Bazer FW 2000*b* Fibroblast growth factor-10: a stromal mediator of epithelial function in the ovine uterus. *Biology of Reproduction* **63** 959–966. (doi:10.1095/biolreprod63.3.959)

Chen SC, Kung ML, Hu TH, Chen HY, Wu JC, Kuo HM, Tsai HE, Lin YW, Wen ZH, Liu JK *et al.* 2012 Hepatoma-derived growth factor regulates breast cancer cell invasion by modulating epithelial–mesenchymal transition. *Journal of Pathology* **228** 158–169. (doi:10.1002/path.3988)

Dobbs KB, Khan FA, Sakatani M, Moss JI, Ozawa M, Ealy AD & Hansen PJ 2013 Regulation of pluripotency of inner cell mass and growth and differentiation of trophectoderm of the bovine embryo by colony stimulating factor 2. *Biology of Reproduction* **89** 141. (doi:10.1095/biolreprod.113.113183)

Enomoto H, Yoshida K, Kishima Y, Kinoshita T, Yamamoto M, Everett AD, Miyajima A & Nakamura H 2002 Hepatoma-derived growth factor is highly expressed in developing liver and promotes fetal hepatocyte proliferation. *Hepatology* **36** 1519–1527. (doi:10.1002/hep.1840360629)

Everett AD 2001 Identification, cloning, and developmental expression of hepatoma-derived growth factor in the developing rat heart. *Developmental Dynamics* **222** 450–458. (doi:10.1002/dvdy.1204)

Everett AD, Stoops T & McNamara CA 2001 Nuclear targeting is required for hepatoma-derived growth factor-stimulated mitogenesis in vascular smooth muscle cells. *Journal of Biological Chemistry* **276** 37564–37568. (doi:10.1074/jbc.M105109200)

Fields SD, Hansen PJ & Ealy AD 2011 Fibroblast growth factor requirements for *in vitro* development of bovine embryos. *Theriogenology* **75** 1466–1475. (doi:10.1016/j.theriogenology.2010.12.007)

Francis GL 2010 Albumin and mammalian cell culture: implications for biotechnology applications. *Cytotechnology* **62** 1–16. (doi:10.1007/s10616-010-9263-3)

Gallitzendoerfer R, Abouzied MM, Hartmann D, Dobrowolski R, Gieselmann V & Franken S 2008 Hepatoma-derived growth factor (HDGF) is dispensable for normal mouse development. *Developmental Dynamics* **237** 1875–1885. (doi:10.1002/dvdy.21589)

Gjørret JO, Knijn HM, Dieleman SJ, Avery B, Larsson LI & Maddox-Hyttel P 2003 Chronology of apoptosis in bovine embryos produced *in vivo* and *in vitro*. *Biology of Reproduction* **69** 1193–1200. (doi:10.1095/biolreprod.102.013243)

Gómez E, Rodríguez A, Muñoz M, Caamaño JN, Carrocera S, Martín D, Facal N & Díez C 2008 Development and quality of bovine morulae cultured in serum-free medium with specific retinoid receptor agonists. *Reproduction, Fertility, and Development* **20** 884–891. (doi:10.1071/RD08103)

Gómez E, Caamaño JN, Corrales FJ, Díez C, Correia-Álvarez E, Martín D, Trigal B, Carrocera S, Mora MI, Pello-Palma J *et al.* 2013 Embryonic sex induces differential expression of proteins in bovine uterine fluid. *Journal of Proteome Research* **12** 1199–1210. (doi:10.1021/pr300845e)

Goossens K, Van Poucke M, Van Soom A, Vandesompele J, Van Zeveren A & Peelman L 2005 Selection of reference genes for quantitative real-time PCR in bovine preimplantation embryos. *BMC Developmental Biology* **5** 27. (doi:10.1186/1471-213X-5-27)

Gopichandran N & Leese HJ 2006 The effect of paracrine/autocrine interactions on the *in vitro* culture of bovine preimplantation embryos. *Reproduction* **131** 269–277. (doi:10.1530/rep.1.00677)

Hansen PJ 2010 Medawar redux – an overview on the use of farm animal models to elucidate principles of reproductive immunology. *American Journal of Reproductive Immunology* **64** 225–230. (doi:10.1111/j.1600-0897.2010.00900.x)

Hellemans J, Mortier G, De Paepe A, Speleman F & Vandesompele J 2007 qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biology* **8** R19. (doi:10.1186/gb-2007-8-2-r19)

Hu TH, Huang CC, Liu LF, Lin PR, Liu SY, Chang HW, Changchien CS, Lee CM, Chuang JH & Tai MH 2003 Expression of hepatoma-derived growth factor in hepatocellular carcinoma. *Cancer* **98** 1444–1456. (doi:10.1002/cncr.11653)

Khan DR, Dubé D, Gall L, Peynot N, Ruffini S, Laffont L, Le Bourhis D, Degrelle S, Jouneau A & Duranthon V 2012 Expression of pluripotency master regulators during two key developmental transitions: EGA and early lineage specification in the bovine embryo. *PLoS ONE* **7** e34110. (doi:10.1371/journal.pone.0034110)

Klagsbrun M, Sasse J, Sullivan R & Smith JA 1986 Human tumor cells synthesize an endothelial cell growth factor that is structurally related to basic fibroblast growth factor. *PNAS* **83** 2448–2452. (doi:10.1073/pnas.83.8.2448)

Kung ML, Tsai HE, Hu TH, Kuo HM, Liu LF, Chen SC, Lin PR, Ma YL, Wang EM, Liu GS *et al.* 2012 Hepatoma-derived growth factor stimulates podosome rosettes formation in NIH/3T3 cells through the activation of phosphatidylinositol 3-kinase/Akt pathway. *Biochemical and Biophysical Research Communications* **425** 169–176. (doi:10.1016/j.bbrc.2012.07.060)

Larson RC, Ignotz GG & Currie WB 1992*a* Transforming growth factor β and basic fibroblast growth factor synergistically promote early bovine embryo development during the fourth cell cycle. *Molecular Reproduction and Development* **33** 432–435. (doi:10.1002/mrd.1080330409)

Larson RC, Ignotz GG & Currie WB 1992*b* Platelet derived growth factor (PDGF) stimulates development of bovine embryos during the fourth cell cycle. *Development* **115** 821–826.

Lee KH, Choi EY, Kim MK, Lee SH, Jang BI, Kim TN, Kim SW, Kim SW, Song SK, Kim JR *et al.* 2010 Hepatoma-derived growth factor regulates the bad-mediated apoptotic pathway and induction of vascular endothelial growth factor in stomach cancer cells. *Oncology Research* **19** 67–76. (doi:10.3727/096504010X12864748215043)

Leidenfrost S, Boelhauve M, Reichenbach M, Güngör T, Reichenbach HD, Sinowatz F, Wolf E & Habermann FA 2011 Cell arrest and cell death in mammalian preimplantation development: lessons from the bovine model. *PLoS ONE* **6** e22121. (doi:10.1371/journal.pone.0022121)

Liao F, Liu M, Lv L & Dong W 2010 Hepatoma-derived growth factor promotes the resistance to anti-tumor effects of nordihydroguaiaretic acid in colorectal cancer cells. *European Journal of Pharmacology* **645** 55–62. (doi:10.1016/j.ejphar.2010.07.033)

Lim KT, Jang G, Ko KH, Lee WW, Park HJ, Kim JJ, Lee SH, Hwang WS, Lee BC & Kang SK 2007 Improved *in vitro* bovine embryo development and increased efficiency in producing viable calves using defined media. *Theriogenology* **67** 293–302. (doi:10.1016/j.theriogenology.2006.07.011)

Loureiro B, Bonilla L, Block J, Fear JM, Bonilla AQ & Hansen PJ 2009 Colony-stimulating factor 2 (CSF-2) improves development and post-transfer survival of bovine embryos produced *in vitro*. *Endocrinology* **150** 5046–5054. (doi:10.1210/en.2009-0481)

Loureiro B, Block J, Favoreto MG, Carambula S, Pennington KA, Ealy AD & Hansen PJ 2011 Consequences of conceptus exposure to colony-stimulating factor 2 on survival, elongation, interferon-τ secretion, and gene expression. *Reproduction* **141** 617–624. (doi:10.1530/REP-10-0511)

Madan P, Calder MD & Watson AJ 2005 Mitogen-activated protein kinase (MAPK) blockade of bovine preimplantation embryogenesis requires inhibition of both p38 and extracellular signal-regulated kinase (ERK) pathways. *Reproduction* **130** 41–51. (doi:10.1530/rep.1.00554)

Mansouri-Attia N, Aubert J, Reinaud P, Giraud-Delville C, Taghouti G, Galio L, Everts RE, Degrelle S, Richard C, Hue I *et al.* 2009 Gene expression profiles of bovine caruncular and intercaruncular endometrium at implantation. *Physiological Genomics* **39** 14–27. (doi:10.1152/physiolgenomics.90404.2008)

Mao J, Xu Z, Fang Y, Wang H, Xu J, Ye J, Zheng S & Zhu Y 2008 Hepatoma-derived growth factor involved in the carcinogenesis of gastric epithelial cells through promotion of cell proliferation by Erk1/2 activation. *Cancer Science* **99** 2120–2127. (doi:10.1111/j.1349-7006.2008.00928.x)

Mitko K, Ulbrich SE, Wenigerkind H, Sinowatz F, Blum H, Wolf E & Bauersachs S 2008 Dynamic changes in messenger RNA profiles of bovine endometrium during the oestrous cycle. *Reproduction* **135** 225–240. (doi:10.1530/REP-07-0415)

Muñoz M, Peirson SN, Hankins MW & Foster RG 2005 Long-term constant light induces constitutive elevated expression of mPER2 protein in the murine SCN: a molecular basis for Aschoff's rule? *Journal of Biological Rhythms* **20** 3–15. (doi:10.1177/0748730404272858)

Muñoz M, Rodriguez A, De Frutos C, Caamano JN, Diez C, Facal N & Gomez E 2008 Conventional pluripotency markers are unspecific for bovine embryonic-derived cell-lines. *Theriogenology* **69** 1159–1164. (doi:10.1016/j.theriogenology.2008.02.014)

Muñoz M, Corrales FJ, Caamaño JN, Díez C, Trigal B, Mora MI, Martín D, Carrocera S & Gómez E 2012 Proteome of the early embryo–maternal dialogue in the cattle uterus. *Journal of Proteome Research* **11** 751–766. (doi:10.1021/pr200969a)

Muñoz M, Uyar A, Correia E, Díez C, Fernandez-Gonzalez A, Caamaño JN, Trigal B, Carrocera S, Seli E & Gomez E 2014*a* Non-invasive assessment of embryonic sex in cattle by metabolic fingerprinting of *in vitro* culture medium. *Metabolomics* **443** 451. (doi:10.1007/s11306-013-0587-9)

Muñoz M, Uyar A, Correia E, Ponsart C, Guyader-Joly C, Martínez-Bello D, Marquant-Le Guienne B, Fernandez-Gonzalez A, Díez C, Caamaño JN *et al.* 2014*b* Metabolomic prediction of pregnancy viability in super-ovulated cattle embryos and recipients with Fourier transform infrared spectroscopy. *BioMed Research International* **2014** 608579. (doi:10.1155/2014/608579)

Muñoz M, Uyar A, Correia E, Díez C, Fernandez-Gonzalez A, Caamaño JN, Martínez-Bello D, Trigal B, Humblot P, Ponsart C *et al.* 2014*c* Prediction of pregnancy viability in bovine *in vitro* produced embryos and recipient plasma with Fourier transform infrared spectroscopy. *Journal of Dairy Science*. In press. (doi:10.3168/jds.2014-8067)

Nakamura H, Izumoto Y, Kambe H, Kuroda T, Mori T, Kawamura K, Yamamoto H & Kishimoto T 1994 Molecular cloning of complementary DNA for a novel human hepatoma-derived growth factor. Its homology with high mobility group-1 protein. *Journal of Biological Chemistry* **269** 25143–25149.

Okada M, Matsumori A, Ono K & Miyamoto T 1999 Hepatocyte growth factor is a major mediator in heparin-induced angiogenesis. *Biochemical and Biophysical Research Communications* **255** 80–87. (doi:10.1006/bbrc.1999.0150)

Oliver JA & Al-Awqati Q 1998 An endothelial growth factor involved in rat renal development. *Journal of Clinical Investigation* **102** 1208–1219. (doi:10.1172/JCI785)

Persaud SD, Lin YW, Wu CY, Kagechika H & Wei LN 2013 Cellular retinoic acid binding protein I mediates rapid non-canonical activation of ERK1/2 by all-*trans* retinoic acid. *Cellular Signalling* **25** 19–25. (doi:10.1016/j.cellsig.2012.09.002)

Rodríguez A, Diez C, Ikeda S, Royo LJ, Caamaño JN, Alonso-Montes C, Goyache F, Alvarez I, Facal N & Gomez E 2006 Retinoids during the *in vitro* transition from bovine morula to blastocyst. *Human Reproduction* **21** 2149–2157. (doi:10.1093/humrep/del099)

Rodríguez A, Díez C, Caamaño JN, de Frutos C, Royo LJ, Muñoz M, Ikeda S, Facal N, Alvarez-Viejo M & Gómez E 2007*a* Retinoid receptor-specific agonists regulate bovine *in vitro* early embryonic development, differentiation and expression of genes related to cell cycle arrest and apoptosis. *Theriogenology* **68** 1118–1127. (doi:10.1016/j.theriogenology.2007.08.007)

Rodríguez A, De Frutos C, Díez C, Caamaño JN, Facal N, Duque P, García-Ochoa C & Gómez E 2007*b* Effects of human versus mouse leukemia inhibitory factor on the *in vitro* development of bovine embryos. *Theriogenology* **67** 1092–1095. (doi:10.1016/j.theriogenology.2006.11.015)

Sakagami N, Umeki H, Nishino O, Uchiyama H, Ichikawa K, Takeshita K, Kaneko E, Akiyama K, Kobayashi S & Tamada H 2012 Normal calves produced after transfer of embryos cultured in a chemically defined medium supplemented with epidermal growth factor and insulin-like growth factor I following ovum pick up and *in vitro* fertilization in Japanese black cows. *Journal of Reproduction and Development* **58** 140–146. (doi:10.1262/jrd.11-050M)

Sasaki Y, Negishi H, Idogawa M, Yokota I, Koyama R, Kusano M, Suzuki H, Fujita M, Maruyama R, Toyota M *et al.* 2011 p53 negatively regulates the hepatoma growth factor HDGF. *Cancer Research* **71** 7038–7047. (doi:10.1158/0008-5472.CAN-11-1053)

Satterfield MC, Hayashi K, Song G, Black SG, Bazer FW & Spencer TE 2008 Progesterone regulates FGF10, MET, IGFBP1, and IGFBP3 in the endometrium of the ovine uterus. *Biology of Reproduction* **79** 1226–1236. (doi:10.1095/biolreprod.108.071787)

Thirant C, Galan-Moya EM, Dubois LG, Pinte S, Chafey P, Broussard C, Varlet P, Devaux B, Soncin F, Gavard J *et al.* 2012 Differential proteomic analysis of human glioblastoma and neural stem cells reveals HDGF as a novel angiogenic secreted factor. *Stem Cells* **30** 845–853. (doi:10.1002/stem.1062)

Trigal B, Gómez E, Díez C, Caamaño JN, Martín D, Carrocera S & Muñoz M 2011 *In vitro* development of bovine embryos cultured with activin A. *Theriogenology* **75** 584–588. (doi:10.1016/j.theriogenology.2010.09.010)

Trigal B, Gómez E, Caamaño JN, Muñoz M, Moreno J, Carrocera S, Martín D & Diez C 2012 *In vitro* and *in vivo* quality of bovine embryos *in vitro* produced with sex-sorted sperm. *Theriogenology* **78** 1465–1475. (doi:10.1016/j.theriogenology.2012.06.018)

Tsai HE, Wu JC, Kung ML, Liu LF, Kuo LH, Kuo HM, Chen SC, Chan EC, Wu CS, Tai MH *et al.* 2013 Up-regulation of hepatoma-derived growth factor facilities tumor progression in malignant melanoma. *PLoS ONE* **8** e59345. (doi:10.1371/journal.pone.0059345)

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A & Speleman F 2002 Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3** RESEARCH0034. (doi:10.1186/gb-2002-3-7-research0034)

Wang CH, Davamani F, Sue SC, Lee SC, Wu PL, Tang FM, Shih C, Huang TH & Wu WG 2011 Cell surface heparan sulfates mediate internalization of the PWWP/HATH domain of HDGF via macropinocytosis to fine-tune cell signalling processes involved in fibroblast cell migration. *Biochemical Journal* **433** 127–138. (doi:10.1042/BJ20100589)

Wydooghe E, Vandaele L, Beek J, Favoreel H, Heindryckx B, De Sutter P & Van Soom A 2001 Differential apoptotic staining of mammalian blastocysts based on double immunofluorescent CDX2 and active caspase-3 staining. *Analytical Biochemistry* **416** 228–230. (doi:10.1016/j.ab.2011.05.033)

Wydooghe E, Heras S, Dewulf J, Piepers S, Van den Abbeel E, De Sutter P, Vandaele L & Van Soom A 2013 Replacing serum in culture medium with albumin and insulin, transferrin and selenium is the key to successful bovine embryo development in individual culture. *Reproduction, Fertility, and Development* **26** 717–724. (doi:10.1071/RD13043)

Yamakoshi S, Bai R, Chaen T, Ideta A, Aoyagi Y, Sakurai T, Konno T & Imakawa K 2012 Expression of mesenchymal-related genes by the bovine trophectoderm following conceptus attachment to the endometrial epithelium. *Reproduction* **143** 377–387. (doi:10.1530/REP-11-0364)

Yang Y, Li H, Zhang F, Shi H, Zhen T, Dai S, Kang L, Liang Y, Wang J & Han A 2013 Clinical and biological significance of hepatoma-derived growth factor in Ewing's sarcoma. *Journal of Pathology* **231** 323–334.

Zhao J, Yu H, Lin L, Tu J, Cai L, Chen Y, Zhong F, Lin C, He F & Yang P 2011 Interactome study suggests multiple cellular functions of hepatoma-derived growth factor (HDGF). *Journal of Proteomics* **75** 588–602. (doi:10.1016/j.jprot.2011.08.021)

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CAPÍTULO 5 EMBRYONIC SEX INDUCES DIFFERENTIAL EXPRESSION OF PROTEINS IN BOVINE UTERINE FLUID

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RESUMEN EN CASTELLANO

El endometrio bovino reconoce a los embriones tempranos y reacciona de manera diferente dependiendo del potencial de desarrollo del embrión. Sin embargo, se desconoce si el endometrio puede distinguir el sexo del embrión. Nuestro objetivo fue analizar el dimorfismo sexual en el útero en respuesta a embriones macho y hembra. En el el fluido uterino (FU) se analizaron las proteínas expresadas diferencialmente (DE), los niveles de hexosas y las propiedades embriotróficas. Tras la transferencia de embriones macho o hembra a novillas en Día 5, el análisis proteómico del FU en Día 8 indentificó 23 proteínas DE. La regulación de las subunidades de la proteína proteasoma/inmunoproteasoma indicó diferencias en el procesamiento de antígenos entre FU portadores de embriones macho (FU-macho) o embriones hembra (FU-hembra). Varias enzimas involucradas en la glicolisis/gluconeogenésis y en respuestas antioxidativas/antiestrés fueron más abundantes en el FU-hembra. La concentración de fructosa fue mayor en el FU-hembra que en el FU-macho, mientras que los niveles de glucosa fueron similares. Los cultivos *in vitro* de embriones macho con moléculas aisladas del FU-macho mejoraron el desarrollo en comparación con los cultivos de embriones hembra con moléculas aisladas del FU-hembra. Según estos resultados, proponemos que en el animal vivo, los embriones macho inducen cambios en el endometrio para ayudar a asegurar su supervivencia. En contraste, los embriones hembra no parecen inducir tales cambios.

