UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE VETERINARIA
Departamento de Fisiología Animal

TESIS DOCTORAL

Bovine embryo-maternal interaction: implications for embryo development and quality

Interacción materno-embrionaria en la especie bovina : implicaciones para el desarrollo y la calidad embrionaria

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Director

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El doctor Dimitrios Rizos, Investigador Titular del Departamento de Reproducción Animal del Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) hace constar:

Que la memoria de Tesis Doctoral titulada: "**Bovine Embryo-Maternal Interaction: Implications For Embryo Development and Quality" / "Interacción Materno Embrionaria en la Especie Bovina: Implicaciones Para el Desarrollo y la Calidad Embrionaria**", ha sido realizada por Dña. Meriem Hamdi en el Departamento de Reproducción Animal del INIA bajo mi dirección, y que tras su revisión, considero que reúne los requisitos exigidos y la debida calidad para su presentación y defensa para optar el título de Doctor en Veterinaria por la Universidad Complutense de Madrid.

Madrid, 15 de Febrero de 2018

Fdo. D. Dimitrios Rizos

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“Cuando deseas alcanzar un objetivo en la vida,

El universo entero conspira para que lo logres.”

Paulo Coelho

“Ose rêver, Ose essayer, Ose te tromper,

... Ose avoir du succès”

Kingsley Ward

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LIST OF ABBREVIATIONS

3D	Three-dimensional
ANOVA	One-way analysis of variance
ART	Assisted reproductive technology
BMP	Bone morphogenetic protein
BOEC	Bovine oviduct epithelial cells
BSA	Bovine serum albumin
C3	Complement protein 3
cDNA	Complementary dna
CL	Corpus luteum
CM	Conditioned media
CM-H2DCFDA	Chloromethyl-20-70-dichlorodihydrofluorescein diacetate
COCs	Cumulus–oocyte complexes
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
E2	Estradiol
EGA	Embryonic genome activation
EGF	Epidermal growth factor
ET	Embryo transfer
EV	Extracellular vesicles
FCS	Fetal calf serum
HA	Hyaluronic acid
ICM	Inner cell mass
IFNT	Interferon tau
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVP	*In vitro* embryo production
LOS	Large Offspring Syndrome
LH	Luteinizing hormone
MII	Metaphase II
MET	Maternal-to-embryonic transition
MMLV	Moloney murine leukaemia virus
mRNA	Messenger Ribonucleic acid
NEFA	Non esterified fatty acid
OF	Oviductal fluid
OVGP1	Oviductin, or oviduct-specific glycoprotein
P4	Progesterone
PBS	Phosphate-buffered saline
PGF2α	Prostaglandin F2α
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SOF	Synthetic oviductal fluid
TALP	Tyrode's albumin lactate pyruvate
TCM-199	Tissue culture medium 199
TE	Trophectoderm cells
UF	Uterine fluid
ZP	Zona pellucida

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SUMMARY

The early preimplantation period represents a critical period during embryo development. *In vivo,* the bovine embryo spends the first four days in the oviduct where it is in contact with epithelia cells and oviductal fluid (OF). During this period, important morphological and metabolic changes occur, including the first mitotic division and embryonic genome activation (EGA). Then, the embryo enters the uterus and remains free floating in the uterine fluid (UF) until the initiation of implantation at approximately Days 19–22. In cattle, the high failure rate to maintain pregnancy is generally attributed to early embryonic loss prior to maternal recognition of pregnancy. Thus, the study of physiological mechanisms and interactions during the preimplantational period is essential to understand early embryo development process and to decrease embryonic loss.