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Embryonic Sex Induces Differential Expression of Proteins in Bovine Uterine Fluid

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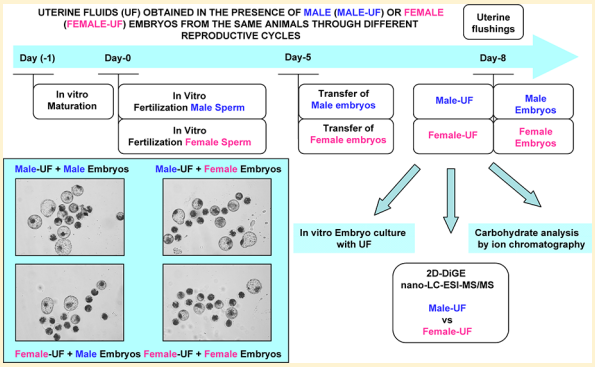
Supporting Information

ABSTRACT: The bovine endometrium recognizes early embryos and reacts differently depending on the developmental potential of the embryo. However, it is unknown whether the endometrium can distinguish embryonic sex. Our objective was to analyze sexual dimorphism in the uterus in response to male and female embryos. Differentially expressed (DE) proteins, different levels of hexoses, and other embryotrophic differences were analyzed in uterine fluid (UF). Proteomic analysis of day-8 UF recovered from heifers after the transfer of day-5 male or female embryos identified 23 DE proteins. Regulated proteasome/immunoproteasome protein subunits indicated differences in antigen processing between UF carrying male embryos (male-UF) or female embryos (female-UF). Several enzymes involved in glycolysis/gluconeogenesis and antioxidative/antistress responses were up-regulated in female-UF. Fructose concentration was increased in female-UF versus male-UF, while glucose levels were similar. *In vitro* cultures with molecules isolated from male-UF were found to improve male embryo development compared to female embryos cultured with molecules isolated from female-UF. We postulated that, *in vivo*, male embryos induce changes in the endometrium to help ensure their survival. In contrast, female embryos do not appear to induce these changes.



KEYWORDS: *bovine, embryo, sex, uterus, dimorphism, proteomics*

INTRODUCTION

As gestation progresses in mammals, the trophectoderm (TE) becomes a functional placenta that regulates exchanges between the embryo and the maternal blood. However, during oviductal and early uterine development, the mother and embryo enter into direct molecular "cross-talk", which includes different endometrial reactions depending on the potential of the embryo to develop to term and beyond.[1,2] This dialogue may affect the outcome of the pregnancy and have consequences in adulthood.[3−5]

In the cattle uterus, day-8 embryos overcome pro-inflammatory conditions by down-regulation of the nuclear factor kappa-B system (NFκB;[6]). This mechanism has also been described in pig embryos.[7] In rats, increased NFκB expression in the uterus is detrimental for embryonic development.[5] These experiments using pigs,[7] rats,[5] and cattle[6] were performed with multiple embryos in the uterus. The presence of tens of embryos led to detectable changes in bovine uterine fluid (UF) as determined by proteome analysis.[6] However, in cattle, a species that normally carries only one or two embryos, no studies have detected changes induced by embryos in endometrial gene expression before day 15.[8,9] Therefore, early endometrial responses to zona-enclosed embryos seem to be conserved among polytocus and monotocus species, despite the difference between cattle and pigs in the signaling molecules used for maternal recognition of pregnancy.[10]

During the cow preimplantation period, after embryonic genome activation and before X-chromosome inactivation,[11] both X chromosomes are active, with higher expression of X-linked and autosomal X-linked regulated genes in female embryos.[12] Thus, male and female embryos differ not only in their chromosomal complement but also in their epigenetic status[13] and transcriptional activity.[12,14−16] Sexually dimorphic transcription affects metabolism[17−19] and pregnancy recog-

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nition molecules, such as interferon tau (IFNT), which has a higher level of transcription and release in female embryos than in male embryos, both *in vivo* and *in vitro*.[20−22] Phenotypically, sexual dimorphism can be observed during *in vitro* development such that, in general, males cleave[23] and progress through blastulation faster than females.[19,24] However, suboptimal culture conditions, such as elevated glucose concentration, can bias embryo development in favor of females[25] or males[19,26,27] depending on the concentrations of the hexose in culture. In contrast, fructose neither skews the blastocyst sex ratio toward males[27] nor causes detrimental effects.[28] Interestingly, *in vivo*-derived male and female embryos do not show developmental differences.[29]

Mammalian development may undergo sex selection at several stages, from the sperm stage to late pregnancy, although convincing evidence of a biological mechanism is lacking.[11,30] A sex-selective embryo/fetal loss mechanism could initially require maternal recognition of the embryo gender.[11] The endometrium distinguishes between embryos based on their developmental potential,[1,2] and there is clear evidence of embryonic sexual dimorphism, so it is conceivable that males and females could be recognized in the uterus. Our objective in the present study was to analyze sexual dimorphism in the uterine fluid in response to male and female embryos.

We first compared the proteomes of UF collected from heifers following transfer on day 5 with either male or female embryos throughout nonconsecutive estrous cycles. After embryo and UF recovery on day 8, the proteomic profiles of the UF following exposure to either male or female embryos were compared, and DE proteins were identified. Subsequently, after functional UF analysis and endometrial morphological studies, we identified sexually dimorphic interactions between the uterus and the embryos and investigated the metabolic pathways involved.

■ MATERIALS AND METHODS

Animals and Embryos

All experimental procedures with animals were performed according to the European Community Directive 86/609/EC (Spanish Regulation 1201/2005) and were sanctioned by the Animal Research Ethics Committee of SERIDA. Procedures involving animal feeding and management, estrus synchronization and embryo transfer (ET), and progesterone (P4) blood sampling and analysis have been described elsewhere.[6]

Embryos were *in vitro* produced (IVP) with frozen male or female sex-sorted sperm (Sexing Technologies; Navasota, TX, USA) as previously reported.[31] Early morulae were transferred to the ipsilateral horn of estrus-synchronized, cross-bred beef heifers on day 5 after *in vitro* fertilization (IVF) and were recovered on day 8 (see below) or sustained *in vitro* up to day 8.

Uterine Flushings on Day 8

Recovery of uterine fluid and embryos has been described in detail elsewhere.[6] Briefly, the ipsilateral horns of recipients were first flushed with 45 mL of recovery medium (RM), consisting of PBS + 10 μL/mL protease inhibitor (Protease Arrest; GE Healthcare, Madrid, Spain). Recovery of diluted UF was performed by aspirating with syringe only while a steady flow could be achieved. Those recipients transferred with embryos were then extensively flushed with PBS + 1 mg/mL poly vinyl-pyrrolidone. The embryos were identified using a stereomicroscope and were rapidly separated from the UF, which was centrifuged (2000*g*) at 4 °C, aliquoted, and stored at −145 °C.

Two-Dimensional Difference Gel Electrophoresis (2D-DIGE) and Imaging

All proteomic procedures were conducted at the Proteomics Core Facility of the Center for Medical Applied Research (CIMA), an affiliate of ProteoRed, the Spanish National Institute of Proteomic Facilities.

2D-DIGE analysis was performed with paired male-UF and female-UF samples that contained blastocysts upon collection ($n = 6 \times 2$ samples). UF samples were processed through Concentrators Spin 5K MWCO 4 mL (Agilent Technologies) with 200 μL of 3.5 M urea, 1 M thiourea, 2% CHAPS, and 20 mM DTT at 5,000*g* and 12 °C. Protein concentrations were determined using the Bradford assay (Bio-Rad). Proteins were precipitated using a Clean Up kit (Bio-Rad). Fifty micrograms of protein was labeled with 400 pmol of CyDye DIGE Fluor minimal dyes (GE Healthcare) and incubated on ice in the dark for 30 min, according to the manufacturer's instructions. Cy3 and Cy5 were used with the samples; Cy2 was used with an internal control mixture composed of equal amounts of protein from all samples. Paired samples were reverse-labeled to prevent potential dye-labeling bias. The reaction was stopped by the addition of 1 μL of 10 mM lysine and then incubated on ice for 10 min. Samples were cup-loaded onto IPG strips (24 cm long, pH 3−11 NL, from GE Healthcare) and subjected to isoelectrofocusing in an IPGphor IEF System (GE Healthcare) according to the manufacturer's recommendations. Upon completion of IEF, the strips were incubated for 15 min in equilibration buffer (50 mM Tris-HCl at pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and a trace of bromophenol blue) containing 0.5% DTT and were subsequently incubated for 15 min in equilibration buffer with 4.5% iodoacetamide. For the second dimension, strips were loaded onto 12.5% polyacrylamide gels and run at 1 W/gel for 12−14 h, until the bromophenol blue reached the bottom of the gel. Subsequently, 2D gels were scanned using a Typhoon Trio Imager (GE Healthcare) at 100-μm resolution with a $\lambda_{ex}/\lambda_{em}$ ratio of 488/520 nm for Cy2, 532/580 nm for Cy3, and 633/670 nm for Cy5. The photomultiplier tube was set to ensure that the maximum pixel intensity was between 90,000 and 99,000 pixels. Image analysis was performed using DeCyder 6.5 software (GE Healthcare) as specified in the user's manual and briefly summarized below. The differential in-gel analysis (DIA) module was used for spot detection, spot volume quantification, and volume ratio normalization of different samples in the same gel statistically. The Biological Variation Analysis (BVA) module was then used to match protein spots on different gels and identify protein spots with statiscally significant differences. Manual editing was performed in the BVA module to ensure that spots were correctly matched on different gels and to remove streaks and speckles. Differentially expressed spots were considered for mass spectrometry (MS) analysis if the corresponding *t* test *p*-value was <0.05. To account for the problem of multiple hypotheses testing, *q*-values, representing the false discovery rate (FDR) adjusted *p*-values, were determined for each matched spot using the Benjamini−Hochberg correction from Decyder at a 0.20 cutt-off. Differences in standardized abundance between protein spots were expressed as average ratio (av ratio, fold change).

Proteins were visualized by staining with SYPRO Ruby Protein Gel Stain (Bio-Rad), and images were acquired with a

Typhoon Trio Imager at 100-μm resolution using a $\lambda_{ex}/\lambda_{em}$ ratio of 532/560 nm. The total number of spots detected in the 2D-DIGE experiments was n = 3274 ± 181. Seven biological replicates were used for image analysis. The desired spots were manually excised, and gel specimens were processed with a MassPrep station (Waters) as described elsewhere.[32] In-gel tryptic digestion was performed with 12.5 ng/μL trypsin in 50 mM ammonium bicarbonate for 12 h at 37 °C. The resulting peptides were extracted with 1% formic acid and 50% acetonitrile. Samples were then concentrated in a speed-vac before MS analysis.

LC–ESI-MS/MS Analysis

Microcapillary reverse-phase LC was performed with a CapLC (Waters) capillary system. Reverse-phase separation of trypsin digests was performed with an Atlantis, C18, 3 μm, 75 μm × 10 cm Nano Ease fused silica capillary column (Waters) in Solvent A (95% water/5% acetonitrile/0.2% formic acid). After injection of 6 μL of sample, the column was washed for 5 min with Solvent A, and the peptides were eluted using a linear gradient of 5–50% acetonitrile over a period of 30 min at a constant flow rate of 0.2 μL/min. The column was coupled online with a Q-TOF Micro (Waters) using a PicoTip nanospray ionization source (Waters). The heated capillary temperature was 80 °C, and the spray voltage was 1.8–2.2 kV. MS/MS data were collected in an automated, data-dependent mode. The three most intense ions in each survey scan were sequentially fragmented by collision-induced dissociation (CID) using an isolation width of 2.5 and a relative collision energy of 35%. Data processing was performed with MassLynx 4.0. Database searching was conducted with Phenyx 2.2 (GeneBio) against the Uniprot knowledgebase Release 12.3, consisting of UniprotKB/Swiss-Prot Release 54.3 and UniprotKB/TrEMBL Release 37.3, with 285,335 and 4,932,421 entries, respectively. Phenyx incorporates the true probabilistic and flexible scoring system "OLAV" developed at GeneProt, Inc.[33] The search was enzymatically constrained for trypsin and allowed for one missed cleavage site. Further search parameters were as follows: minimum significant scores for Phenyx were 5 and 6 for peptides and proteins, respectively; the mass error tolerance was 50 ppm for parent and 0.1 Da for fragments; there were no restrictions on molecular weight or isoelectric point; the fixed modification was carbamidomethylation of cysteine; and the variable modification was oxidation of methionine. To integrate the results into a general model, DE proteins were classified according to Gene Ontology (GO), and functional associations were investigated using the Ingenuity Pathway Analysis Network (IPA).

Western Blotting (WB) in Uterine Fluids

Targeted proteins were detected following previously described procedures[6] with antibodies raised against bovine proteins (NFκB-P65, HSPA5) or proteins from other species sharing high sequence identity with the bovine proteins (clusterin, immunoproteasome 20S Beta5i subunit, TXN and PARK7). Briefly, 5 μg of UF protein per biological replicate was boiled in SDS sample buffer for 5 min and electrophoresed on 4–20% SDS-PAGE gels for 120 min at 80 V. Separated proteins were then electrically (0.150 A, 120 min) transferred to a PVDF membrane. Antisera specific to (1) NFκB, 2 μg/mL (ab72555; Abcam); (2) clusterin, 2 μg/mL (Sc-5289; Santa Cruz Biotechnologies, Santa Cruz); (3) HSPA5, 1 μg/mL (ab3148; Abcam); (4) immunoproteasome 20S β5i subunit, 1:10000 (BML-PW8355; Enzo Life Sciences, Axxora); (5) TXN, 1:1500 (ab86255; Abcam); and (6) PARK7/DJ1, 1:2000 (ab131591; Abcam) were used at the previously stated dilutions. Protein concentrations were quantified by scanning densitometry (Image J) for 3 replicates. Differences in mean protein abundance were analyzed and expressed as fold change (LSM ± SE). Equal loading of the gels was assessed by Coomassie staining.

Carbohydrate Analysis by Ion Chromatography

Glucose and fructose concentrations were analyzed in male-UF and female-UF by an ion chromatography method. We used an ion chromatography system, the Dionex ICS-5000 (Thermo Scientific Dionex), comprising a metal-free gradient pump coupled to a pulsed amperometric detector. Samples (100 μL) were diluted with 380 μL of ultrapure water, 10 μL of Carrez I solution, and 10 μL of Carrez II solution. After centrifugation and filtration (0.22 μm), samples were injected with a metal-free rotary injection valve (Dionex) equipped with a 10 μL injection loop, and sample fractionation was performed with a CarboPac PA20 anion-exchange column (150 mm × 3 mm i.d.) coupled to a guard column (30 mm × 3 m i.d.). The flow-through detection cell was made from a gold working electrode and a pH-AglAgCl combination reference electrode; the titanium body of the cell served as a counter electrode. The potential waveform parameters applied at the gold working electrode were adapted from Rocklin and co-workers.[35] The column and detector were maintained at 30 °C. The gradient conditions are shown in Supporting Information.

Isolation of High Molecular Weight Factors from Uterine Fluid for *in Vitro* Embryo Culture

A functional analysis of UFs was performed from day 5 to day 8 in *in vitro* culture with male and female embryos in synthetic oviduct fluid (SOF) containing a >3-kDa dialysate from three paired male-UF and female-UF samples

High-MW uterine factors were isolated from frozen/thawed UF samples by 3-kDa dialyzation against SOF, as described by Muñoz et al.[6] Retentates were adjusted to an estimated final protein concentration of 1.0 mg/mL (SOF-UF). Washing droplets (2 × 150 μL) and a culture droplet (50 μL) of media were layered under mineral oil, and day-5 IVP morulae were cultured up to day 8. Blastocyst development was recorded on day 8.

Blastocysts Differential Cell Counts

Embryonic cells were differentially counted in the inner cell mass (ICM) and the trophectoderm of day-8 expanded and hatched blastocysts with a propidium iodide/bisbenzimide based method.[36]

Caspase-3 Staining in Blastocysts

Following a procedure modified from Wydooghe and co-workers,[37] day-8 blastocysts were fixed in 4% paraformaldehyde and stored in phosphate-buffered saline (PBS) containing 0.2 mg/mL polyvinyl alcohol (PBS–PVA) at 4 °C until staining. The blastocysts were permeabilized overnight in 0.5% Triton X-100 and 0.05% Tween-20 in PBS at 4 °C. On the second day, the DNA of blastomeres was denatured by exposure to 2 N HCl in PBS-PVA. After denaturation, the embryos were transferred to a blocking solution. Blocking occurred overnight in 10% goat serum (S-1000, Vectorlabs) and 0.05% Tween-20 in PBS at 4 °C. The target embryos were incubated overnight at 4 °C with a ready-to-use primary rabbit active caspase-3 antibody (no. 9661, Cell Signaling Technology, IZASA) while embryos serving as negative controls were kept in the blocking

Table 1. Description and Identification of 23 Proteins Found to Be Present in Different Amounts in Day-8 Uterine Fluid That Contained Male or Female Embryos[a]

spot	q values	t test[b]	av ratio[c]	ID	AC	network	score[d]	peptides[e]	% cov[f]	pI[g]	M_w[h]
645	20	0.022	1.5	78 kDa glucose-regulated protein	Q0VCX	HSPA5	114.5	13	26.6	5.09	72,400
941	20	0.01	1.34	glucose-6-phosphate isomerase	Q3ZBD7	GPI	80.4	14	22.0	7.75	62,871
1101	13	0.016	1.27	alpha-enolase	Q9XSJ4	ENO1	183.5	18	38.6	6.63	47,194
1186	17	0.047	1.25	tryptophan-tRNA ligase	P17248	WARS	83.7	11	31.7	5.57	53,182
1211	11	0.005	1.47	40S ribosomal protein SA	P26452	RPSA	113.0	11	42.0	4.85	32,884
1329	14	0.02	1.38	poly(rC)-binding protein 1	Q5E9A3	PCPB1	63.5	8	28.1	7.03	37,497
1429	12	0.012	1.45	annexin A2	P04272	ANXA2	140.2	15	46.4	7.53	38,480
1439	13	0.019	1.51	annexin A5	P81287	ANXA5	10.7	2	6.5	4.88	36,088
1440	14	0.022	1.33	elongation factor 1-delta	A5D989	EEF1D	13.4	2	8.6	4.99	31,141
1547	16	0.042	1.33	actin-related protein 2/3 complex subunit 2	Q3MHR7	ARPC2	49.1	7	23.3	7.19	34,349
1594	20	0.046	1.24	dimethylarginine dimethylaminohydrolase 2	Q3SX44	DDAH2	74.1	9	43.5	5.82	29,780
1617	12	0.013	1.35	chloride intracellular channel protein 1	Q5E9B7	CLIC1	119.5	13	66.2	5.27	26,991
1649	17	0.047	1.31	proteasome activator complex subunit 1	Q4U5R3	PSME1	92.2	10	40.2	5.97	28,663
1664	11	0.006	1.38	proteasome subunit alpha type-3	Q58DU5	PSMA3	39.8	5	23.6	5.28	28,273
1682	19	0.047	1.28	beta-enolase	Q3ZC09	ENO3	23.5	3	10.8	8.06[I]	47,096[j]
1717	16	0.039	1.69	phosphoglycerate mutase 1	Q3SZ62	PGAM1	28.8	4	19.8	7.25	28,720
1748	18	0.049	1.28	Peroxiredoxin-6	O77834	PRDX6	76.6	10	37.2	6.43	24,935
1865	17	0.046	1.26	glutathione *S*-transferase P	P28801	GSTP1	132.6	14	52.4	7.65	23,613
1872	9	0.002	1.54	protein DJ-1	Q5E946	PARK7	34.3	4	34.9	7.68	20,035
1892	19	0.047	1.39	peroxiredoxin-2	Q9BGI3	PRDX2	63.1	8	37.2	5.54	21,946
1927	9	0.002	1.44	proteasome subunit beta type-2	Q5E9K0	PSMB2	18.6	3	16.9	6.96	22,896
2492	11	0.01	1.46	peroxiredoxin-5, mitochondrial	Q9BGI1	PRDX5	44.9	6	39.5	6.30	17,362
2834	11	0.005	1.56	thioredoxin	O97680	TXN	19.1	2	21.0	5.16	11,812

[a]Positive average ratio indicate protein up-regulation in female uterine fluid. [b]Student *t* test *P*-value. [c]Average volume ratio as calculated by the DeCyder BVA analysis. [d]Phenyx score. [e]Number of matched peptides. [f]Percent protein sequence coverage for the most probable candidate as provided by Phenyx. [g]Theoretical pI. [h]Theoretical molecular weight. [I]Theoretical and experimental pI values differed. [j]Theoretical and experimental MW values differed.

solution. After washing, target embryos and negative controls were transferred to goat anti-rabbit Alexa 488 antibody (in blocking solution, Molecular Probes, Invitrogen) for 2 h at RT. Finally, the embryos were washed and transferred to Vectashield mounting media with DAPI. Evaluation of the embryos was performed by confocal fluorescence microscopy (Leica Confocal microscope TCS SP2 AOBS). The staining showed a clear detection of apoptotic cells by caspase positivity in the cytoplasm. Negative control embryos showed no positive staining.

Endometrial Localization of Proteins by Immunocytochemical Analysis (ICQ)

Animals on day 8 of the estrous cycle were sacrificed in a nearby slaughterhouse. The genital apparatus was transported to the laboratory at 4 °C. After visual inspection and corpus luteum assignment, the ipsilateral horn was dissected from adjacent tissues, clamped at the utero-tubal junction, and ligated at the base. The horn was flushed 5 times with PBS and longitudinally dissected, and the medial and cranial regions were marked and separated with a scalpel. Endometrial samples (~1 cm^2) were taken from the caruncular and intercaruncular regions, fixed in 4% paraformaldehyde in PBS (pH 7.2) overnight and then embedded in paraffin.

Cell-specific expression of various targeted proteins was detected in endometrial cross sections following a classical immunostaining protocol[34] using primary antibodies at the stated dilutions: anti-NFκB, 5 μg/mL (ab72555; Abcam); (2) anti-clusterin, 4 μg/mL (Sc-5289; Santa Cruz Biotechnologies); (3) anti-HSPA5, 6 μg/mL (ab3148; Abcam); (4) anti-immunoproteasome 20S βi subunit, 1:7000 (BML-PW8355; Enzo Life Sciences); (5) anti-TXN, 1:1500 (ab86255; Abcam); and (6) Anti-PARK7/DJ1, 1:2000 (ab131591; Abcam).

Immunostained sections were then rinsed in tap water, counterstained using modified Harris Hematoxylin solution (AHHSS16, Sigma-Aldrich) and Eosin Yellowish hydro-alcoholic solution (251301.1609, Panreac), cleared, and mounted.

Images of representative fields were recorded under bright-field illumination using an Olympus BX51 microscope fitted with an Olympus DP70 digital camera. Positive immunostaining was scored semiquantitatively using two independent observers blind to the nature of the tissue. Negative control sections showed no positive staining.

Experimental Design

Recipients (n = 7) were subjected to multiple ET[6] with either male or female day-5 IVP morulae (n = 37.8 on average) within nonconsecutive estrous cycles. Four recipients received males first, 3 recipients received females first and sham transfers were alternated with ETs. The use of the same animals prevented genetic variability. The animals were flushed on day 8 with 45 mL of recovery medium, consisting of PBS + 10 μL/mL protease inhibitor. Diluted UFs with live male or female embryos were recovered from all animals and then analyzed for 2D-DIGE once the embryos were removed. Total protein was measured in UFs. P4 was measured on days 5 and 8 in plasma samples.

A 2D-DIGE analysis was performed only with paired male-UF and female-UF samples that contained blastocysts upon collection (n = 6 × 2 samples). Proteins were labeled, mixed, and analyzed by DIGE. Protein spots showing differential

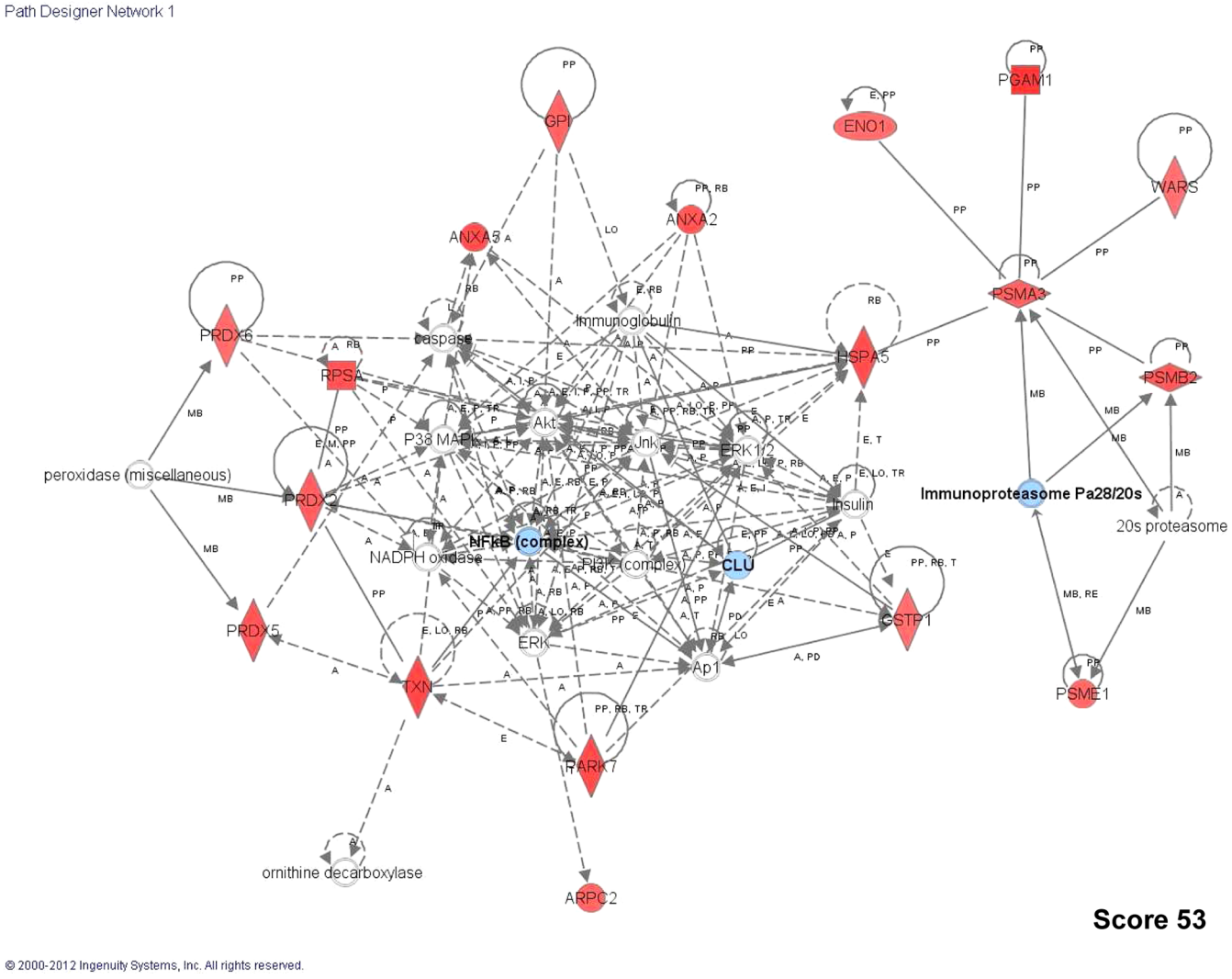

Figure 1. Functional clustering of n = 18 differentially expressed proteins in uterine fluid containing male or female embryos. Proteomic data were grouped according to Ingenuity interaction parameters (score network = 53). The selected proteins correspond to nodes, which are represented by different symbols. Red up-regulated proteins in female compared to male-UF. Blue nodes represent proteins (nuclear factor beta-kappa complex (NFκB complex), clusterin (CLU), and immunoproteasome Pa28/20S) not identified by 2D-DIGE-MS but included in the molecular network generated by a Core Analysis-IPA. As candidates to be involved in the observed effects, such proteins were validated by Western blotting. The nodes are interconnected by specific connectors according to the type of interaction between the linked nodes.

expression were identified by nano-LC−ESI-MS/MS. To integrate the results into a general model, DE proteins were classified according to Gene Ontology, and functional associations were investigated using the IPA Network.

Subsequently, glucose and fructose concentrations were analyzed in male-UF and female-UF.

A functional analysis of UFs was performed from day 5 to day 8 in *in vitro* culture with male and female embryos in synthetic oviduct fluid containing a >3-kDa dialysate from three paired male-UF and female-UF samples. On day 8, blastocysts were subjected to differential cell counts or analyzed for apoptosis by caspase-3 staining

Flushed animals were subjected to either AI or ET to confirm that their ability to become pregnant was unaffected after the experiments. Finally, synchronized heifers (n = 3) carrying multiple embryos were sacrificed on day 8, and the endometrial localization of 3 DE proteins and 3 proteins not identified by DIGE but shown in the IPA network was assessed by immunocytochemical analysis (adapted from ref 34)

Statistical Analysis

Sources of variation affecting total recoverable protein, P4, abundance of single and total DE proteins, glucose and fructose concentrations, embryo recovery, embryo development, cell counts and apoptosis were identified by fitting linear models using the Proc GLM module of SAS/STAT (Version 9.2; SAS Institute Inc., Cary, NC). The models included the following fixed effects: embryonic sex within UF, animal, and replicate. Least squares means (LSM) and their errors (±SEM) were estimated for each level of fixed effects with a significant F value. The Ryan−Einot−Gabriel−Welsch q-test was used to compare the raw means of the levels from the fixed effects.

■ RESULTS

Proteomics

The proteomes of UF from heifers exposed to male or female embryos were compared. Quantitative proteomic analysis (2D-DIGE-MS) identified 23 up-regulated proteins in the female-UF compared to the male-UF. A description of the regulated

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proteins is shown in Table 1. Proteasome/immunoproteasome proteins (PSME1, PSMA3, and PSMB2), antioxidative/anti-stress response proteins (PRDX2, PRDX5, PRDX6, TXN, GSTP1, and HSPA5), and several enzymes involved in glycolysis (ENO1, ENO3, GPI, and PGAM1) were up-regulated in female-UF. No protein was found up-regulated in male-UF.

Integrated functional pathway analysis of the DE proteins was performed using IPA. IPA generated a network with a high score of 53 (Figure 1), and a functional element, the NFκB complex, was directly targeted by 2 DE proteins, suggesting its involvement in sex-mediated effects. The activation of proteasome/immunoproteasome (P/IP) subunits suggests sex differences in recognition and antigen processing. Associated molecular and cellular network functions, as well as the main canonical pathways, are shown in Figure 2A and B, respectively.

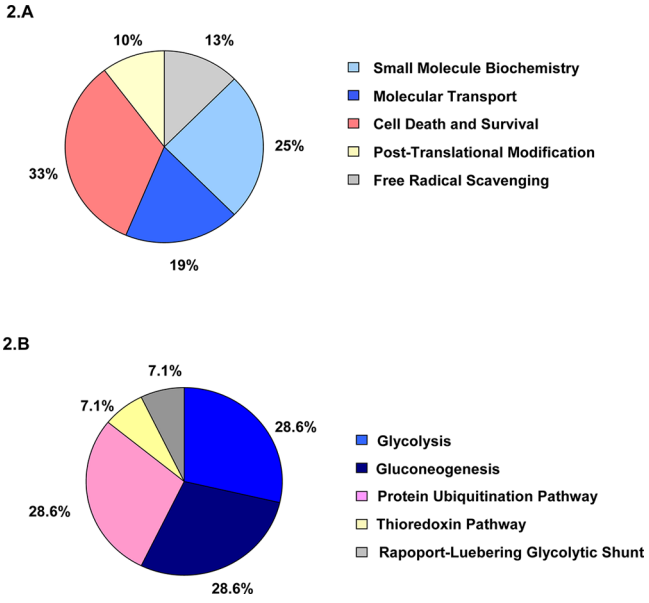

Figure 2. Broad classification of the proteins differentially expressed in uterine fluid that contained male or female embryos according to (A) their molecular and cellular functions or to (B) the main canonical pathways represented.

To confirm the results obtained by DIGE, 3 DE proteins (HSPA5, PARK7, and TXN) and 3 proteins not identified by DIGE but shown in the IPA network as likely to be involved in the observed effects were examined by Western blotting (Table 2). The latter proteins included subunit p65 of the NFκB complex, which is involved in early embryo endometrial interactions;[6] immunoproteasome subunit 20S (β5i) [I-20S], which distinguishes proteasome activity from immunoproteasome activity; and clusterin, which plays a role in hexose metabolism, antioxidative activity, and protease activity. The selected proteins were identified and quantified in male- and female-UF (Table 2). The validation data for HSPA5 (up-regulated; $p < 0.05$), PARK7 (up-regulated; $p < 0.05$), and TXN (up-regulated; $p < 0.01$) were consistent with the DIGE data for female-UF (Table 2). I-20S and NFκB were not significantly regulated ($p = 0.35$, and $p = 0.49$, respectively). In male-UF, the clusterin pro-form and the mature form of clusterin (36−39 kDa) were up-regulated ($p = 0.03$, both isoforms). All of the selected proteins were identified in uterine necropsies by ICQ in endometrial glands and other locations (Figure 3). These results suggest that although not all proteins in the UF have a uterine origin, those that differentially respond to male and female embryos, as detected by DIGE, are localized to the endometrium.

Glucose and Fructose Concentrations in Male-UF and Female-UF

Male and female embryos cultured *in vitro* show differences in glucose metabolism, although fructose metabolism did not seem to differ. We analyzed the concentrations of D-glucose and D-fructose. Measurements were normalized by total protein, and glucose was detected in equal concentrations in male-UF and female-UF (Table 3). Fructose, however, was found to be increased in female-UF. This increase is consistent with the decreased glucose/fructose ratio found in the raw data (i.e., un-normalized data).

Embryo Development

Recovered UF factors conditioned by live embryos are embryotrophic *in vitro*.[6] In this work, development rates did not differ between male and female embryos that were not exposed to UF molecules (i.e., entirely cultured with BSA) (Table 4) or those that developed *in vivo*, as determined by uterine recovery rates (Table 4). However, *in vitro* exposure of male embryos to male-UF significantly improved blastocyst ($p < 0.01$) and expanded blastocyst ($p < 0.01$) rates (Table 5). These effects were associated with UF origin ($p < 0.01$) and embryonic sex ($p < 0.01$). *In vitro* culture in SOF-UF decreased development in females compared to males.

Table 2. WB Validation of Proteomic Data[a]

	WB				DIGE	
	LSM ± SEM					
protein	female-UF	male-UF	*P* value	ratio female/male	ratio female/male	*P* value
HSPA5	35.29 ± 5.6	20.44 ± 4.07	0.04	1.68	1.50	0.022
PARK7	11.76 ± 0.63	9.63 ± 0.63	0.04	1.26	1.54	0.002
TXN	68.29 ± 3.83	32.33 ± 3.83	0.002	3.51	1.56	0.005
I-20S	31.66 ± 4.5	25.99 ± 3.27	0.35	1.27		
clusterin proform	40.67 ± 3.68	52.70 ± 2.67	0.03	−1.16		
clusterin mature	22.99 ± 6.11	43.72 ± 3.45	0.03	−1.51		
NFκB	30.20 ± 5.35	35.06 ± 3.87	0.49	1.03		

[a]Differences in mean protein abundance between uterine fluid that carried female embryos (female-UF) and uterine fluid that carried male embryos (male-UF) were analyzed and expressed as fold change (LSM ± SE). Proteins up-regulated in female-UF show positive values, while proteins down-regulated show negative values.

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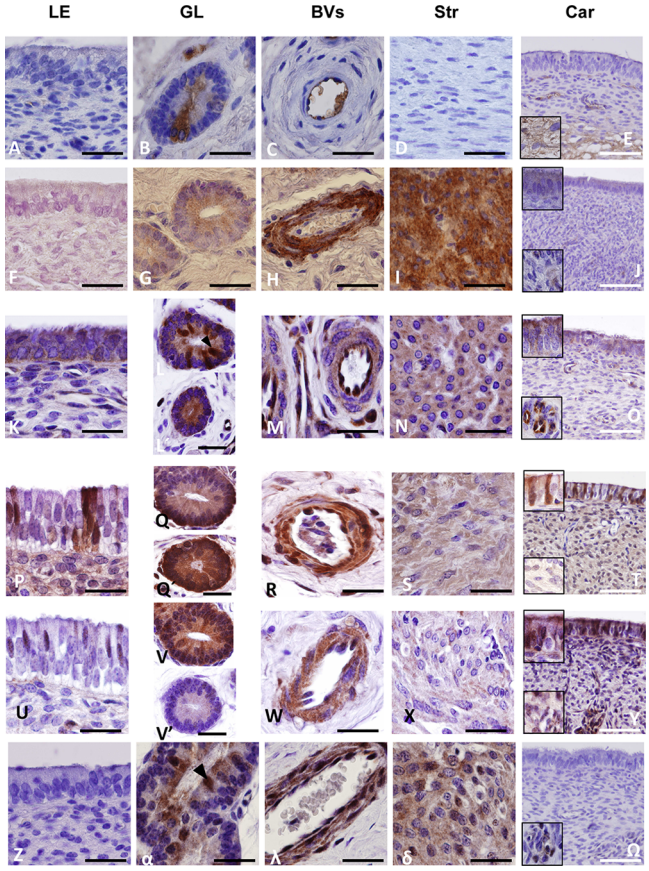

Figure 3. Immunocytochemical staining of clusterin (A-E), NFκB (F–J), immunoproteasome subunit 20S (β5i) (K–O), PARK7 (P–T), TXN (U–Y), and HSPA5 (Z–Ω) performed on individual endometrial samples taken from slaughtered cows. Positive labeling of glandular (B, G, L, L′, Q, Q′, V, α) and/or luminal epithelium, (K, P, U) cells suggests that these proteins may all be secreted into the uterine lumen. Immunoproteasome subunit 20S (β5i) staining in glandular epithelium is frequently present in vesicles (L, black arrowhead). PARK7 and TXN staining patterns differ between the glandular epithelium from functional (Q, V) and basal (Q′, V′) layers. No differences in staining patterns were found between the caruncular (E, J, O, T, Y, Ω) and intercaruncular (A–D, F–I, K–N, P–S, U–X, Z–δ) areas. Black scale bars = 25 μm; white scale bars = 50 μm. No staining was observed when the primary antibody was omitted (data not shown). LE, luminal epithelium; GL, endometrial glands; BVs, blood vessels; Str, deep stroma; Car, caruncle.

Table 3. Glucose and Fructose Concentrations Analyzed in Male Uterine Fluid and Female Uterine Fluid by Ion Chromatography

embryos in uterus	N^a	glucose (μg/mL)	fructose^b (μg/mL)	glucose/fructose^b
male	13	13.5 ± 2.1	1.5 ± 0.4^a	9.5 ± 1.2^a
female	12	13.0 ± 2.7	2.7 ± 0.4^b	5.8 ± 1.4^b

^{a}N = number of uterine fluid samples. ^{b}Superscripts (a, b) indicate significant differences ($p < 0.05$).