Moreover, the advances in such studies may support the improvement of assisted reproductive techniques such as *in vitro* embryo production (IVP) that seeks to mimic the physiological conditions in order to develop an embryo in a proper stage and quality. Despite, many advances have been achieved in recent decades in IVP; *in vitro* conditions are suboptimal as evidenced by lower blastocyst yields (30–40%) and *in vitro* embryo still displaying numerous marked differences from their *in vivo* counter-parts with regard to morphology, timing of development, cryotolerance, embryonic metabolism, and gene expression. Various *in vitro* culture (IVC) systems have been used to support early embryo development. However, embryo requirements are far from to be completely defined. The aim of the first chapter was to reproduce the physiological conditions of the oviductal and uterine environment and assessing the effects of both OF and UF supplementation during IVC on bovine embryo development and quality. The results revealed that *in vitro* sequential culture with OF and UF at low concentration (1.25%) in serum-free culture media supports embryo development and improves blastocyst quality which was reflected by an increasing of embryo survival rate after vitrification/warming process, an upregulation of epigenetic genes (*DNMT3A* and *IGF2R*) primarily indicating a better control of embryo methylation, predominantly due to OF effect. In addition to a decreased abundance of oxidative stress genes (*CLIC1* and *GPX1*) accompanied with a low accumulation of reactive oxygen species (ROS) in the produced blastocyst, primarily attributed to the effect of the UF. This study may provide a step towards the characterization of relevant proteins for embryo development present in the physiological environment of the reproductive tract (OF and UF) to improve assissted reproductives techniques (ART) in mammals.

The conclusion from the first chapter supports the importance of embryo exposure to the maternal environment, highlighting the necessity to understand the embryo-maternal crosstalk during the pre-implantation period. The uterus has been extensively studied in relation to embryo-maternal communication where it has been identified complex signals to achieve a

normal pregnancy. However, little is known about the interaction between the oviduct and the embryo during the first stages of embryo development. Recent *in vivo* research suggested that interactions of the embryo with the oviduct epithelium may be very local make it difficult to detect. Therefore, *in vitro* model would be an easier alternative to understand the complex interactions between the embryo and the maternal environment.

In the second chapter of this thesis using nontoxic polyester mesh we established an *in vitro* co-culture system allowing a local and temporal interaction between early bovine embryos or their conditioned media (CM) and isthmus epithelial cells in order to know if the embryo affect bovine oviduct epithelial cell (BOEC) transcriptome during early stages and whether it is due to a contact-depending signaling, or the result of BOEC interaction with embryo secretions. Finally we wanted to elucidate if the early embryo induces changes on energy and amino acids metabolism of BOEC.

The result revealed that *in vitro* culture of BOEC provides a suitable model for the study of the mechanisms involved in the interaction of gametes and embryos with the mammalian oviduct. Moreover, results of gene expression revealed that early embryo has an effect on BOEC transcriptome differentially depending on embryo stage. The effect seems to be due to a direct contact with embryo or with their secretions released into the media. Furthermore, energy substrates and amino acid analysis revealed that BOEC metabolism was not affected by presence of early embryos (2-cell and 8-cell stage) or by their CM. Interestingly, our result suggest that embryo metabolism is inactive before EGA and supports previous studies providing that embryo may have sufficient endogenous energy to support its early development

Taking into account the exciting results of the two previous chapters, we decided to go a step further. In fact, different signaling pathways could mediate the embryo-maternal crosstalk. Among the candidate signaling pathways, we focused our attention on the signaling mediated by bone morphogenetic proteins (BMPs). BMPs are expressed in an anatomically and temporally regulated fashion in bovine oviduct. However, a local response of this signaling to the presence of the embryo has yet to be elucidated. Therefore, the objective of the third chapter was to investigate changes in the gene expression of BMP signaling components after early embryo-oviduct interaction *in vitro*. BOEC were co-cultured with early embryos during the main phase of EGA using the same system of Chapter 2. Exposure to embryos, irrespective of the period, significantly reduced the relative abundance of *BMPR1B*, *BMPR2*, *SMAD1*, *SMAD6* and *ID2* mRNAs in BOEC. In contrast, embryos that interacted with BOEC before EGA showed a significant increase in the relative abundance of *SMAD1* while embryos that interacted with BOEC during EGA showed a significant increase in *BMPR1B*, *BMPR2* and *ID2* mRNA. These results demonstrate that embryo-oviduct interaction *in vitro* induces a bidirectional response of BMP signaling, by reducing their expression in the oviductal cells while increases

them in the early embryo. Finally, results from Chapter 3 suggest that BMP signaling pathway may be involved in an early cross talk between the bovine embryo and the oviduct during the first stages of development.