Combinations of male- and female-UF with male and female embryos had no effect on cell counts (Table 6), although the cumulative data showed significantly higher ($p < 0.05$) cell numbers in the TE of males in comparison to females. Interestingly, female embryos cultured in female-UF had a higher incidence of apoptosis in the ICM than male embryos cultured in male-UF ($p < 0.05$) (Table 6). Within the ICM, this

Table 4. Effects of Embryonic Sex on *in Vitro* Development, Embryo Recovery Rates from Host-Recipients, Protein Contents in Flushes, and P4 Concentration Increase from Day 5 (Embryo Transfer) to Day 8 (Flushing)

	in vitro development					uterine recovery (*in vivo* development)						
			Day 6	Day 8			embryos^f (%)		flushed protein^g			
sex	N^a	R^b	% morulae	% blastocysts	R^c	N^d	flushed volume^e	total	blastocysts	μg/100 μL	total (μg)	P4 (ng/mL) Day 8–Day 5
female	527	7	29.9 ± 2.6	12.6 ± 1.7	7	37.8	60.9 ± 2.3	12.0 (27.7 ± 3.3)	6.2 (14.2 ± 2.3)	9.2 ± 2.5^x	2,563 ± 920^a	11.9 ± 3.5
male	769	7	30.0 ± 2.0	10.9 ± 1.3	7	37.8	57.8 ± 2.9	7.4 (18.6 ± 2.5)	4.6 (11.6 ± 1.7)	17.2 ± 2.1	4,892 ± 841	8.6 ± 3.6
sham					12					24.4 ± 2.2^y	6,375 ± 814^b	12.0 ± 2.3

^{a}Number of fertilized oocytes. ^{b}Replicates. ^{c}Replicates over 7 animals. ^{d}Number of day-5 IVP morulae transferred per flushing (range: males, 29–44; females, 23–51). ^{e}As a proportion of infused volume (45 mL). ^{f}In parentheses, as a percentage of transferred morulae. ^{g}Superscripts indicate significant differences; a, b: $p < 0.05$; x, y: $p < 0.01$.

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Table 5. *In Vitro* Development within Blastocysts Derived of Day-5 IVP Morulae Cultured in mSOF with a Dyalizate of Flushes Recovered on Day 8 from Uterus Containing Male and Female Embryos[a]

uterine fluid containing embryos	sex of embryos in culture	N^b	R^c	day-8 blastocysts[d] (%) blastocysts	expanded
male	male	201	10	57.5 ± 3.1^{x}	49.7 ± 3.2^{xa}
female	male	181	9	50.0 ± 3.3	37.0 ± 3.3^{b}
male	female	195	10	40.7 ± 3.1^{y}	30.1 ± 3.2^{y}
female	female	178	9	35.5 ± 3.3^{y}	25.1 ± 3.3^{y}

[a]Data are LSM ± SE. Flushes with male and female embryos were taken from 3 cows and 2 bulls. [b]Number of day-5 cultured morulae. [c]Number of replicates. [d]Superscripts indicate significant differences: x, y: $p < 0.01$; a, b: $p < 0.05$.

effect was the result of specific interactions between the embryonic sex and its cognate sex-conditioned environment; UF and embryonic sex alone had no effect on apoptosis. In contrast, within the TE, males showed lower apoptosis rates than females ($p < 0.05$).

The procedures used did not affect the capacity of animals to become pregnant; 6 out of 7 heifers delivered healthy calves. The P4 increase during embryo development in the uterus did not differ ($p = 0.23$) between males and females. Interestingly, recoverable total protein in the female-UF decreased both in concentration ($p < 0.05$) and in total protein ($p < 0.01$) compared to the sham control (Table 4).

DISCUSSION

Uterine fluid is a highly dynamic environment containing blood plasma, endometrial proteins, and epithelial cells, which under constant renewal are continuously extruded into the lumen.[38] In our experimental context, DE UF proteins seem to be mostly a product of local responses within an embryo-maternal "cross-talk". Thus, our UF proteome analysis may reliably reflect endometrial reproductive processes.[6,39] In this work, a pathway analysis of DE proteins, a representative sample set of which was effectively localized to endometrial cells, provided evidence of uterine recognition of embryonic sex. The pathway differences included regulation in antigen processing, biosynthesis, antioxidation, apoptosis, glycolysis, and functional properties of the UF. In addition, recent work shows that some of our UF DE proteins (PRDX2, 5 and 6; TXN; DJ-1; PGAM1; and ENO1) were also found in plasma and regulated by the endometrium in the UF.[40]

Sexual dimorphism involving antigen processing in the uterus is linked to embryonic recognition. The abundance of PSMA3, PSME1, and PSMB2, components of the P/IP, and DJ-1, a transcriptional regulator that prevents proteasome inhibition,[41] supports the observation of increased P/IP activity in female-UF. In the uterus, the P/IP proteolysis of paternal embryonic antigens generates peptides that can be presented by maternal MHC class I molecules, increasing the risk of rejection.[42] Thus, the presentation of different antigens by males and females would involve dimorphic proteolysis and would in turn cause differences in embryo tolerance. IPs form upon exposure of cells to proinflammatory cytokines (IFNγ, IFNα, IFNβ, and TNFα), NO, H_2O_2, and hyperglycemia.[43,44] TNF-α, which can be produced by the embryo,[6] mediates the expression of IFN-stimulated endometrial genes and indirectly interacts with the IP and MHC class 1 complex.[8] In our work, the exclusive IP subunit I-20S was not differentially expressed, which might indicate that sex recognition operates through constitutively expressed proteasomes. However, IP transient induction might be difficult to detect, as the IP shows a much shorter half-life than the standard P to permit cells to return to a normal situation once the IP is no longer required.[45] Furthermore, in cells that express both the inducible and constitutive β-subunits, which make up the catalytic core of the IP/P, immunosubunits are preferentially assembled.[45,46] In our study, upregulation of PSME1 (PA28α), a subunit of the PA28α/β complex (11S regulator complex subunit alpha) that associates to IPs upon IFN-γ treatment,[47] suggests a role for IPs. Therefore, we should not discard some maternal responses to male or female embryos involving IPs, although constitutive activity was conducted by Ps. In ruminants, it is unclear whether the antiluteolytic IFNT induces IP formation. If so, the distinct IFNT levels of males and females[20−22] may explain the dimorphism in the context of P/IP proteins.

IP inducers can also induce NFκB, a molecule found not regulated by WB. Thus, the absence of regulation we observed for both I-20S and NFκB are consistent. NFκB was localized to the endometrial glands, endothelium, stroma and UF. In cattle and pig uteri,[6,7] NFκB down-regulation could explain how the embryo induces a developmentally improved local environment.[6] In contrast, male and female embryos seem to modify the UF through mechanisms that do not involve participation of NFκB. In cattle, female embryos overexpress *NKRF*, an NFκB transcriptional repressor[12] that can diminish the effects of NFκB inducers in UF upon binding to embryos. Thus, females would not need an improved environment to develop in the uterus. Conversely, the more stress-sensitive males[21,48] would need maternal cooperation to develop (i.e., an improved

Table 6. Differential Cell Counts and Apoptotic Nuclei within Blastocysts Derived of Day-5 IVP Morulae Cultured in mSOF with a Dyalizate of Flushes Recovered on Day 8 from Uterus Containing Male and Female Embryos

uterine fluid containing embryos	sex of embryos in culture	cell numbers N^a	ICM[b]	TE[c]	apoptotic nuclei (%) N^a	ICM[b]	TE[c]
male	male	36	32.2 ± 4.2	94.7 ± 10.4	17	10.3 ± 1.2^{a}	5.6 ± 0.9
male	female	31	36.6 ± 4.4	79.6 ± 10.9	11	13.5 ± 1.4	7.1 ± 1.1
female	male	30	33.8 ± 4.1	86.3 ± 10.0	16	13.8 ± 1.3	4.9 ± 1.0
female	female	26	32.7 ± 4.5	80.2 ± 11.1	14	14.7 ± 1.3^{b}	7.9 ± 1.0
cumulative by embryonic sex							
male		66	33.0 ± 3.9	90.5 ± 9.6^{a}	33	12.0 ± 0.9	5.3 ± 0.7^{a}
female		57	34.6 ± 4.2	80.0 ± 10.4^{b}	25	14.0 ± 0.9	7.5 ± 0.7^{b}

[a]Embryos analyzed (4 replicates). [b]Inner cell mass. [c]Trophectoderm.

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embryotrophic profile: male-UF). The presence of the NFκB inducers IL-1B and TNFα in the cyclic, native uterine environment[6] represents a stress source for male embryos. This hypothesis would explain why embryo development rates *in vivo* (the present work and ref 29) and *in vitro* (i.e., no NFκB inducers) are comparable between male and female embryos, despite the superior embryotrophic properties of male-UF *in vitro*. Stress sensitivity and developmental speed are linked.[11,30] Thus, the uterus would compensate males with a more embryotrophic UF and/or adapt its secretion to the slower development of females.[26] Maternal nutrition may also be an important factor for sex selection. Our animals had good body condition scores (BCS), and Trivers and Willard[49] predicted that it would be advantageous for mothers with the best BCS and/or enhanced caloric intakes[29] to produce male offspring. However, this passive nutritional effect on sex selection could be reinforced by early recognition of the embryonic sex, thereby allowing for more complex and adaptable responses.

Changes in hexoses and enzymes between male-UF and female-UF can reflect different nutritional requirements for males and females.[11] Glucose is a major energy source for bovine embryos,[50−52] which also metabolize fructose.[50] *In vitro* studies have shown that male and female embryos differ in glucose metabolism, especially in some of the enzymes involved in the PPP and glycolysis.[11] However, unlike glucose, *in vitro* embryo culture with fructose neither skews the blastocyst sex ratio toward males[27] nor causes detrimental effects.[28] In our DIGE study we showed regulation of GPI, an enzyme that can reversibly isomerize D-glucose to D-fructose. Therefore, UF dimorphism in glycolytic activity (GPI in particular), with more fructose being available to females without changes in glucose, suggests that the *in vitro* effects of glucose could be an artifact. Glucose regulation, though not yet well understood, is thought to be one of the mechanisms involved in intrauterine sex selection.[11] The potential benefits for *in vitro* culture using a balanced glucose/fructose ratio warrant further investigation.

Many of the proteins up-regulated in female-UF, such as PRDX2, 5 and 6; TXN; DDAH2;[53] DJ-1;[54] and GSTP1,[55] are H_2O_2 responsive, suggesting that hydrogen peroxide might be produced in different amounts in male and female embryos.[11] Under H_2O_2 regulation,[56] DDAH2 interferes with (NO) synthase inhibition by L-arginine.[53] Arginine uptake is higher in females than males,[18] and such dimorphism may affect NO/H_2O_2 signaling. Peroxiredoxins, TXN and GSTP1 are associated with the in utero development of ruminant embryos,[6,52] and TXN and SOD1 improve *in vitro* blastocyst development.[57,58] PRDX6 is involved in the metabolism of phenylalanine, which is taken up preferentially by male embryos.[18]

Regulated annexins may anticipate differences in implantation between males and females. Clusterin expression is associated with receptivity in mice,[59] consistent with the up-regulation of mature and pro-form clusterin in male-UF.

In our work, IVP was believed to be a better choice than superovulation to obtain the necessary tens of embryos for multiple transfer. *In vivo* fertilization with male- and female-sorted sperm would be impractical, as its efficiency within superovulated donors is low.[60] With regards to untreated sperm, the use of sex-sorted sperm leads to reduced *in vitro* development rates, but it seems not to affect the quality of embryos produced.[60-62] However, there are transcriptomic differences between IVP and *in vivo* blastocysts,[63] which are greatly influenced by the *in vitro* or *in vivo* occurrence of major genome activation.[64] By contrast, IVP embryos and *in vivo* embryos that result in births share similar genetic signatures,[65] suggesting that individual embryonic viability is more important than culture systems. In our laboratory, IVP embryos produced with sex-sorted sperm show 52% pregnancy rates as fresh and 42% after cryopreservation.[6] It cannot be dismissed that the use of IVP embryos could have contributed to a proportion of the observed effects in our work.

The presence of day-8 embryos in the uterus reduces the histotroph volume.[6] However, an extreme reduction (as seen in female-UF) did not result in improved embryotrophic effects (as seen in male-UF). Collectively, the absence of NFκB regulation indicates that uterine recognition of male and female embryos does not operate through the NFκB system. Down-regulation of NFκB would be shared by embryos developing in the uterus[6] independent of embryonic sex.

We confirmed the endometrial origin of 3 DE proteins. These proteins can be secreted into the uterine lumen from the glandular endometrium. Our results support an immune role for endometrial cells, consistent with the low numbers of uterine immune cells present during early development.[41,66]

■ CONCLUSIONS

Bovine UF has a dynamic nature and can distinguish embryonic sex at early stages, as shown by 23 DE proteins. In addition, the uterine response induced by males, but not by females, leads to a favorable uterine environment in accordance with sex-selection mechanisms operating through males. This response could depend on stress sensitivity, nutritional prerequisites, or both. Dimorphism between sex-specific UFs may involve signaling molecules that can differ in expression between male and female embryos, such as IFNT, TNFα, H_2O_2, or NO, and differences in carbohydrate balances could also play a role. We expect that this information will lead to improvements in embryo culture media design and will enable further investigations of sex selection.

■ ASSOCIATED CONTENT

Supporting Information

Extended information on all peptides identified for each target protein, searching parameters and acceptance criteria; gradient conditions used for carbohydrate analysis by ion chromatography; ingenuity pathway analysis network legends. Representative 2D-DiGE gel image of recovered uterine protein showing spots identified as up-regulated in female uterine fluid. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

AI, artificial insemination; BCS, body condition score; BVA, biological variation analysis; CID, collision-induced dissociation; 2D- DiGE, two-dimensional difference gel electrophoresis; DE, differentially expressed; DIA, differential in-gel analysis; ET, embryo transfer; FDR, false discovery rate; female-UF, uterine fluid that carried female embryos; GPI, glucose-6 phosphate isomerase; GO, gene ontology; ICQ, immunocytochemical analysis; DO, indoleamine 2,3-dioxygenase; IFNT, interferon-τ; ICM, inner cell mass; I-20S, immunoproteasome subunit 20S (β5i); IPA, igenuity pathway analysis; IVP, *in vitro* produced; IVF, *in vitro* fertilized; male-UF, uterine fluid that carried male embryos; MS, mass spectrometry; NFκB, nuclear factor kappa-B system; PBS, phosphate buffer saline; PVA, polyvinyl-alcohol; P4, progesterone; P/IP, proteasome/immunoproteasome; RM, recovery medium; SOF, synthetic oviduct fluid; TE, trophectoderm; UF, uterine fluid; WB, Western blotting

■ REFERENCES

(1) Mansouri-Attia, N.; Sandra, O.; Aubert, J.; Degrelle, S.; Everts, R. E.; Giraud-Delville, C.; Heyman, Y.; Galio, L.; Hue, I.; Yang, X.; Tian, X. C.; Lewin, H. A.; Renard, J. P. Endometrium as an early sensor of in vitro embryo manipulation technologies. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 5687−5692.

(2) Sandra, O.; Mansouri-Attia, N.; Lea, R. G. Novel aspects of endometrial function: a biological sensor of embryo quality and driver of pregnancy success. *Reprod., Fertil. Dev.* **2011**, *24*, 68−79.

(3) Barker, D. J. P. *Mothers, Babies and Health in Later Life*; Churchill Livingstone: Edinburgh, 1998; p 232.

(4) Vickers, M. H.; Krechowec, S. O.; Breier, B. H. Is later obesity programmed in utero? *Curr. Drug Targets* **2007**, *8*, 923−934.

(5) Shankar, K.; Zhong, Y.; Kang, P.; Lau, F.; Blackburn, M. L.; Chen, J. R.; Borengasser, S. J.; Ronis, M. J.; Badger, T. M. Maternal obesity promotes a proinflammatory signature in rat uterus and blastocyst. *Endocrinology* **2011**, *152*, 4158−4170.

(6) Muñoz, M.; Corrales, F. J.; Caamaño, J. N.; Díez, C.; Trigal, B.; Mora, M. I.; Martín, D.; Carrocera, S.; Gómez, E. Proteome of the early embryo-maternal dialogue in the cattle uterus. *J. Proteome Res.* **2012**, *11*, 751−766.

(7) Almiñana, C.; Heath, P. R.; Wilkinson, S.; Sanchez-Osorio, J.; Cuello, C.; Parrilla, I.; Gil, M. A.; Vazquez, J. L.; Vazquez, J. M.; Roca, J.; Martinez, E. A.; Fazeli, A. Early developing pig embryos mediate their own environment in the maternal tract. *PLoS One* **2012**, *7*, e33625.

(8) Forde, N.; Carter, F.; Spencer, T. E.; Bazer, F. W.; Sandra, O.; Mansouri-Attia, N.; Okumu, L. A.; McGettigan, P. A.; Mehta, J. P.; McBride, R.; O'Gaora, P.; Roche, J. F.; Lonergan, P. Conceptus-induced changes in the endometrial transcriptome: how soon does the cow know she is pregnant? *Biol. Reprod.* **2011**, *85*, 144−156.

(9) Bauersachs, S.; Ulbrich, S. E.; Reichenbach, H. D.; Reichenbach, M.; Büttner, M.; Meyer, H. H.; Spencer, T. E.; Minten, M.; Sax, G.; Winter, G.; Wolf, E. Comparison of the effects of early pregnancy with human interferon, alpha 2 (IFNA2), on gene expression in bovine endometrium. *Biol. Reprod.* **2012**, *86*, 46.

(10) Ostrup, E.; Hyttel, P.; Ostrup, O. Embryo-maternal communication: signalling before and during placentation in cattle and pig. *Reprod., Fertil. Dev.* **2011**, *23*, 964−975.

(11) Bermejo-Alvarez, P.; Rizos, D.; Lonergan, P.; Gutierrez-Adan, A. Transcriptional sexual dimorphism during preimplantation embryo development and its consequences for developmental competence and adult health and disease. *Reproduction* **2011**, *141*, 563−570.

(12) Bermejo-Alvarez, P.; Lonergan, P.; Rath, D.; Gutierrez-Adan, A.; Rizos, D. Developmental kinetics and gene expression in male and female bovine embryos produced in vitro with sex-sorted spermatozoa. *Reprod., Fertil. Dev.* **2010a**, *22*, 426−436.

(13) Bermejo-Alvarez, P.; Rizos, D.; Rath, D.; Lonergan, P.; Gutierrez-Adan, A. Epigenetic differences between male and female bovine blastocysts produced in vitro. *Physiol Genomics* **2008**, *32*, 264−272.

(14) Gutierrez-Adan, A.; Oter, M.; Martinez-Madrid, B.; Pintado, B.; De La Fuente, J. Differential expression of two genes located on the X chromosome between male and female in vitro-produced bovine embryos at the blastocyst stage. *Mol. Reprod. Dev.* **2000**, *55*, 146−151.

(15) Peippo, J.; Farazmand, A.; Kurkilahti, M.; Markkula, M.; Basrur, P. K.; King, W. A. Sex-chromosome linked gene expression in in-vitro produced bovine embryos. *Mol. Hum. Reprod.* **2002**, *8*, 923−929.

(16) Bermejo-Alvarez, P.; Rizos, D.; Rath, D.; Lonergan, P.; Gutierrez-Adan, A. Sex determines the expression level of one third of the actively expressed genes in bovine blastocysts. *Proc. Natl. Acad. Sci. U.S.A.* **2010b**, *107*, 3394−3399.

(17) Kimura, K.; Iwata, H.; Thompson, J. G. The effect of glucosamine concentration on the development and sex ratio of bovine embryos. *Anim. Reprod. Sci.* **2008**, *103*, 228−238.

(18) Sturmey, R. G.; Bermejo-Alvarez, P.; Gutierrez-Adan, A.; Rizos, D.; Leese, H. J.; Lonergan, P. Amino acid metabolism of bovine blastocysts: a biomarker of sex and viability. *Mol. Reprod. Dev.* **2010**, *77*, 285−296.

(19) Rubessa, M.; Boccia, L.; Campanile, G.; Longobardi, V.; Albarella, S.; Tateo, A.; Zicarelli, L.; Gasparrini, B. Effect of energy source during culture on in vitro embryo development, resistance to cryopreservation and sex ratio. *Theriogenology* **2011**, *76*, 1347−1355.

(20) Larson, M. A.; Kimura, K.; Kubisch, H. M.; Roberts, R. M. Sexual dimorphism among bovine embryos in their ability to make the transition to expanded blastocyst and in the expression of the signaling molecule IFN-tau. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 9677−9682.

(21) Kimura, K.; Spate, L. D.; Green, M. P.; Roberts, R. M. Effects of oxidative stress and inhibitors of the pentose phosphate pathway on sexually dimorphic production of IFN-tau by bovine blastocysts. *Mol. Reprod. Dev.* **2004a**, *68*, 88−95.

(22) Kimura, K.; Spate, L. D.; Green, M. P.; Murphy, C. N.; Seidel, G. E., Jr.; Roberts, R. M. Sexual dimorphism in interferon-tau production by in vivo-derived bovine embryos. *Mol. Reprod. Dev.* **2004b**, *67*, 193−199.

(23) Beyhan, Z.; Johnson, L. A.; First, N. L. Sexual dimorphism in IVM-IVF bovine embryos produced from X and Y chromosome-bearing spermatozoa sorted by high speed flow cytometry. *Theriogenology* **1999**, *52*, 35−48.

(24) Nedambale, T. L.; Dinnyes, A.; Yang, X.; Tian, X. C. Bovine blastocyst development in vitro: timing, sex, and viability following vitrification. *Biol. Reprod.* **2004**, *71*, 1671−1676.

(25) Jiménez, A.; Madrid-Bury, N.; Fernández, R.; Pérez-Garnelo, S.; Moreira, P.; Pintado, B.; de la Fuente, J.; Gutiérrez-Adán, A. Hyperglycemia-induced apoptosis affects sex ratio of bovine and murine preimplantation embryos. *Mol. Reprod. Dev.* **2003**, *65*, 180−187.

(26) Bredbacka, K.; Bredbacka, P. Glucose controls sex-related growth rate differences of bovine embryos produced in vitro. *J. Reprod. Fertil.* **1996**, *106*, 169−172.

(27) Kimura, K.; Spate, L. D.; Green, M. P.; Roberts, R. M. Effects of D-glucose concentration, D-fructose, and inhibitors of enzymes of the pentose phosphate pathway on the development and sex ratio of bovine blastocysts. *Mol. Reprod. Dev.* **2005**, *72*, 201−207.
(28) Kwun, J.; Chang, K.; Lim, J.; Lee, E.; Lee, B.; Kang, S.; Hwang, W. Effects of exogenous hexoses on bovine in vitro fertilized and cloned embryo development: Improved blastocyst formation after glucose replacement with fructose in a serum-free culture medium. *Mol. Reprod. Dev.* **2003**, *65*, 167−174.
(29) King, W. A.; Yadav, B. R.; Xu, K. P.; Picard, L.; Sirard, M. A.; Verini Supplizi, A.; Betteridge, K. J. The sex ratios of bovine embryos produced in vivo and in vitro. *Theriogenology* **1991**, *36*, 779−788.
(30) Rosenfeld, C. S. Periconceptional influences on offspring sex ratio and placental responses. *Reprod., Fertil. Dev.* **2012**, *24*, 45−58.
(31) Trigal, B.; Gómez, E.; Díez, C.; Caamaño, J. N.; Muñoz, M.; Moreno, J. F.; Carrocera, S.; Martín, D.; Goyache, F.; Álvarez, I. Comparative study of PCR-sexing procedures using bovine embryos fertilized with sex-sorted spermatozoa. *Span. J. Agric. Res.* **2012**, *10*, 353−359.
(32) Santamaría, E.; Avila, M. A.; Latasa, M. U.; Rubio, A.; Martin-Duce, A.; Lu, S. C.; Mato, J. M.; Corrales, F. J. Functional proteomics of nonalcoholic steatohepatitis: mitochondrial proteins as targets of S-adenosylmethionine. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 3065−3070.
(33) Colinge, J.; Masselot, A.; Giron, M.; Dessingy, T.; Magnin, J. OLAV: towards high-throughput tandem mass spectrometry data identification. *Proteomics* **2003**, *3*, 1454−1463.
(34) Muñoz, M.; Peirson, S. N.; Hankins, M. W.; Foster, R. G. Long-term constant ight induces constitutive elevated expression of mPER2 protein in the murine SCN: a molecular basis for Aschoff's rule? *J. Biol. Rhythms* **2005**, *20*, 3−14.
(35) Rocklin, R. D.; Clarke, A. P.; Weitzhandler, M. Improved long-term reproducibility for pulsed amperometric detection of carbohydrates via a new quadruple-potential waveform. *Anal. Chem.* **1998**, *70*, 1496−1501.
(36) Thouas, G. A.; Korfiatis, N. A.; French, A. J.; Jones, G. M.; Trounson, A. O. Simplified technique for differential staining of inner cell mass and trophectoderm cells of mouse and bovine blastocysts. *Reprod. Biomed. Online* **2001**, *3*, 25−29.
(37) Wydooghe, E.; Vandaele, L.; Beek, J.; Favoreel, H.; Heindryckx, B.; De Sutter, P.; Van Soom, A. Differential apoptotic staining of mammalian blastocysts based on double immunofluorescent CDX2 and active caspase-3 staining. *Anal. Biochem.* **2001**, *416*, 228−230.
(38) Velazquez, M. A.; Parrilla, I.; Van Soom, A.; Verberckmoes, S.; Kues, W.; Niemann, H. Sampling techniques for oviductal and uterine luminal fluid in cattle. *Theriogenology* **2010**, *73*, 758−767.
(39) Hannan, N. J.; Stephens, A. N.; Rainczuk, A.; Hincks, C.; Rombauts, L. J.; Salamonsen, L. A. 2D-DiGE analysis of the human endometrial secretome reveals differences between receptive and nonreceptive states in fertile and infertile women. *J. Proteome Res.* **2010**, *9*, 6256−6264.
(40) Faulkner, S.; Elia, G.; Mullen, M. P.; O'Boyle, P.; Dunn, M. J.; Morris, D. A comparison of the bovine uterine and plasma proteome using iTRAQ proteomics. *Proteomics* **2012**, *12*, 2014−2023.
(41) Yokota, T.; Sugawara, K.; Ito, K.; Takahashi, R.; Ariga, H.; Mizusawa, H. Down regulation of DJ-1 enhances cell death by oxidative stress, ER stress, and proteasome inhibition. *Biochem. Biophys. Res. Commun.* **2003**, *312*, 1342−1348.
(42) Groebner, A. E.; Schulke, K.; Schefold, J. C.; Fusch, G.; Sinowatz, F.; Reichenbach, H. D.; Wolf, E.; Meyer, H. H.; Ulbrich, S. E. Immunological mechanisms to establish embryo tolerance in early bovine pregnancy. *Reprod., Fertil. Dev.* **2011**, *23*, 619−632.
(43) Sijts, E. J.; Kloetzel, P. M. The role of the proteasome in the generation of MHC class I ligands and immune responses. *Cell. Mol. Life Sci.* **2011**, *68*, 1491−1502.
(44) Angeles, A.; Fung, G.; Luo, H. Immune and non-immune functions of the immunoproteasome. *Front. Biosci.* **2012**, *17*, 1904−1916.
(45) Heink, S.; Ludwig, D.; Kloetzel, P. M.; Kruger, E. IFN-gamma-induced immune adaptation of the proteasome system is an accelerated and transient response. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102* (26), 9241−9246.
(46) Griffin, T. A.; Nandi, D.; Cruz, M.; Fehling, H. J.; Kaer, L. V.; Monaco, J. J.; Colbert, R. A. Immunoproteasome assembly: cooperative incorporation of interferon gamma (IFN-gamma)-inducible subunits. *J. Exp. Med.* **1998**, *187* (1), 97−104.
(47) Bose, S.; Brooks, P.; Mason, G. G.; Rivett, A. J. gamma-Interferon decreases the level of 26 S proteasomes and changes the pattern of phosphorylation. *Biochem. J.* **2011**, *353* (Pt 2), 291−297.
(48) Pérez-Crespo, M.; Ramírez, M. A.; Fernández-González, R.; Rizos, D.; Lonergan, P.; Pintado, B.; Gutiérrez-Adán, A. Differential sensitivity of male and female mouse embryos to oxidative induced heat-stress is mediated by glucose-6-phosphate dehydrogenase gene expression. *Mol. Reprod. Dev.* **2005**, *72*, 502−510.
(49) Trivers, R. L.; Willard, D. E. Natural selection of parental ability to vary the sex ratio of offspring. *Science* **1973**, *179*, 90−92.
(50) Guyader-Joly, C.; Khatchadourian, C.; Menezo, Y. Comparative glucose and fructose incorporation and conversion by in vitro produced bovine embryos. *Zygote* **1996**, *4*, 85−91.
(51) Hugentobler, S. A.; Humpherson, P. G.; Leese, H. J.; Sreenan, J. M.; Morris, D. G. Energy substrates in bovine oviduct and uterine fluid and blood plasma during the oestrous cycle. *Mol. Reprod. Dev.* **2008**, *75*, 496−503.
(52) Gao, H.; Wu, G.; Spencer, T. E.; Johnson, G. A.; Li, X.; Bazer, F. W. Select nutrients in the ovine uterine lumen. I. Amino acids, glucose, and ions in uterine lumenal flushings of cyclic and pregnant ewes. *Biol. Reprod.* **2009**, *80*, 86−93.
(53) Knipp, M. How to control NO production in cells: N(omega),N(omega)-dimethyl-L-arginine dimethylaminohydrolase as a novel drug target. *ChemBioChem* **2006**, *7*, 879−889.
(54) Rai, P.; Shivaji, S. The role of DJ-1 in the pathogenesis of endometriosis. *PLoS One* **2011**, *6*, e18074.
(55) Laborde, E. Glutathione transferases as mediators of signaling pathways involved in cell proliferation and cell death. *Cell Death Differ.* **2010**, *17*, 1373−1380.
(56) Tain, Y. L.; Kao, Y. H.; Hsieh, C. S.; Chen, C. C.; Sheen, J. M.; Lin, I. C.; Huang, L. T. Melatonin blocks oxidative stress-induced increased asymmetric dimethylarginine. *Free Radic. Biol. Med.* **2010**, *49*, 1088−1098.
(57) Bing, Y. Z.; Hirao, Y.; Takenouchi, N.; Che, L. M.; Nakamura, H.; Yodoi, J.; Nagai, T. Effects of thioredoxin on the preimplantation development of bovine embryos. *Theriogenology* **2003**, *59*, 863−873.
(58) Takahashi, M. Oxidative stress and redox regulation on in vitro development of mammalian embryos. *J. Reprod. Dev.* **2012**, *58*, 1−9.
(59) Lam, K. K.; Pang, R. T.; Lee, C. L.; Koistinen, H.; Seppala, M.; Ho, P. C.; Yeung, W. S.; Chiu, P. C. Glycodelin-A protein interacts with Siglec-6 protein to suppress trophoblast invasiveness by down-regulating extracellular signal-regulated kinase (ERK)/c-Jun signaling pathway. *J. Biol. Chem.* **2011**, *286*, 37118−37127.
(60) Schenk, J. L.; Suh, T. K.; Seidel, G. E., Jr. Embryo production from superovulated cattle following insemination of sexed sperm. *Theriogenology* **2006**, *65*, 299−307.
(61) Trigal, B.; Gómez, E.; Caamaño, J. N.; Muñoz, M.; Moreno, J.; Carrocera, S.; Martín, D.; Diez, C. In vitro and in vivo quality of bovine embryos in vitro produced with sex-sorted sperm. *Theriogenology* **2012**, *787*, 1465−1475.
(62) Garcia-Herreros, M.; Carter, T. F.; Villagómez, D. A.; Macaulay, A. D.; Rath, D.; King, W. A.; Lonergan, P. Incidence of chromosomal abnormalities in bovine blastocysts derived from unsorted and sex-sorted spermatozoa. *Reprod., Fertil. Dev.* **2010**, *22*, 1272−1278.
(63) Driver, A. M.; Peñagaricano, F.; Huang, W.; Ahmad, K. R.; Hackbart, K. S.; Wiltbank, M. C.; Khatib, H. RNA-Seq analysis uncovers transcriptomic variations between morphologically similar in vivo- and in vitro-derived bovine blastocysts. *BMC Genomics* **2012**, *13*, 118.
(64) Gad, A.; Hoelker, M.; Besenfelder, U.; Havlicek, V.; Cinar, U.; Rings, F.; Held, E.; Dufort, I.; Sirard, M. A.; Schellander, K.; Tesfaye,

D. Molecular mechanisms and pathways involved in bovine embryonic genome activation and their regulation by alternative in vivo and in vitro culture conditions. *Biol. Reprod.* **2012**, *87*, 100.

(65) Ghanem, N.; Salilew-Wondim, D.; Gad, A.; Tesfaye, D.; Phatsara, C.; Tholen, E.; Looft, C.; Schellander, K.; Hoelker, M. Bovine blastocysts with developmental competence to term share similar expression of developmentally important genes although derived from different culture environments. *Reproduction* **2011**, *142*, 551−564.

(66) Cronin, J. G.; Turner, M. L.; Goetze, L.; Bryant, C. E.; Sheldon, I. M. Toll-like receptor 4 and MYD88-dependent signaling mechanisms of the innate immune system are essential for the response to lipopolysaccharide by epithelial and stromal cells of the bovine endometrium. *Biol. Reprod.* **2012**, *86*, 51.

CAPÍTULO 6 DISCUSIÓN Y CONCLUSIONES

DISCUSIÓN GENERAL

En esta tesis hemos identificado varias proteínas que intervienen en la comunicación materno-embrionaria temprana. Para ello, hemos utilizado dos enfoques, uno específico y otro global. Mediante el enfoque específico hemos caracterizado la expresión de varias proteínas seleccionadas por su regulación diferencial en el fluido uterino (FU) en función de la presencia de embriones. A partir del enfoque global, hemos descrito la influencia del sexo del embrión en el proteoma del FU. Asimismo, el estudio funcional de HDGF ha permitido demostrar que el empleo de estas proteínas puede mejorar el cultivo *in vitro* de embriones bovinos.

Comunicación materno-embrionaria

Papel de las proteínas TNF, IL1B y HDGF

Un estudio previo encontró que la presencia de embriones producía cambios en la concentración relativa de las proteínas TNF, IL1B y HDGF en el FU bovino (Muñoz et al. 2012). Estas evidencias nos indujeron a investigar si el endometrio o el embrión expresan dichas proteínas, y también sus receptores, durante el desarrollo temprano. Si bien la presencia de TNF, IL1B y sus receptores había sido ya descrita en el endometrio y embriones de mamíferos, nunca se había analizado de forma simultánea la expresión conjunta de tales ligandos y receptores durante el desarrollo temprano. Por otro lado, y hasta donde sabemos, la presencia de HDGF en el tracto reproductivo de mamíferos no había sido nunca investigada.

Nuestros resultados muestran que tanto el endometrio como el embrión contienen mRNA y proteína de TNF, IL1B y HDGF. En el endometrio, todas las proteínas se localizan mayoritariamente en la parte más apical del epitelio glandular, lo que sugiere secreción hacia el lumen uterino y eventualmente FU. En concordancia, el epitelio endometrial humano produce y secreta TNF e IL1B tanto *in vivo* (Simón et al. 1996, von Wolff et al. 1999) como *in vitro* (Jokhi et al. 1997, Tabibzadeh et al. 1995). Nosotros hemos encontrado que TNF, IL1B y HDGF no sólo se encuentran en el epitelio glandular sino también en otros tipos celulares (epitelio luminal, estroma, carúncula y, en el caso de TNF y HDGF, también endotelio). De manera análoga, TNF e IL1B han sido localizados durante el ciclo estral en el epitelio, estroma y endotelio del endometrio bovino (Okuda et al. 2010, Paula-Lopes et al. 1999).

En el embrión bovino, hemos encontrado que TNF, IL1B y HDGF se localizan mayoritariamente en las células del trofectodermo. Del mismo modo, TNF e IL1B predominan en el trofectodermo de humano y ratón (Ben-Yair et al. 1997, De los Santos et al. 1996). El trofectodermo es un mediador decisivo en la comunicación materno-embrionaria y la implantación (Ozawa et al. 2012, Spencer et al. 2004). Al igual que el endometrio humano, los embriones humanos también pueden secretar TNF e IL1B al medio de cultivo *in vitro* (Barañao et al. 1997, Witkin et al. 1991). La IL1B es también producida y secretada por el embrión porcino durante la elongación (Ross et al. 2003).

Hemos demostrado que tanto las células endometriales como las embrionarias pueden responder a TNF e IL1B durante el desarrollo temprano, ya que expresan los receptores de estas citoquinas, TNFR2 e IL1R1. En concordancia con lo reportado previamente durante el ciclo estral bovino (Okuda et al. 2010), nosotros hemos obtenido un alto grado de colocalización ligando/receptor en todos los tipos celulares, tanto en el endometrio cíclico como preñado. La localización de IL1R1 en células endometriales no había sido investigada en bovino. Sin embargo, en humano, la expresión de IL1R1 es intensa en el epitelio luminal y glandular, débil en el endotelio, y no se observa en el estroma (Simón et al. 1993). La localización de HDGF en el núcleo de las células epiteliales sugiere que la proteína podría ser captada del FU e internalizada tras su unión a proteoglicanos heparán sulfato (HS) presentes en la superficie celular, según uno de los mecanismos de señalización conocidos para este factor (Wang et al. 2011) del cual no se conoce receptor. Conviene señalar que FGF2, factor de crecimiento con un importante papel en la comunicación materno-embrionaria, también se une a HS en la matriz extracelular antes de su unión al correspondiente receptor de membrana (Abouzied et al. 2005). También hemos demostrado que los embriones bovinos responden a HDGF recombinante (rHDGF) en cultivos *in vitro*. Nuestros resultados sugieren que cada uno de los tres factores estudiados, TNF, IL1B y HDGF, regulan el crecimiento del embrión y la función uterina durante el desarrollo temprano de una forma autocrina y paracrina.

La presencia de embriones aumenta la expresión de TNF, IL1B y TNFR2 en el endometrio. TNF e IL1B son potentes reguladores del sistema NFKB, una familia de factores de transcripción que regulan las respuestas inmune e inflamatoria (Ghosh and Hayden 2008, Hayden and Ghosh 2011). La actividad de NFKB aumenta en vaca, oveja y cerdo poco antes de la implantación (Bauersachs et al. 2006, Koch et al. 2010, Roberts

et al. 2008, Ross et al. 2010). Sin embargo, durante estadios tempranos, la regulación a la baja de NFKB en el útero bovino y porcino explicaría el privilegio inmunológico del embrión (Almiñana et al. 2012, Muñoz et al. 2012). En concordancia, en esta tesis hemos descrito una disminución en el número de leucocitos CD45 en las células endometriales durante el desarrollo temprano. Este descenso de leucocitos es más pronunciado en las carúnculas, lo que podría tener una significación biológica dado que las carúnculas soportarán la placentación más adelante en el desarrollo. De forma similar, la abundancia de leucocitos disminuye en el epitelio glandular funcional y en el estroma subepitelial antes de la implantación (Día 18) (Groebner et al. 2011). Nuestros resultados, con un aumento de TNF e IL1B y una disminución de leucocitos en el endometrio, sugieren que existen mecanismos que limitan los efectos proinflamatorios de TNF e IL1B en el endometrio durante el desarrollo temprano.