RESUMEN

El período preimplantacional representa una etapa crítica durante el desarrollo embrionario. *In vivo*, el embrión bovino pasa los primeros cuatros días en el oviducto donde está en contacto con el fluido oviductal (*oviductal fluid*, OF). Durante este período ocurren importantes cambios morfológicos y metabólicos, que incluyen la primera división mitótica y la activación del genoma embrionario (*embryo genome activation,* EGA). Luego, el embrión entra en el útero y permanece flotando libremente en el fluido uterino (*uterine fluid,* UF) hasta el inicio de la implantación a los 19-22 días aproximadamente. Generalmente, en ganadería, la alta tasa de perdida gestacional se atribuye a la pérdida embrionaria temprana, es decir, antes del reconocimiento materno del embarazo. Por lo tanto, el estudio de los mecanismos fisiológicos y las interacciones durante ese periodo es esencial para comprender el proceso de desarrollo embrionario temprano y para disminuir la pérdida embrionaria.

Los avances en tales estudios pueden además mejorar las técnicas de reproducción asistida, como la producción embrionaria *in vitro* (*in vitro embryo production,* IVP), que busca imitar las condiciones fisiológicas para desarrollar embriones de buena calidad. A pesar de los avances logrados en las últimas décadas en la IVP, las condiciones *in vitro* siguen siendo subóptimas. En la actualidad la tasa de desarrollo embrionario hasta el estadio de blastocisto se encuentra entorno al 30-40%. Además existen varias diferencias entre los embriones producidos *in vitro* e *in vivo* que se ven reflejadas en la morfología, el tiempo de desarrollo, la criotolerancia, el metabolismo embrionario y la expresión génica. Con el objetivo de mejorar el desarrollo embrionario temprano, se han utilizado varios sistemas de cultivo *in vitro* (*in vitro culture*, IVC). Sin embargo, existen todavía muchos interrogantes sobre las necesidades embrionarias durante este periodo. El objetivo del primer capítulo fue por lo tanto imitar las condiciones fisiológicas del ambiente oviductal y uterino durante la fase preimplantacional, evaluando los efectos de la suplementación del OF y UF durante el IVC sobre el desarrollo y la calidad embrionaria. Los resultados revelaron que el cultivo de embriones en medios de cultivo sin suero suplementados con una baja concentración de OF y UF (1.25%) permite el desarrollo embrionario y mejora la calidad de los blastocitos obtenidos. Esta mejoría se reflejó por un aumento de la tasa de supervivencia embrionaria tras el proceso de vitrificación. Así mismo, se observó un incremento en la expresión de genes implicados en procesos epigenéticos (*DNMT3A* e *IGF2R*) indicando un mejor control de la metilación del embrión, predominantemente debido al efecto del OF. Además, se obtuvo una disminución de la expresión de genes relacionados con el estrés oxidativo (*CLIC1* y *GPX1*), acompañada de una disminución en la acumulación de radicales libres de oxigeno (*reactive oxygen species,* ROS), atribuidas principalmente al efecto del UF. Este estudio supone un paso hacia la caracterización de proteínas presentes en el entorno fisiológico del tracto reproductivo (OF y UF) y relevantes para el desarrollo

embrionario, permitiendo la mejoría de las técnicas de reproducción asistida (*assissted reproductives techniques*, ART) en mamíferos.

La conclusión del primer capítulo confirma la importancia de la exposición embrionaria al ambiente materno, además de destacar la necesidad de estudiar la comunicación materno-embrionaria durante el período preimplantacional. Tal comunicación ha sido ampliamente estudiada a nivel del útero, donde se han identificado algunas de las señales implicadas en el desarrollo de una gestación normal. Sin embargo, existe un escaso conocimiento acerca de esta comunicación a nivel del oviducto. Recientes trabajos *in vivo* sugieren que las interacciones que tienen lugar entre el embrión y el epitelio del oviducto pueden tener un efecto muy local y, por tanto, son difíciles de detectarse. Como consecuencia, un modelo *in vitro* podría ser una alternativa más adaptada para estudiar dichas interacciones.

En el segundo capítulo de esta tesis, establecimos un sistema de co-cultivo *in vitro* permitiendo una interacción local y temporal entre embriones de estadios tempranos o sus medios condicionados (*conditioned media,* CM) con las células epiteliales de la parte del istmo del oviducto bovino (*bovine oviduct epithelial cells* BOEC). El objetivo fue, en primer lugar, evaluar el efecto del embrión sobre el transcriptoma de las BOEC y si tal efecto se debe a una señalización dependiente del contacto o es debido a la interacción con las secreciones embrionarias; en segundo lugar, el objetivo fue evaluar si el embrión en estadio temprano induce cambios en el metabolismo energético y de amino ácidos de las BOEC.