Hemos constatado diferencias locales en la regulación de la actividad de TNF e IL1B en el endometrio. Así, al igual que los leucocitos, TNF e IL1B se expresan con mayor intensidad en las carúnculas que en las intercarúnculas. Otros estudios en rumiantes también han descrito que la expresión génica es diferente en las carúnculas respecto a las intercarúnculas desde el Día 12 de preñez (Al-Gubory et al. 2014, Mansouri-Attia et al. 2009, Walker et al. 2010, Wang et al. 2013). Por tanto, las carúnculas muestran una función definida mucho antes del inicio de la implantación, y sugerimos analizarlas siempre por separado. Además, la receptividad a TNF e IL1B de los distintos tipos celulares del endometrio parece estar sujeta a estricta regulación. Así, IL1B regula la producción endometrial de prostaglandinas en células estromales durante la fase luteal temprana (Día 2 - Día 5) (Tanikawa et al. 2009), en células epiteliales durante el reconocimiento materno de la preñez (Día 16) (Betts and Hansen 1992) y en ambos tipos celulares durante la fase luteal media-tardía (Días 11, 14 y 17) (Davidson et al. 1995). De forma similar, el aumento de estradiol durante la preñez reduce el número de receptores IL1R1 en células epiteliales humanas (Schaefer et al. 2005). En este contexto, nuestros resultados muestran que la localización nuclear de TNFR2 en algunas células endometriales podría responder a un mecanismo de disminución de la respuesta celular a TNF (Fischer et al. 2011). Además, el aumento endometrial de TNFR2 producido por la presencia de embriones ocurre específicamente en el epitelio glandular basal, lo que pone de manifiesto la importancia de analizar la expresión de

proteínas utilizando técnicas que permitan diferenciar los distintos tipos celulares presentes en el endometrio.

En esta tesis no hemos obtenido cambios en la expresión endometrial de IL1R1 y HDGF en respuesta a embriones. En cerdo y humano, sí se han señalado cambios en la expresión de IL1R1 durante el ciclo estral y la preñez (Bourdiec et al. 2014, Ross et al. 2003), aunque estas especies presentan un tipo de implantación diferente a la bovina (Peter 2013). Hay que tener en cuenta que en el sistema IL hay otros elementos que nosotros no hemos estudiado y que están regulados en el endometrio de otras especies, como por ejemplo IL1RAP (Ross et al. 2003). Varios estudios han analizado qué factores regulan la expresión de HDGF. Uno de ellos es el factor de crecimiento de hepatocitos (HGF), el cual induce la expresión de HDGF en células tumorales (Lee et al. 2010, Mao et al. 2008) y además regula la receptividad uterina y el crecimiento del embrión en la oveja (Chen et al. 2000). Curiosamente, la expresión de *HGF* no varía en los endometrios de vaca, durante el ciclo estral, y de oveja, bajo el estímulo de progesterona (Mitko et al. 2008, Satterfield et al. 2008).

En el endometrio, los cambios que hemos visto afectan a las proteínas, pero no a su mRNA. Es posible que en estadios muy precoces del desarrollo uterino el embrión no precise de respuestas que impliquen la transcripción de mRNA y su traducción. La materialización de estas respuestas en forma de cambios perceptibles por el embrión puede tardar días en producirse, como se ha demostrado en células endometriales cultivadas *in vitro* (Ulbrich et al. 2009). Dado que el embrión de mamífero no mantiene una posición estática en el cuerno uterino, sino que desciende describiendo bucles (Chen et al. 2013). Los cambios locales en proteína o metabolitos mediados por transcripción inducida por el embrión no no serían aprovechados, dado que el embrión estaría ya probablemente lejos del lugar de la respuesta. Por tanto, para su desarrollo uterino, el embrión podría requerir cambios rápidos postranscripcionales, que no impliquen la transcripción de mRNA y la síntesis de proteínas.

Por el contrario, la transcripción en el embrión sí está regulada por el endometrio. Tanto los embriones producidos enteramente *in vitro* como aquellos que pasan por el tracto materno contienen mRNA y proteína de TNF, TNFR2, IL1B, IL1R1 y HDGF. Sin embargo, los blastocistos que pasaron por el tracto materno presentan niveles más bajos de *HDGF*, *TNFR2*, y probablemente de *TNF* (en nuestro trabajo la baja expresión de *TNF* en los embriones que pasaron por el tracto materno ha impedido su cuantificación).

Por tanto, aunque el endometrio no parece ser esencial para la síntesis de estas proteínas por parte del embrión, sí juega un importante papel modulando su expresión. De forma similar a nuestros resultados, el IFNT puede ser sintetizado por los embriones producidos enteramente *in vitro*, si bien el endometrio actúa regulando su expresión embrionaria *in vivo* (Neira et al. 2011). La figura 6.1 muestra un esquema de la posible regulación de TNF, IL1B, HDGF y sus receptores en el tracto reproductivo bovino durante el desarrollo temprano.

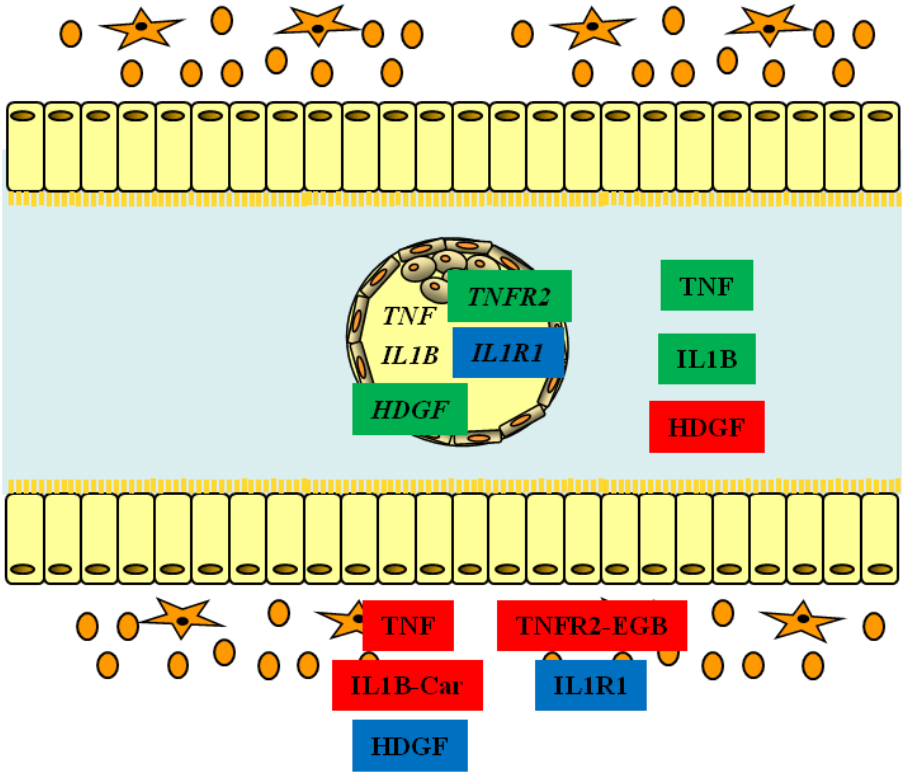

Figura 6.1 Regulación de la expresión de las citoquinas TNF e IL1B, sus receptores TNFR2 e IL1R1, y del factor de crecimiento HDGF durante el desarrollo embrionario temprano bovino. En el fluido uterino y endometrio se representan los niveles de estas proteínas tras la transferencia embrionaria, en comparación con la transferencia sin embriones. En el blastocisto se representan los niveles de transcripción tras el pasaje temporal por el tracto materno, en comparación con el cultivo enteramente *in vitro*. El color rojo representa un aumento, el verde una disminución y el azul ausencia de variación. En el caso de IL1B y TNFR2, el aumento se produce específicamente en las carúnculas (Car) y en el epitelio glandular basal (EGB), respectivamente. En el embrión, los niveles de mRNA de *TNF* e *IL1B* permanecieron por debajo del límite de detección en algunas muestras, lo que impidió su cuantificación.

Dimorfismo sexual

Hasta donde sabemos, la presente tesis es la primera en demostrar que el sexo del embrión condiciona el proteoma del FU bovino. Cuatro de las proteínas con expresión diferencial (DE) en el FU (PARK7, TXN, HSPA5, clusterina) y dos de las proteínas incluidas en la red de interacciones moleculares (NFKB, subunidad β5i del inmunoproteasoma) se localizan en el epitelio glandular y otras células endometriales,

lo que sugiere que estas proteínas tienen un origen uterino. De forma similar, el endometrio cíclico bovino de Día 7 regula la presencia en el FU bovino de varias de las proteínas DE en nuestro estudio (PRDX, 2 y 5; TXN; DJ-1; PGAM1; y ENO1) (Faulkner et al. 2012). Por tanto, nuestros resultados sugieren que el útero es capaz de reconocer el sexo del embrióndurante el desarrollo temprano y responder secretando proteínas adecuadas a las necesidades del embrión macho o hembra.

La clasificación funcional de las 23 proteínas DE en este trabajo indica que las vías moleculares implicadas en el reconocimiento materno del dimorfismo sexual incluyen procesamiento de antígenos, glicolisis, biosíntesis, antioxidación y apoptosis. No obstante, el complejo NFKB uterino no presentó dimorfismo sexual en nuestro estudio, por lo que son ambos tipos de embriones, machos y hembras, quienes son capaces de reducir la expresión de NFKB en el FU (Muñoz et al. 2012). Mediante el análisis funcional *in vitro* de los FUs condicionados por embriones hembra (FU-hembra) o macho (FU-macho), hemos obtenido que el FU-macho presenta más capacidad embriotrófica *in vitro* que su homólogo hembra.

En nuestro estudio, la vía de procesamiento de antígenos muestra cuatro proteínas con expresión incrementada en el FU-hembra. Se trata de PSMA3 y PSMB2, dos de las subunidades catalíticas del complejo proteasoma (Collavoli et al. 2011); PSME1 o PA28A, una de las subunidades reguladoras del complejo inmunoproteasoma (Pla et al. 2014); y PARK7 o DJ-1, regulador transcripcional que previene la inhibición del proteasoma (Yokota et al. 2003). El proteasoma, especialmente su isoforma denominada inmunoproteasoma, tiene como función regular la presentación de antígenos por las moléculas MHC (Mishto et al. 2014).

Aunque sólo hemos detectado cambios en las subunidades catalíticas del proteasoma, la regulación al alza de PSME1 indica que el inmunoproteasoma también podría estar implicado en el reconocimiento materno del dimorfismo sexual. De hecho, hemos determinado que la subunidad catalítica del inmunoproteasoma, B5i, se localiza en la mayoría de las células endometriales, incluyendo el epitelio glandular superficial donde forma vesículas de secreción. La escasa vida media de las subunidades catalíticas del inmunoproteasoma (Heink et al. 2005) podría dificultar la detección de cambios en su expresión. La inducción del inmunoproteasoma está mediada por citoquinas (IFNA, IFNB, IFNG y TNF), NO, H_2O_2 e hiperglucemia (Angeles et al. 2011, Sijts and Kloetzel 2011). Sin embargo, se desconoce si IFNT, citoquina producida

diferencialmente por los embriones macho y hembra, también induce la expresión del inmunoproteasoma. Durante el desarrollo temprano bovino, la presentación de antígenos embrionarios de origen paterno por las moléculas MHC maternas regula el rechazo inmunológico del embrión (Groebner et al. 2011). Por tanto, nuestros resultados sugieren que los blastocistos hembra y macho modulan la expresión y secreción del proteasoma e inmunoproteasoma, regulando así diferencialmente la tolerancia inmunológica materna.

El metabolismo de la glucosa es otra de las vías sujetas a dimorfismo sexual en nuestro estudio. Los embriones de rumiantes pueden metabolizar tanto la glucosa como la fructosa (Gao et al. 2009, Guyader-Joly et al. 1996, Hugentobler et al. 2008). Los embriones macho y hembra producidos *in vitro* difieren en el metabolismo de la glucosa, especialmente en varias enzimas implicadas en la vía de las pentosa fosfato y glucolisis (Bermejo-Álvarez et al. 2011). Como consecuencia, la utilización de glucosa a concentraciones superiores a 2.5 mM reduce el desarrollo embrionario y desvia la proporción de sexos hacia los machos (Kimura et al. 2005). Por el contrario, el cultivo *in vitro* con fructosa no conlleva ninguno de estos efectos (Kimura et al. 2005) e incluso puede mejorar el desarrollo embrionario a determinadas concentraciones, en comparación con la glucosa (Kwun et al. 2003).

En nuestro estudio DIGE observamos una regulación al alza en el FU-hembra de la enzima GPI, encargada de convertir glucosa en fructosa por medio de una reacción reversible (Rengaraj et al. 2013). La expresión diferencial de GPI en el FU nos indujo a determinar la concentración de hexosas, y vimos que los FUs macho y hembra no difieren en su concentración de glucosa mientras que la de fructosa es más abundante en el FU-hembra. Por tanto, los efectos de la glucosa que se han descrito en cultivos de embriones *in vitro* podrían ser en realidad un artefacto. La regulación de la glucosa en el útero parece ser uno de los factores implicados en la selección del sexo, aunque los mecanismos subyacentes no han sido claramente determinados (Bermejo-Álvarez et al. 2011). Nuestros resultados indican que el balance glucosa/fructosa podría participar en la regulación diferencial del desarrollo de embriones macho y hembra en el animal vivo.

Muchas de las proteínas reguladas al alza en el FU-hembra protegen a las células del estrés oxidativo y muerte celular mediante la regulación redox y el control de la concentración de especies reactivas que pueden causar daño celular. Estas proteínas son: PRDX 2, 5 y 6; TXN; GSTP1 (Griffiths et al. 2014, Laborde 2010); DDAH2 (Tain et al.

2010); y PARK7 (Rai and Shivaji 2011). Es probable que NO se produzca diferencialmente por los embriones macho y hembra, ya que su síntesis depende de L-arginina, cuya tasa de captación es mayor en las hembras que en los machos (Sturmey et al. 2010). De igual modo, nuestros resultados sugieren que los embriones macho y hembra pueden producir H_2O_2 en diferentes cantidades. Es de resaltar que la presencia de PRDX2, TXN y glutatión, el sustrato de GSTP1 (Armstrong 1997), aumenta en el FU de rumiantes como consecuencia de la preñez (Gao et al. 2009, Ledgard et al. 2009, Muñoz et al. 2012). Además, TXN y glutatión mejoran el desarrollo de blastocistos de rumiantes *in vitro* (Bing et al. 2003, Ozawa et al. 2006). PRDX6 participa en el metabolismo de la fenilalanina, aminoácido que los embriones bovinos incorporan a distintos niveles según su sexo (Sturmey et al. 2010).

Por último, las anexinas, proteínas implicadas en la adhesión del embrión al epitelio luminal (Garrido-Gómez et al. 2012), reguladas al alza en el FU-hembra según nuestro estudio, podrían anticipar diferencias en la implantación de embriones macho y hembra. La expresión de ANXA4 aumenta en el FU de vacas preñadas (Muñoz et al. 2012). Por otro lado, la clusterina, única proteína cuya expresión aumenta en el FU-macho, se asocia con la receptividad en ratón (Vitiello et al. 2008).

A partir del estudio funcional del FU *in vitro* observamos que los embriones machos, frente asus homólogos hembras, indujeron un ambiente uterino más favorable para su desarrollo. Los embriones macho son más sensibles que las hembras a condiciones subóptimas (Kimura et al. 2004b, Pérez-Crespo et al. 2005). Por tanto, la desviación de la proporción de sexos suele realizarse a través de los machos y depende de la sensibilidad al estrés o del estado nutricional de la madre (Gutiérrez-Adán et al. 2006). Sin embargo, este mecanismo nutricional pasivo podría complementarse con otras respuestas más complejas y adaptables que impliquen el reconocimiento temprano del sexo del embrión en el útero. La respuesta materna al dimorfismo sexual podría involucrar a moléculas de señalización cuya expresión cambia entre embriones macho y hembra, como IFNT (Kimura et al. 2004a, Kimura et al. 2004b, Larson et al. 2001), y posiblemente NO y H_2O_2.

Perspectivas para el cultivo in vitro de embriones bovinos

Entre las proteínas DE en el FU con y sin embriones, hemos elegido al HDGF para su ensayo funcional en el cultivo *in vitro*. Dicha elección se basa en que otros factores de

crecimiento que comparten rutas de señalización similares al HDGF mejoran el desarrollo embrionario *in vitro*. Tal es el caso del GF insulínico (IGF1); el GF epidérmico (EGF); activina; el factor estimulador de colonias de granulocitos-macrófagos (GMCSF o CSF2); ácido hialurónico; el GF de fibroblastos 2 (FGF2); el factor activador de plaquetas (PAF), el GF transformante alfa y beta (TGFA, TGFB) y el GF derivado de plaquetas alfa (PDGFA) (Block et al. 2011, Bonilla et al. 2011, Dobbs et al. 2013, Fields et al. 2011, Gopichandran and Leese 2006, Larson et al. 1992a, Larson et al. 1992b, Sakagami et al. 2012, Trigal et al. 2011).

Mediante el ensayo de proliferación de fibroblastos bovinos se observó que el péptido recombinante rHDGF resultó ser bioactivo tanto en presencia como en ausencia de suero. El suero puede contener cofactores que ayudan a los GF a ejercer sus efectos fisiológicos (Francis 2010). Sin embargo, los medios de cultivo químicamente definidos permiten normalizar las condiciones de cultivo y evitar riesgos sanitarios propios de los componentes de origen animal (Wydooghe et al. 2013).

Una vez determinada la concentración idónea de bioactividad del rHDGF, analizamos el efecto del GF sobre el desarrollo embrionario *in vitro*. Los efectos de rHDGF dependieron del estadio de desarrollo. Así, cuando rHDGF se añade a partir del Día 5, la proporción de blastocistos expandidos en Día 7 disminuye drásticamente y el número de células permanece invariable. Por el contrario, cuando se añade a partir del Día 6, rHDGF promueve el desarrollo embrionario y la proliferación celular. Estos efectos específicos del estadio de desarrollo del embrión *in vitro* también se han observado con IGF1, EGF, activina, CSF2, FGF2, TGFA, TGFB y PDGF (Dobbs et al. 2013, Fields et al. 2011, Larson et al. 1992a, Larson et al. 1992b, Loureiro et al. 2009, Trigal et al. 2011). Una posible explicación para estos efectos reside en la expresión también temporal de los receptores de factores de crecimiento en el embrión (Schultz and Heyner 1992).

Podría ocurrir que HDGF regule la transición epitelio-mesenquimal en las células embrionarias, al igual que ocurre en células cancerígenas y sanas (Chen et al. 2012, Tsai et al. 2013). En estas células, HDGF regula la expresión de E-cadherina y vimentina, proteínas con un papel específico en la formación del blastocisto (Yamakoshi et al. 2012). En el embrión, dada la localización de HDGF solo en células del trofectodermo, los efectos de rHDGF sobre la proliferación celular en el trofectodermo podrían producirse directamente, mientras que la estimulación del crecimiento de las células de

la ICM podría requerir la intervención de un segundo mediador. Curiosamente, HGF regula la diferenciación de células madre embrionarias humanas (Schuldiner et al. 2000), aunque se desconoce si se localiza en la ICM del blastocisto bovino. En nuestro estudio, la adición de rHDGF no afectó a los índices de apoptosis. Estos resultados contrastan con otros estudios en células cancerígenas, según los cuales HDGF inhibe la apoptosis (Hsu et al. 2012, Liao et al. 2010, Song et al. 2014). Sin embargo, en fibroblastos sanos HDGF es prescindible para el desencadenamiento de la apoptosis (Gallitzendoerfer et al. 2008).

En esta tesis hemos demostrado los efectos positivos de rHDGF tras su adición al medio de cultivo libre de proteína de embriones, en grupos o individuales. El diseño de sistemas que mejoren el cultivo individual de embriones es de gran interés ya que éste presenta varias ventajas respecto al cultivo en grupo. En primer lugar, el cultivo individual evita interacciones paracrinas que pueden llevar a conclusiones erróneas en el ensayo de proteínas durante el cultivo *in vitro* de embriones. Además, el análisis espectroscópico del medio de cultivo condicionado por el embrión permite detectar, de forma no invasiva.el sexo del embrión y la viabilidad de la preñez (Muñoz et al. 2014a, Muñoz et al. 2014c). En bovino, el cultivo individual tiene un importante interés comercial ya que es frecuente que animales con alto mérito genético sólo puedan donar uno o dos ovocitos viables por ciclo (Wydooghe et al. 2013). Por último, el cultivo individual es el único posible a la hora de establecer modelos para el cultivo de embriones humanos (Hansen 2010, Van Soom et al. 2011).

La producción de embriones bovinos mediante IVF y cultivo *in vitro* es una valiosa herramienta para la investigación y en la industria. Sin embargo, la calidad de los EPIV continúa siendo menor que la de los embriones que se desarrollan en el animal vivo (Driver et al. 2012, Rizos et al. 2002). La presente tesis ha demostrado que el estudio proteómico del FU durante el desarrollo temprano puede identificar proteínas, como el HDGF, que mejoran el desarrollo embrionario *in vitro*. Además, la magnitud de los procesos postranscripcionales que hemos identificado durante el desarrollo embrionario resalta la importancia de analizar el proteoma y no sólo el transcriptoma .En contraste con nuestras observaciones, otros estudios no han identificado diferencias en el transcriptoma o proteoma del tracto reproductivo materno antes de los Días 13 ó 10 (Forde et al. 2011, Forde et al. 2012, Forde et al. 2014, Spencer et al. 2013). Nuestro modelo experimental, que implica la presencia de varios embriones en el útero, podría

amplificar la respuesta uterina, que de otro modo sería difícil de cuantificar. Este modelo ha sido validado en un estudio previo (Muñoz et al. 2012) y las respuesta observada en el FU es coherente con la producida por un único embrión de manera fisiológica.

ESTUDIOS FUTUROS

Las proteínas y vías moleculares descritas en esta tesis pueden continuar siendo caracterizadas en estudios futuros para tratar de comprender su funcionalidad. Adaptar el medio de cultivo *in vitro* a las condiciones óptimas para el desarrollo embrionario, teniendo en cuenta además el sexo del embrión, será un paso decisivo en el progreso de las técnicas de reproducción asistida.

Por ejemplo, en el caso de HDGF, aún existen numerosas incógnitas acerca de su expresión durante el desarrollo temprano ya que hasta donde sabemos, su presencia en el tracto reproductivo no había sido reportada antes de la publicación de nuestro artículo. Entre los posibles estudios a realizar sugerimos: (1) evaluar la expresión de proteoglicanos heparán sulfato, las moléculas a las que se une HDGF en la superficie celular (Wang et al. 2011), en el endometrio y embrión bovinos; (2) analizar si HGF, como posible mediador en la señalización de HDGF, se expresa en los mismos tipos celulares que HDGF en el endometrio y embrión bovinos; y (3) investigar si los efectos estadio-específicos de HDGF sobre el desarrollo *in vitro* se correlacionan con una expresión temporal de E-cadherina y vimentina en el blastocisto.

En el desarrollo temprano, encontramos de interés investigar la función de proteínas del FU que varían con el sexo del embrión. Algunas de ellas, como la tioredoxina y el glutatión, mejoran el desarrollo embrionario *in vitro* (Ozawa et al. 2006). Por tanto, los efectos positivos de estas proteínas y de otras aún por ensayar, podrían verse incrementados al tener en cuenta el sexo del embrión. Otro aspecto por aclarar es el efecto de la concentración de glucosa y fructosa sobre el desarrollo embrionario. Se precisaría estudiar si el uso de bajas concentraciones de glucosa en combinación con concentraciones de fructosa adaptadas a las necesidades de los embriones macho y hembra puede ayudar a mejorar el desarrollo embrionario *in vitro*.

Por último, aún se desconocen los mecanismos por los cuales el útero podría reconocer el sexo del embrión. El IFNT es un posible candidato dada su expresión diferencial por embriones macho y hembra. Por tanto, es interesante analizar si esta citoquina, al igual que otros miembros de su familia, induce la expresión del inmunoproteasoma. Igualmente, sería útil investigar la expresión diferencial según el sexo del embrión de otras moléculas de señalización candidatas como TNF, H_2O_2 y NO.

CONCLUSIONES

Los resultados de esta tesis permiten concluir que durante la comunicación materno-embrionaria temprana en el útero bovino intervienen los siguientes factores y procesos:

1. Expresión de citoquinas y factores de crecimiento.

1.1. Las citoquinas TNF e IL1B; sus receptores TNFR2 e IL1R1; y el factor de crecimiento HDGF, se expresan en el endometrio y embrión.

1.2. El mRNA de las citadas proteínas se expresa en el endometrio y el embrión.

2. Localización de citoquinas y factores de crecimiento.

2.1. TNF, IL1B y HDGF se localizan en el epitelio luminal, epitelio glandular, estroma, y trofectodermo. En el endotelio, también se localizan TNF y HDGF.

2.2. TNF e IL1B colocalizan con sus receptores, TNFR2 e IL1R1, en todos los tejidos descritos.

2.3. Todas las proteínas se presentan en el citoplasma. TNFR2 se encuentra en el núcleo y el citoplasma. HDGF presenta dos patrones de localización, nuclear en los epitelios luminal y glandular superficial y citoplasmático en el epitelio glandular basal.

3. Respuesta de las proteínas del endometrio a la presencia de embriones.

3.1. Los ligandos TNF e IL1B aumentan su expresión en el endometrio.

3.2. La expresión de TNF tiende a ser mayor específicamente en el epitelio luminal, epitelio glandular basal y endotelio. La expresión de IL1B aumenta localmente en las carúnculas.

3.3. La expresión del receptor TNFR2 aumenta en el endometrio, específicamente en su epitelio glandular basal.

3.4. La respuesta se limita, en efecto, a cambios en proteínas, sin afectar a los niveles de mRNA.

4. Respuesta de las proteínas del embrión tras su pasaje por el tracto uterino.

4.1. La expresión del mRNA de *TNF* permanece por debajo de los límites de detección. La expresión del mRNA de *HDGF* disminuye en el embrión.

4.2. La expresión del mRNA del receptor *TNFR2* disminuye en el embrión.

5. Respuesta inmune.

5.1. El número de los leucocitos endometriales disminuye en el epitelio glandular funcional y en el estroma subepitelial debido a la presencia de embriones.

6. Efecto funcional de HDGF.

6.1. La adición de una proteína recombinante de HDGF, rHDGF, al cultivo *in vitro* de embriones bovinos incrementa el desarrollo de blastocistos y el número de células en la masa celular interna y el trofectodermo.

6.2. Estos efectos dependen del estadio en el que este factor de crecimiento es añadido.

7. Los efectos postivos de rHDGF se producen cuando es añadido al medio de cultivo sin suero de embriones, tanto en grupo como individuales.

8. Dimorfismo sexual.

8.1. El endometrio puede reconocer el sexo del embrión, según reflejan los cambios en la composición del fluido uterino.

8.2. Las vías moleculares involucradas incluyen: regulación en el procesamiento de antígeno, glicolisis, biosíntesis, antioxidación y apoptosis.

8.3. La concentración de fructosa es mayor en el fluido uterino de las hembras en comparación con el fluido uterino de los machos, mientras que los niveles de glucosa son similares.

8.4. Los embriones machos inducen un ambiente uterino más favorable para su desarrollo que el de los embriones hembras.

CONCLUSIONS

Based on the results of this thesis, we conclude that during the early embryo-maternal communication in the bovine uterus, there are the following factors and processes involved:

1. Expression of cytokines and growth factors.

1.1. Cytokines TNF e IL1B; their receptors TNFR2 e IL1R1; and the growth factor HDGF, are expressed in the endometrium and embryo.

1.2. The mRNA of mentioned proteins is expressed in the endometrium and embryo.

2. Localization of cytokines and growth factors.

2.1. TNF, IL1B and HDGF are localized in the luminal epithelium, glandular epithelium, stroma and trophectoderm. In the endothelium, are also localized TNF and HDGF.

2.2. TNF e IL1B colocalize with their receptors, TNFR2 e IL1R1, in all the tissues described above.

2.3. All proteins are localized in the cytoplasm. TNFR2 is localized in the nucleus and cytoplasm. HDGF has two localization patterns, nuclear in the luminal and superficial glandular ephitelia, and cytoplasmic in the basal glandular epithelium.

3. Response of endometrial proteins to the embryo presence

3.1. The ligands TNF e IL1B increase their protein expression in the endometrium.

3.2. The expression of TNF tends to be higher in the luminal epithelium, basal glandular epithelium and endothelium. The expression of IL1B increases locally in the caruncles.

3.3. The receptor TNFR2 increases its expression in endometrium, specifically in the basal glandular epithelium.

3.4. The endometrial response is limited to protein changes, without affecting levels of mRNA.

4. Response of embryonic proteins after its passage through the uterine tract.

4.1. The mRNA expression of *TNF* remains undetectable in the embryo. The mRNA expression of *HDGF* decreases in the embryo.

4.2. The mRNA expression of the receptor *TNFR2* decreases in the embryo.

5. Immune response.

5.1. The number of endometrial leukocytes decreases in the functional glandular epithelium and in the subepithelial stroma due to the presence of embryos.

6. Functional effect of HDGF.

6.1. The addition of a recombinant protein of HDGF, rHDGF, to the *in vitro* culture of bovine embryos increases blastocyst development and number of cells in the inner cell mass and trophectoderm.

6.2. These effects depend on the stage at wich the factor is added.

6.3. The positive effects of rHDGF occur when this growth factor is added to defined culture media of embryos, both in groups and individuals.

7. Sexual dimorphism.

7.1. Endometrium can recognise the embryonic sex, as reflected by changes in the composition of uterine fluid.

7.2. The molecular pathways involved include: regulation of antigen processing, glycolysis, biosynthesis, antioxidation and apoptosis.

7.3. Fructose concentration is higher in female uterine fluid compared with male uterine fluid, whereas glucose levels are similar.

7.4. Male embryos induce a more favorable uterine environment for their development than female embryos do.

REFERENCIAS

Abouzied, M.M., El-Tahir, H.M., Prenner, L., Häberlein, H., Gieselmann, V., Franken, S., 2005. Hepatoma-derived Growth Factor: significance of amino acid residues 81-100 in cell surface interaction and proliferative activity. Journal of Biological Chemistry. 280, 10945-10954.

Al-Gubory, K.H., Arianmanesh, M., Garrel, C., Bhattacharya, S., Cash, P., Fowler, P.A., 2014. Proteomic analysis of the sheep caruncular and intercaruncular endometrium reveals changes. Reproduction.147, 599-614.

Almiñana, C., Heath, P.R., Wilkinson, S., Sanchez-Osorio, J., Cuello, C., Parrilla, I., Gil, M.A., Vazquez, J.L., Vazquez, J.M., Roca, J., 2012. Early developing pig embryos mediate their own environment in the maternal tract. PloS One. 7, e33625.

Angeles, A., Fung, G., Luo, H., 2011. Immune and non-immune functions of the immunoproteasome. Frontiers in bioscience (Landmark edition). 17, 1904-1916.

Armstrong, R.N., 1997. Structure, catalytic mechanism, and evolution of the glutathione transferases. Chemical Research in Toxicology. 10, 2-18.

Barañao, R., Piazza, A., Rumi, L., Fried, E., 1997. Determination of IL-1 and IL-6 Levels in Human Embryo Culture-Conditioned Media. American Journal of Reproductive Immunology. 37, 191-194.

Bauersachs, S., Ulbrich, S., Gross, K., Schmidt, S., Meyer, H., Wenigerkind, H., Vermehren, M., Sinowatz, F., Blum, H., Wolf, E., 2006. Embryo-induced transcriptome changes in bovine endometrium reveal species-specific and common molecular markers of uterine receptivity. Reproduction. 132, 319 - 331.

Ben-Yair, E., Less, A., Lev, S., Ben-Yehoshua, L., Tartakovsky, B., 1997. Tumour necrosis factor alpha binding to human and mouse trophoblast. Cytokine. 9, 830-836.

Bermejo-Álvarez, P., Rizos, D., Lonergan, P., Gutierrez-Adan, A., 2011. Transcriptional sexual dimorphism during preimplantation embryo development and its consequences for developmental competence and adult health and disease. Reproduction. 141, 563-570.

Betts, J., Hansen, P., 1992. Regulation of prostaglandin secretion from epithelial and stromal cells of the bovine endometrium by interleukin-1 beta, interleukin-2, granulocyte-macrophage colony stimulating factor and tumor necrosis factor-alpha. Life Sciences. 51, 1171-1176.

Bing, Y., Hirao, Y., Takenouchi, N., Che, L., Nakamura, H., Yodoi, J., Nagai, T., 2003. Effects of thioredoxin on the preimplantation development of bovine embryos. Theriogenology. 59, 863-873.

Block, J., Hansen, P., Loureiro, B., Bonilla, L., 2011. Improving post-transfer survival of bovine embryos produced *in vitro*: Actions of insulin-like growth factor-1, colony stimulating factor-2 and hyaluronan. Theriogenology. 76, 1602-1609.

Bonilla, A., Ozawa, M., Hansen, P., 2011. Timing and dependence upon mitogen-activated protein kinase signaling for pro-developmental actions of insulin-like growth factor 1 on the preimplantation bovine embryo. Growth Hormone and IGF Research. 21, 107-111.

Bourdiec, A., Martel, V., Akoum, A., 2014. Synchronous regulation of the determinants of endometrial receptivity to interleukin 1 at key stages of early embryo implantation in vivo. Fertility and Sterility. 101, 1183-1193.

Collavoli, A., Comelli, L., Cervelli, T., Galli, A., 2011. The Over-expression of the B2 Catalytic Subunit of the Proteasome Decreases Homologous Recombination and Impairs DNA Double-Strand Break Repair in Human Cells. Journal of Biomedicine and Biotechnology. 2011, 7.

Chen, C., Spencer, T.E., Bazer, F.W., 2000. Expression of Hepatocyte Growth Factor and its Receptor c-metin the Ovine Uterus. Biology of Reproduction. 62, 1844-1850.

Chen, Q., Zhang, Y., Elad, D., Jaffa, A.J., Cao, Y., Ye, X., Duan, E., 2013. Navigating the site for embryo implantation: biomechanical and molecular regulation of intrauterine embryo distribution. Molecular Aspects of Medicine. 34, 1024-1042.

Chen, S.C., Kung, M.L., Hu, T.H., Chen, H.Y., Wu, J.C., Kuo, H.M., Tsai, H.E., Lin, Y.W., Wen, Z.H., Liu, J.K., 2012. Hepatoma-derived growth factor regulates breast cancer cell invasion by modulating epithelial–mesenchymal transition. The Journal of pathology. 228, 158-169.

Davidson, J., Tiemann, U., Betts, J., Hansen, P., 1995. DNA synthesis and prostaglandin secretion by bovine endometrial cells as regulated by interleukin-1. Reproduction, Fertility and Development. 7, 1037-1043.

De Los Santos, M.J., Mercader, A., Francés, A., Portoles, E., Remohí, J., Pellicer, A., Simon, C., 1996. Role of endometrial factors in regulating secretion of components of the immunoreactive human embryonic interleukin-1 system during embryonic development. Biology of Reproduction. 54, 563-574.

Dobbs, K.B., Khan, F.A., Sakatani, M., Moss, J.I., Ozawa, M., Ealy, A.D., Hansen, P.J., 2013. Regulation of pluripotency of inner cell mass and growth and differentiation of trophectoderm of the bovine embryo by colony stimulating factor 2. Biology of Reproduction. 89, 141, 141-110.

Driver, A.M., Peñagaricano, F., Huang, W., Ahmad, K.R., Hackbart, K.S., Wiltbank, M.C., Khatib, H., 2012. RNA-Seq analysis uncovers transcriptomic variations between morphologically similar in vivo-and in vitro-derived bovine blastocysts. BMC Genomics. 13, 118-127.

Faulkner, S., Elia, G., Mullen, M.P., O'boyle, P., Dunn, M.J., Morris, D., 2012. A comparison of the bovine uterine and plasma proteome using iTRAQ proteomics. Proteomics. 12, 2014-2023.

Fields, S.D., Hansen, P.J., Ealy, A.D., 2011. Fibroblast growth factor requirements for in vitro development of bovine embryos. Theriogenology. 75, 1466-1475.

Fischer, R., Maier, O., Naumer, M., Krippner-Heidenreich, A., Scheurich, P., Pfizenmaier, K., 2011. Ligand-induced internalization of TNF receptor 2 mediated by a di-leucin motif is dispensable for activation of the NFκB pathway. Cellular Signalling. 23, 161-170.

Forde, N., Carter, F., Spencer, T., Bazer, F., Sandra, O., Mansouri-Attia, N., Okumu, L., Mcgettigan, P., Mehta, J., Mcbride, R., 2011. Conceptus-induced changes in the endometrial transcriptome: how soon does the cow know she is pregnant? Biology of Reproduction. 85, 144-156.

Forde, N., Duffy, G.B., Mcgettigan, P.A., Browne, J.A., Mehta, J.P., Kelly, A.K., Mansouri-Attia, N., Sandra, O., Loftus, B.J., Crowe, M.A., Fair, T., Roche, J.F., Lonergan, P., Evans, A.C.O., 2012. Evidence for an early endometrial response to pregnancy in cattle: both dependent upon and independent of interferon tau. Physiological Genomics. 44, 799-810.

Forde, N., Mcgettigan, P.A., Mehta, J.P., O'hara, L., Mamo, S., Bazer, F.W., Spencer, T., Lonergan, P., 2014. Proteomic analysis of uterine fluid during the pre-implantation period of pregnancy in cattle. Reproduction. 147, 575-587.

Francis, G.L., 2010. Albumin and mammalian cell culture: implications for biotechnology applications. Cytotechnology. 62, 1-16.

Gallitzendoerfer, R., Abouzied, M.M., Hartmann, D., Dobrowolski, R., Gieselmann, V., Franken, S., 2008. Hepatoma-derived growth factor (HDGF) is dispensable for normal mouse development. Developmental Dynamics. 237, 1875-1885.

Gao, H., Wu, G., Spencer, T.E., Johnson, G.A., Li, X., Bazer, F.W., 2009. Select nutrients in the ovine uterine lumen. I. Amino acids, glucose, and ions in uterine lumenal flushings of cyclic and pregnant ewes. Biology of Reproduction. 80, 86-93.

Garrido-Gómez, T., Dominguez, F., Quiñonero, A., Estella, C., Vilella, F., Pellicer, A., Simon, C., 2012. Annexin A2 is critical for embryo adhesiveness to the human endometrium by RhoA activation through F-actin regulation. The FASEB Journal. 26, 3715-3727.

Ghosh, S., Hayden, M.S., 2008. New regulators of NF-κB in inflammation. Nature Reviews Immunology. 8, 837-848.

Gopichandran, N., Leese, H.J., 2006. The effect of paracrine/autocrine interactions on the in vitro culture of bovine preimplantation embryos. Reproduction. 131, 269-277.

Griffiths, H.R., Dias, I.H.K., Willetts, R.S., Devitt, A., 2014. Redox regulation of protein damage in plasma. Redox Biology. 2, 430-435.

Groebner, A.E., Schulke, K., Schefold, J.C., Fusch, G., Sinowatz, F., Reichenbach, H.D., Wolf, E., Meyer, H.H.D., Ulbrich, S.E., 2011. Immunological mechanisms to establish embryo tolerance in early bovine pregnancy. Reproduction, Fertility and Development. 23, 619-632.