El resultado de este experimento confirmó que el cultivo *in vitro* de las BOEC facilita un modelo adecuado para el estudio de los mecanismos implicados en la interacción materno-embrionaria en el oviducto. Los resultados de la expresión génica revelaron que el embrión afecta el transcriptoma de las BOEC diferencialmente dependiendo de su estadio (antes o durante la EGA). Tal efecto es debido al contacto directo con el embrión, con sus secreciones liberadas en el medio o a ambos. Respecto al metabolismo de BOEC, los análisis de los substratos energéticos y de los aminoácidos revelaron que dicho metabolismo no se vio afectado por la presencia de embriones en estadio temprano (2-cell and 8-cell stage) ni por su CM. Además estos resultados sugieren que el metabolismo embrionario se encuentra inactivo antes de la EGA, apoyando la idea aportada en estudios anteriores de que el embrión tiene suficiente energía endógena para su desarrollo temprano.

Teniendo en cuenta los interesantes resultados de los dos capítulos anteriores, se decidió dar un paso más en el estudio de una de las vías de señalización que puede estar implicada en la comunicación materno-embrionaria en el oviducto. Se centró el interés en la señalización mediada por las proteínas morfogenéticas del hueso (*Bone Morphogenetic Proteins*, BMPs). Las BMP se expresan de una manera regulada anatómica y temporalmente en el oviducto bovino.

Sin embargo, aún no se ha establecido una relación entre su expresión y la presencia del embrión. Por lo tanto, el objetivo del tercer capítulo fue investigar los cambios en la expresión génica de los componentes de la vía de señalización de BMP tras la interacción embrio-oviductal *in vitro*. Las BOEC se co-cultivaron con embriones tempranos durante las principales fases de EGA usando el mismo sistema que en el Capítulo 2. La exposición a embriones, independientemente de su estadio, disminuyó significativamente la expresión génica de *BMPR1B*, *BMPR2*, *SMAD1*, *SMAD6* e *ID2* en las BOEC. Por el contrario, los embriones que interactuaron con BOEC antes de EGA mostraron un incremento significativo del gen *SMAD1*, mientras que los embriones que interactuaron con las BOEC durante EGA mostraron un aumento significativo de los genes *BMPR1B*, *BMPR2* e *ID2*. Estos resultados demuestran que la interacción *in vitro* del embrión con las BOEC induce una respuesta bidireccional de la vía de señalización de BMP, reduciendo su expresión en las BOEC al mismo tiempo que aumenta la expresión en el embrión. Finalmente, los resultados del Capítulo 3 sugieren que la vía de señalización BMP puede estar involucrada en la comunicación materno-embrionaria durante las primeras etapas de desarrollo.

LITERATURE REVIEW

Introduction

A comprehensive understanding of the complex embryo-maternal interactions during the preimplantation period requires the analysis of very early stages of pregnancy: early embryonic development, implantation and maintenance of a pregnancy. So far, only few signals involved in this dialogue have been identified in bovine. Interferon tau (IFNT) is the predominant embryonic pregnancy recognition signal. However, this is just one aspect of the complex process of embryo-maternal signaling and a number of other mechanisms are more likely to be involved (Wolf *et al.* 2003). Furthermore, in cattle, the majority of pregnancy loss is attributed to early embryonic loss prior to maternal recognition of pregnancy (Diskin and Morris 2008) which occurs by approximately day 16 following conception (Northey and French 1980). Therefore, the knowledge of mechanisms controlling embryo-maternal communication should help to increase the pregnancy rate following embryo transfer (ET) and to avoid embryonic losses (Wolf *et al.* 2003).

On the other hand, despite the fact that embryo development until blastocyst stage is somewhat autonomous (i.e., does not need contact with the maternal reproductive tract) and it has been successfully developed *in vitro* using *in vitro* fertilization (IVF) technology (Mamo *et al.* 2012), many researches on ruminant embryo production has focused on the fundamental question of why only 30% to 40% of immature oocytes develop to the blastocyst stage. Furthermore, the quality of such blastocysts continually lags behind that of blastocysts produced *in vivo*. These differences are manifested at the level of morphology, metabolism, gene expression and cryotolerance and may have a knock-on effect further along the developmental axis (Rizos *et al.* 2008). Evidence suggesting that *in vitro* culture conditions, while capable of producing blastocysts in relatively high numbers, are far from optimal with deficiencies being manifested not only in short term effects but also in long terms as the large offspring (LOS) syndrome (Lazzari *et al.* 2002; Rizos *et al.* 2008). Moreover, a low pregnancy rate was observed with *in vitro* produced embryos (16.7 %) compared with those produced *in vivo* (55.2 %) or in a temporary *in vivo* culture (i.e., ewe oviduct) (53.8 %) (Lazzari *et al.* 2002). Certainly, the passage through the genital tract confers to the embryos improved viability and survival to cryopreservation (Rizos *et al.* 2002c; Havlicek *et al.* 2010). Once more, it is important to note the relevance of gaining insight into the embryo−maternal interactions, which would allow the design of novel strategies leading to improve *in vitro* embryo conditions.

Therefore, projects involving specialists in embryology, reproductive biotechnology and functional genome research are necessary to perform a systematic analysis of interactions between preimplantation stage embryos and oviduct or uterine environment. The knowledge of

these mechanisms should help to increase the pregnancy rate following ET and to avoid embryonic losses. Also to determine candidate genes involved in embryo-maternal communication, which could be used as quality criteria for the selection of embryos for transfer to recipients. Another application is the supplementation of embryotrophic factors or components of embryo-maternal signaling in optimized formulations, such as bioartificial matrices. As a long-term goal, signaling mechanisms identified in bovine will also be functionally evaluated in other species, including the human (Wolf et al. 2003). Systematic studies addressing very early embryo–maternal interactions in bovine have now become feasible with a battery of functional genomics technologies (Hiendleder *et al.* 2005). However, holistic transcriptome and proteome analyses, as well as specific experiments targeting embryo–maternal interactions in the reproductive tract, require sufficient numbers of well-defined cells and embryos in a standardized experimental environment. This is often difficult or impossible to achieve *in vivo* (Rottmayer *et al.* 2006). Since experiments are expensive, the amount of material is limited and it is not possible to differentiate between the outcome of fertilization and early embryonic death. In addition, the local interactions of the embryo with the maternal epithelium may not be detectable because of the small size of the embryo and the difficulty of identifying its exact position in the oviduct (Maillo *et al.* 2016). Hence, a suitable *in vitro* systems would greatly aid the analysis of early embryonic and maternal signaling (Wolf *et al.* 2003).

1. Periconception environment *in vivo*

Previous to fusion, the gametes experiment different events, very important for the subsequent embryo development. The generation of mature, developmentally competent oocyte is the culmination of a highly organized process including primordial follicle recruitment, granulosa/theca cell proliferation, oocyte maturation, and ovulation. Since birth, follicular growth occurs in a wave-like pattern: recruitment, selection, dominance and atresia (Driancourt 2001). By the puberty, the anatomical and hormonal conditions required for regular ovulation are established (Ball 2004). Prior to ovulation, luteinizing hormone (LH) peak is responsible of the oocyte maturation, which involves nuclear and cytoplasmic maturation (Ferreira *et al.* 2009) and cumulus cell expansion. Firstly, during nuclear maturation, meiosis is resumed, characterized by chromosome condensation, progress from prophase I to metaphase II (MII) with extrusion of the first polar body (Palma *et al.* 2012). Immature oocytes that have not progressed through meiosis to MII cannot be successfully fertilized (Beall *et al.* 2010). Secondly, cytoplasmic maturation involves organelle redistribution (mitochondria, ribosomes, endoplasmic reticulum, cortical granules and the Golgi complex), cytoskeleton dynamics and molecular maturation that consists of transcription, storage and processing of maternal mRNA which is stored in a stable inactive form until translational recruitment (Ferreira *et al.* 2009).