Gutiérrez-Adán, A., Perez-Crespo, M., Fernandez-Gonzalez, R., Ramirez, M., Moreira, P., Pintado, B., Lonergan, P., Rizos, D., 2006. Developmental Consequences of Sexual Dimorphism During Pre-implantation Embryonic Development. Reproduction in Domestic Animals. 41, 54-62.

Guyader-Joly, C., Khatchadourian, C., Ménézo, Y., 1996. Comparative glucose and fructose incorporation and conversion by in vitro produced bovine embryos. Zygote. 4, 85-91.

Hansen, P.J., 2010. Medawar redux - an overview on the use of farm animal models to elucidate principles of reproductive immunology. American Journal of Reproductive Immunology. 64, 225-230.

Hayden, M.S., Ghosh, S., 2011. NF-κB in immunobiology. Cell Research. 21, 223-244.

Heink, S., Ludwig, D., Kloetzel, P.-M., Krüger, E., 2005. IFN-γ-induced immune adaptation of the proteasome system is an accelerated and transient response.

Proceedings of the National Academy of Sciences of the United States of America. 102, 9241-9246.

Hsu, S.-S., Chen, C.-H., Liu, G.-S., Tai, M.-H., Wang, J.-S., Wu, J.-C., Kung, M.-L., Chan, E.C., Liu, L.-F., 2012. Tumorigenesis and prognostic role of hepatoma-derived growth factor in human gliomas. Journal of Neuro-Oncology. 107, 101-109.

Hugentobler, S., Humpherson, P., Leese, H., Sreenan, J., Morris, D., 2008. Energy substrates in bovine oviduct and uterine fluid and blood plasma during the oestrous cycle. Molecular Reproduction and Development. 75, 496-503.

Jokhi, P.P., King, A., Loke, Y.W., 1997. Cytokine production and cytokine receptor expression by cells of the human first trimester placental - uterine interface. Cytokine. 9, 126-137.

Kimura, K., Spate, L., Green, M., Roberts, R.M., 2005. Effects of D-glucose concentration, D-fructose, and inhibitors of enzymes of the pentose phosphate pathway on the development and sex ratio of bovine blastocysts. Molecular Reproduction and Development. 72, 201-207.

Kimura, K., Spate, L.D., Green, M.P., Murphy, C.N., Seidel, G.E., Michael Roberts, R., 2004a. Sexual dimorphism in interferon-τ production by in vivo-derived bovine embryos. Molecular Reproduction and Development. 67, 193-199.

Kimura, K., Spate, L.D., Green, M.P., Roberts, R.M., 2004b. Effects of oxidative stress and inhibitors of the pentose phosphate pathway on sexually dimorphic production of IFN-τ by bovine blastocysts. Molecular Reproduction and Development. 68, 88-95.

Koch, J.M., Ramadoss, J., Magness, R.R., 2010. Proteomic profile of uterine luminal fluid from early pregnant ewes. Journal of Proteome Research. 9, 3878-3885.

Kwun, J., Chang, K., Lim, J., Lee, E., Lee, B., Kang, S., Hwang, W., 2003. Effects of exogenous hexoses on bovine in vitro fertilized and cloned embryo development: Improved blastocyst formation after glucose replacement with fructose in a serum-free culture medium. Molecular Reproduction and Development. 65, 167-174.

Laborde, E., 2010. Glutathione transferases as mediators of signaling pathways involved in cell proliferation and cell death. Cell Death and Differentiation. 17, 1373-1380.

Larson, M.A., Kimura, K., Kubisch, H.M., Roberts, R.M., 2001. Sexual dimorphism among bovine embryos in their ability to make the transition to expanded blastocyst and in the expression of the signaling molecule IFN-τ. Proceedings of the National Academy of Sciences. 98, 9677-9682.

Larson, R., Ignotz, G., Currie, W., 1992a. Platelet derived growth factor (PDGF) stimulates development of bovine embryos during the fourth cell cycle. Development. 115, 821-826.

Larson, R., Ignotz, G., Currie, W., 1992b. Transforming growth factor β and basic fibroblast growth factor synergistically promote early bovine embryo development during the fourth cell cycle. Molecular Reproduction and Development. 33, 432-435.

Ledgard, A.M., Lee, R.F., Peterson, A.J., 2009. Bovine endometrial legumain and TIMP-2 regulation in response to presence of a conceptus. Molecular Reproduction and Development. 76, 65-74.

Lee, K.H., Choi, E.Y., Kim, M.K., Lee, S.H., Jang, B.I., Kim, T.N., Kim, S.W., Kim, S.W., Song, S.K., Kim, J.-R., 2010. Hepatoma-derived growth factor regulates the bad-mediated apoptotic pathway and induction of vascular endothelial growth factor in stomach cancer cells. Oncology Research Featuring Preclinical and Clinical Cancer Therapeutics. 19, 67-76.

Liao, F., Liu, M., Lv, L., Dong, W., 2010. Hepatoma-derived growth factor promotes the resistance to anti-tumor effects of nordihydroguaiaretic acid in colorectal cancer cells. European Journal of Pharmacology. 645, 55-62.

Loureiro, B., Bonilla, L., Block, J., Fear, J.M., Bonilla, A.Q., Hansen, P.J., 2009. Colony-stimulating factor 2 (CSF-2) improves development and posttransfer survival of bovine embryos produced in vitro. Endocrinology. 150, 5046-5054.

Mansouri-Attia, N.R., Aubert, J., Reinaud, P., Giraud-Delville, C., Taghouti, G.R., Galio, L., Everts, R.E., Degrelle, S.V., Richard, C., Hue, I., Yang, X., Tian, X.C., Lewin, H.A., Renard, J.P., Sandra, O., 2009. Gene expression profiles of bovine caruncular and intercaruncular endometrium at implantation. Physiological Genomics. 39, 14-27.

Mao, J., Xu, Z., Fang, Y., Wang, H., Xu, J., Ye, J., Zheng, S., Zhu, Y., 2008. Hepatoma-derived growth factor involved in the carcinogenesis of gastric epithelial cells through promotion of cell proliferation by Erk1/2 activation. Cancer Science. 99, 2120-2127.

Mishto, M., Liepe, J., Textoris-Taube, K., Keller, C., Henklein, P., Weberruß, M., Dahlmann, B., Enenkel, C., Voigt, A., Kuckelkorn, U., 2014. Proteasome isoforms exhibit only quantitative differences in cleavage and epitope generation. European Journal of Immunology. 44, 3508-3521.

Mitko, K., Ulbrich, S., Wenigerkind, H., Sinowatz, F., Blum, H., Wolf, E., Bauersachs, S., 2008. Dynamic changes in messenger RNA profiles of bovine endometrium during the oestrous cycle. Reproduction. 135, 225 - 240.

Muñoz, M., Corrales, F.J., Caamaño, J.N., Díez, C., Trigal, B., Mora, M.I., Martín, D., Carrocera, S., Gómez, E., 2012. Proteome of the early embryo-maternal dialogue in the cattle uterus. Journal of Proteome Research. 11, 751-766.

Muñoz, M., Uyar, A., Correia, E., Díez, C., Fernandez-Gonzalez, A., Caamaño, J., Martínez-Bello, D., Trigal, B., Humblot, P., Ponsart, C., 2014a. Prediction of pregnancy viability in bovine in vitro-produced embryos and recipient plasma with Fourier transform infrared spectroscopy. Journal of Dairy Science. 97, 5497-5507.

Muñoz, M., Uyar, A., Correia, E., Díez, C., Fernandez-Gonzalez, A., Caamaño, J.N., Trigal, B., Carrocera, S., Seli, E., Gomez, E., 2014b. Non-invasive assessment of embryonic sex in cattle by metabolic fingerprinting of in vitro culture medium. Metabolomics. 10, 443-451.

Muñoz, M., Uyar, A., Correia, E., Ponsart, C., Guyader-Joly, C., Martínez-Bello, D., Marquant-Le Guienne, B., Fernandez-Gonzalez, A., Díez, C., Caamaño, J.N., 2014c. Metabolomic prediction of pregnancy viability in superovulated cattle embryos and recipients with fourier transform infrared spectroscopy. BioMed research international. 2014, article ID 608579.

Neira, J.A., Tainturier, D., L´Haridon, R., Martal, J., 2011. Relationship between IFN-t production by bovines embryos derived ex vivo and completely produced in vitro. Reproduction in Domestic Animals. 42, 68-75.

Okuda, K., Sakumoto, R., Okamoto, N., Acosta, T.J., Abe, H., Okada, H., Sinowatz, F., Skarzynski, D.J., 2010. Cellular localization of genes and proteins for tumor necrosis factor-a (TNF), TNF receptor types I and II in bovine endometrium. Molecular and Cellular Endocrinology. 330, 41-48.

Ozawa, M., Nagai, T., Fahrudin, M., Karja, N.W.K., Kaneko, H., Noguchi, J., Ohnuma, K., Kikuchi, K., 2006. Addition of glutathione or thioredoxin to culture medium reduces intracellular redox status of porcine IVM/IVF embryos, resulting in improved development to the blastocyst stage. Molecular Reproduction and Development. 73, 998-1007.

Ozawa, M., Sakatani, M., Yao, J., Shanker, S., Yu, F., Yamashita, R., Wakabayashi, S., Nakai, K., Dobbs, K.B., Sudano, M.J., 2012. Global gene expression of the inner cell mass and trophectoderm of the bovine blastocyst. BMC Developmental Biology. 12, 33-46.

Paula-Lopes, F.F., Moraes, A.a.D., Hansen, P., 1999. The presence of interleukin-1beta in the bovine reproductive tract. Journal of Interferon & Cytokine Research. 19, 279-285.

Pérez-Crespo, M., Ramirez, M., Fernández-González, R., Rizos, D., Lonergan, P., Pintado, B., Gutiérrez-Adán, A., 2005. Differential sensitivity of male and female mouse embryos to oxidative induced heat-stress is mediated by glucose-6-phosphate dehydrogenase gene expression. Molecular Reproduction and Development. 72, 502-510.

Peter, A.T., 2013. Bovine placenta: a review on morphology, components, and defects from terminology and clinical perspectives. Theriogenology. 80, 693-705.

Pla, A., Pascual, M., Renau-Piqueras, J., Guerri, C., 2014. TLR4 mediates the impairment of ubiquitin-proteasome and autophagy-lysosome pathways induced by ethanol treatment in brain. Cell Death & Disease. 5, e1066.

Rai, P., Shivaji, S., 2011. The role of DJ-1 in the pathogenesis of endometriosis. PloS One. 6, e18074.

Rengaraj, D., Park, T.S., Lee, S.I., Lee, B.R., Han, B.K., Song, G., Han, J.Y., 2013. Regulation of Glucose Phosphate Isomerase by the 3′UTR-Specific miRNAs miR-302b and miR-17-5p in Chicken Primordial Germ Cells. Biology of Reproduction. 89, 33, 31-11.

Rizos, D., Ward, F., Duffy, P., Boland, M.P., Lonergan, P., 2002. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. Molecular Reproduction and Development. 61, 234-248.

Roberts, R.M., Chen, Y., Ezashi, T., Walker, A.M., 2008. Interferons and the maternal–conceptus dialog in mammals. Seminars in Cell & Developmental Biology. 19, 170-177.

Ross, J.W., Ashworth, M.D., Mathew, D., Reagan, P., Ritchey, J.W., Hayashi, K., Spencer, T.E., Lucy, M., Geisert, R.D., 2010. Research activation of the transcription

factor, nuclear factor kappa-B, during the estrous cycle and early pregnancy in the pig. 8, 39-56.

Ross, J.W., Malayer, J.R., Ritchey, J.W., Geisert, R.D., 2003. Characterization of the interleukin-1β system during porcine trophoblastic elongation and early placental attachment. Biology of Reproduction. 69, 1251-1259.

Sakagami, N., Umeki, H., Nishino, O., Uchiyama, H., Ichikawa, K., Takeshita, K., Kaneko, E., Akiyama, K., Kobayashi, S., Tamada, H., 2012. Normal calves produced after transfer of embryos cultured in a chemically defined medium supplemented with epidermal growth factor and insulin-like growth factor I following ovum pick up and in vitro fertilization in Japanese Black cows. Journal of Reproduction and Development. 58, 140-146.

Satterfield, M.C., Hayashi, K., Song, G., Black, S.G., Bazer, F.W., Spencer, T.E., 2008. Progesterone regulates FGF10, MET, IGFBP1, and IGFBP3 in the endometrium of the ovine uterus. Biology of Reproduction. 79, 1226-1236.

Schaefer, T.M., Wright, J.A., Pioli, P.A., Wira, C.R., 2005. IL-1β-mediated proinflammatory responses are inhibited by estradiol via down-regulation of IL-1 receptor type I in uterine epithelial cells. The Journal of Immunology. 175, 6509-6516.

Schuldiner, M., Yanuka, O., Itskovitz-Eldor, J., Melton, D.A., Benvenisty, N., 2000. Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. Proceedings of the National Academy of Sciences. 97, 11307-11312.

Schultz, G., Heyner, S., 1992. Growth factors in preimplantation mammalian embryos. Oxford Reviews of Reproductive Biology. 15, 43-81.

Sijts, E., Kloetzel, P.-M., 2011. The role of the proteasome in the generation of MHC class I ligands and immune responses. Cellular and Molecular Life Sciences. 68, 1491-1502.

Simón, C., Mercader, A., Frances, A., Gimeno, M.J., Polan, M.L., Remohí, J., Pellicer, A., 1996. Hormonal regulation of serum and endometrial IL-1α, IL-1β and IL-1ra: IL-1 endometrial microenvironment of the human embryo at the apposition phase under physiological and supraphysiological steroid level conditions. Journal of Reproductive Immunology. 31, 165-184.

Simón, C., Piquette, G.N., Frances, A., Polan, M.L., 1993. Localization of interleukin-1 type I receptor and interleukin-1 beta in human endometrium throughout the menstrual cycle. The Journal of Clinical Endocrinology & Metabolism. 77, 549-555.

Song, Y., Hu, Z., Long, H., Peng, Y., Zhang, X.A., Que, T., Zheng, S., Li, Z., Wang, G., Yi, L., 2014. A complex mechanism for HDGF-mediated cell growth, migration, invasion, and TMZ chemosensitivity in glioma. Journal of Neuro-Oncology. 119, 285-295.

Spencer, T.E., Forde, N., Dorniak, P., Hansen, T.R., Romero, J.J., Lonergan, P., 2013. Conceptus-derived prostaglandins regulate gene expression in the endometrium prior to pregnancy recognition in ruminants. Reproduction. 146, 377-387.

Spencer, T.E., Johnson, G.A., Bazer, F.W., Burghardt, R.C., 2004. Implantation mechanisms: insights from the sheep. Reproduction. 128, 657-668.

Sturmey, R., Bermejo-Álvarez, P., Gutierrez-Adan, A., Rizos, D., Leese, H., Lonergan, P., 2010. Amino acid metabolism of bovine blastocysts: a biomarker of sex and viability. Molecular Reproduction and Development. 77, 285-296.

Tabibzadeh, S., Zupi, E., Babaknia, A., Liu, R., Marconi, D., Romanini, C., 1995. Physiology: Site and menstrual cycle-dependent expression of proteins of the tumour necrosis factor (TNF) receptor family, and BCL-2 oncoprotein and phase-specific production of TNFalpha in human endometrium. Human Reproduction. 10, 277-286.

Tain, Y.-L., Kao, Y.-H., Hsieh, C.-S., Chen, C.-C., Sheen, J.-M., Lin, I., Huang, L.-T., 2010. Melatonin blocks oxidative stress-induced increased asymmetric dimethylarginine. Free Radical Biology and Medicine. 49, 1088-1098.

Tanikawa, M., Kim, T.S., Okuda, K., Ryoo, Z.Y., Park, S.B., Shin, J.H., Park, C.K., Lee, D.S., 2009. Cell-type specificity of interleukins 1α and 1β on prostaglandin and plasminogen activator production in bovine endometrial cells. Animal Reproduction Science. 114, 32-42.

Trigal, B., Gómez, E., Díez, C., Caamaño, J., Martín, D., Carrocera, S., Muñoz, M., 2011. *In vitro* development of bovine embryos cultured with activin A. Theriogenology. 75, 584-588.

Tsai, H.E., Wu, J.C., Kung, M.L., Liu, L.F., Kuo, L.H., Kuo, H.M., Chen, S.C., Chan, E.C., Wu, C.S., Tai, M.H., 2013. Up-regulation of hepatoma-derived growth factor facilities tumor progression in malignant melanoma. PloS One. 8, e59345.

Ulbrich, S.E., Frohlich, T., Schulke, K., Englberger, E., Waldschmitt, N., Arnold, G.J., Reichenbach, H.-D., Reichenbach, M., Wolf, E., Meyer, H.H., 2009. Evidence for estrogen-dependent uterine serpin (SERPINA14) expression during estrus in the bovine endometrial glandular epithelium and lumen. Biology of Reproduction. 81, 795-805.

Van Soom, A., Wydooghe, E., Heras, S., Vandaele, L., 2011. Alternative models for the study of embryo–maternal cross-talk and signaling molecules from fertilisation to implantation. Reproduction, Fertility and Development. 23, iii-v.

Vitiello, D., Pinard, R., Taylor, H.S., 2008. Gene expression profiling reveals putative HOXA10 downstream targets in the periimplantation mouse uterus. Reproductive Sciences. 15, 529-535.

Von Wolff, M., Classen-Linke, I., Heid, D., Krusche, C.A., Beier-Hellwig, K., Karl, C., Beier, H.M., 1999. Tumour necrosis factor-α (TNF- α) in human endometrium and uterine secretion: an evaluation by immunohistochemistry, ELISA and semiquantitative RT-PCR. Molecular Human Reproduction. 5, 146-152.

Walker, C., Meier, S., Littlejohn, M., Lehnert, K., Roche, J., Mitchell, M., 2010. Modulation of the maternal immune system by the pre-implantation embryo. BMC Genomics. 11, 474.

Wang, C., Davamani, F., Sue, S., Lee, S., Wu, P., Tang, F., Shih, C., Huang, T., Wu, W., 2011. Cell surface heparan sulfates mediate internalization of the PWWP/HATH domain of HDGF via macropinocytosis to fine-tune cell signalling processes involved in fibroblast cell migration. Biochem. J. 433, 127-138.

Wang, Y., Wang, C., Hou, Z., Miao, K., Zhao, H., Wang, R., Guo, M., Wu, Z., Tian, J., An, L., 2013. Comparative analysis of proteomic profiles between endometrial

caruncular and intercaruncular areas in ewes during the peri-implantation period. Journal of animal science and biotechnology. 4, 39-50.

Witkin, S.S., Liu, H.C., Davis, O.K., Rosenwaks, Z., 1991. Tumor necrosis factor is present in maternal sera and embryo culture fluids during in vitro fertilization. Journal of Reproductive Immunology. 19, 85-93.

Wydooghe, E., Heras, S., Dewulf, J., Piepers, S., Van Den Abbeel, E., De Sutter, P., Vandaele, L., Van Soom, A., 2013. Replacing serum in culture medium with albumin and insulin, transferrin and selenium is the key to successful bovine embryo development in individual culture. Reproduction, Fertility and Development. 26, 717-724.

Yamakoshi, S., Bai, R., Chaen, T., Ideta, A., Aoyagi, Y., Sakurai, T., Konno, T., Imakawa, K., 2012. Expression of mesenchymal-related genes by the bovine trophectoderm following conceptus attachment to the endometrial epithelium. Reproduction. 143, 377-387.

Yokota, T., Sugawara, K., Ito, K., Takahashi, R., Ariga, H., Mizusawa, H., 2003. Down regulation of DJ-1 enhances cell death by oxidative stress, ER stress, and proteasome inhibition. Biochemical and Biophysical Research Communications. 312, 1342-1348.