The proteins derived from these mRNAs are involved in maturation, fertilization, pronuclear formation and early embryogenesis. In addition, the cortical granules migrate to the periphery of the oocyte where they contribute to the block of polyspermy after fertilization. Finally, cumulus cells secrete hyaluronic acid (HA) that, when it becomes hydrated causes the spaces between the cumulus cells to enlarge and the cells to be embedded in a sticky, mucified matrix (Eppig 2001). This process is termed cumulus expansion and when it is suppressed artificially *in* vivo, ovulation rate is greatly reduced (Chen et al. 1993). After ovulation, fimbria of the infundibulum that surround the ovary allow the passage of the ovulated oocyte into the oviduct. At this point, both muscle layers and ciliated cells mechanically guide the oocyte into the lumen of the ampulla to the site of fertilization (Hunter 1988). In addition, the oocyte loses the cumulus cells and the zona pellucida (ZP) becomes exposed directly to the OF which prepares it for fertilization and minimizes polyspermy (Coy *et al.* 2012b)

After copulation, semen is deposited in the female reproductive tract; in cranial vagina in cow (Senger 2005). Freshly ejaculated spermatozoa are incapable of fertilization. They require a cascade of changes to acquire the fertilizing ability. This process called capacitation involves a sequence of biochemical changes leading to destabilization of sperm membrane, enabling the acrosome reaction in response to the ZP. It is acquired during the migration of the sperm through the female reproductive tract (Machaty *et al.* 2012). It starts with seminal plasma proteins removal by glycosaminoglycans, present in the uterus and finishes in a characteristic sperm motility called hyperactivation (Lin *et al.* 1994). It enables the spermatozoa to cross the surrounding cumulus cells to arrive to the ZP. The contact between sperm and ZP induces acrosome reaction. Followed by an outwardly secretion of acrosome content (proteolytic enzymes) (Bleil and Wassarman 1983). Sperm binding to ZP is mediated by receptors to stimulate the acrosome reaction and to permit the ZP penetration (O'Toole *et al.* 2000). Normally, only one sperm is able to pass through the ZP, but when more enter, a process known as polyspermy occurs, the resultant embryo is non-viable. Following fusion of sperm and egg the contents of the cortical granules in the egg release into the perivitelline space (the cortical reaction), causing the ZP to become refractory to sperm binding (Sun 2003).

Once fertilization occurs, bovine embryo remains in the oviduct until approximately day 4 of pregnancy, by which time it is at about the 16-cell stage and then enters to the uterus. Several major events occur into the oviduct and the uterus before implantation including: (i) the first cleavage, (ii) activation of embryonic genome, (iii) compaction of the morula, (iv) blastocyst formation, (v) elongation.

First cleavage *in vivo* is observed about 20 h after the estimated time of ovulation (Laurincik *et al.* 1994). First mitotic divisions are supported by the mRNA and proteins synthesized and stored in the oocyte during oogenesis (Memili and First 2000).

In cattle, when the embryo reached the 8-16 cell stage, a switching from maternal to embryonic control is occurred (Barnes and First 1991), during which, the embryo passes a complicated pattern of activation. This process called embryonic genome activation (EGA) is characterized by two different forms, minor and major EGA (Figure 1). Minor EGA in bovine has been observed between the 1-cell to 4-cell stages, suggesting that zygotes and early embryos are transcriptionally and translationally actives (Barnes and Eyestone 1990; Memili and First 1999). The major activation of embryonic genome occurs at the 8- to 16-cell stages and includes a gradual degradation of mRNA molecules of maternal origin (Memili and First 2000), activation of transcription of embryonic genome, developmental arrest in the presence of transcriptional inhibitors and a marked change in the pattern of protein synthesis (Telford *et al.* 1990). This transition is essential for the activation of a large number of genes and for the following pattern of gene expression that is responsible for successful embryonic differentiation and development (Lequarre *et al.* 2003; Lonergan *et al.* 2003a; Lonergan *et al.* 2003b).

Beyond the day 5 and 6 after fertilization is the time of compaction, it occurs at 32-64 cell stage (morula) (Bondioli *et al.* 1990; Van Soom *et al.* 1992), when the embryo has already enter to the uterus (Hackett *et al.* 1993). At this stage the first tight junctions between adjacent blastomeres are formed (Boni *et al.* 1999). Then, the activation of an ion transport favors the generation of a cavity inside the morula called blastocoel and leads to blastocyst formation by day 7 (Fleming *et al.* 2000). During this process the cells start to differentiate, originating the inner cell mass (ICM) that will give rise to the fetus and the trophectoderm cells (TE), which will give rise to extra-embryonic tissue (Van Soom *et al.* 1997). On days 9-10 post-fertilization, the ZP begins to fragment and the blastocyst 'hatches' (Wolf *et al.* 2003). After hatching, the blastocyst develops into an ovoid then tubular form and then elongates on days 14-15 to form a filamentous conceptus that occupies the entire length of the uterine horn (Spencer *et al.* 2008). The elongation is a rapid process where the blastocyst develops from <1 cm (Day 12) to >10 cm (Day 16) (Figure 2), essentially because of the rapid trophoblast growth (Robinson *et al.* 2006). During conceptus elongation, progesterone (P4) is required to regulate the outgrowth of the TE (Spencer *et al.* 2007). The elongation initiates IFNT production by TE cells (Spencer and Bazer 2004), reaching a maximum level between Day 15 and Day 17 (Wolf *et al.* 2003). After Day 19, the elongated conceptus begins implantation with firm apposition and attachment of the TE to the endometrial luminal epithelium.

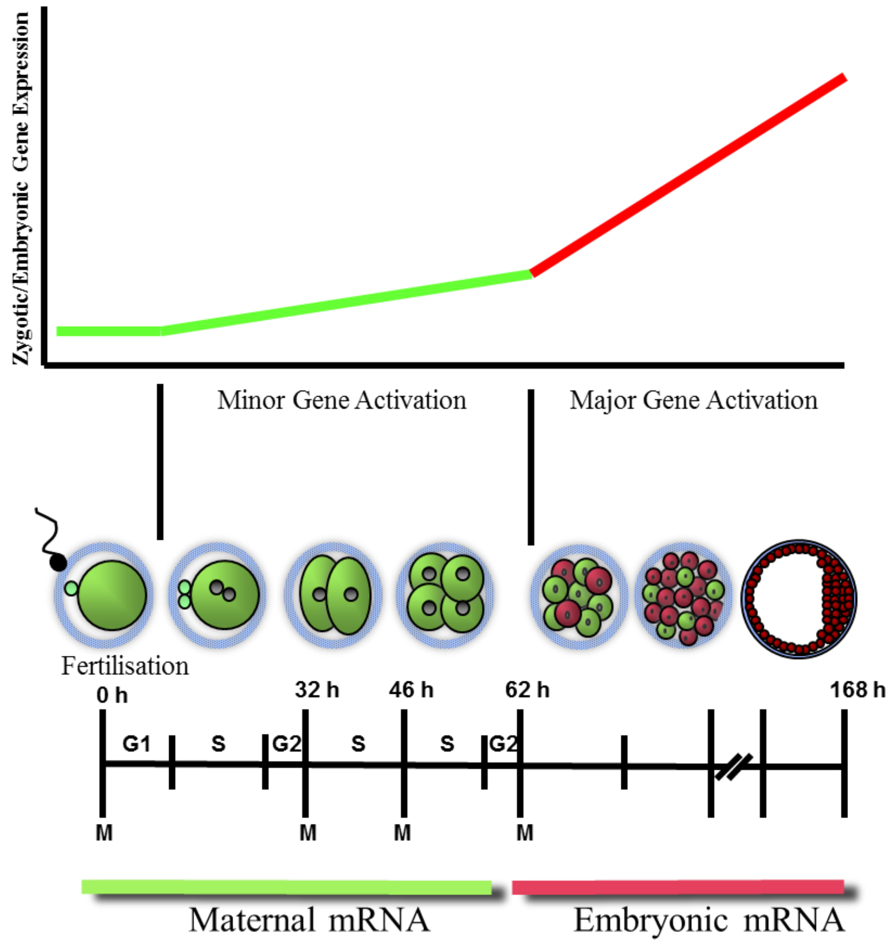

Figure 1. Bovine embryonic cell and zygotic/embryonic gene expression (mRNA synthesis. The cell cycle was adapted from Barnes and Eyestone (1990). There is a "minor gene activation" between the 1-and 4-cell stages and "major gene activation" starting at the 8-cell stage. Changes in the transcriptional machinery and chromatin structure play an important role in the control of early gene expression. Adopted and modified from Memili and First (2000).

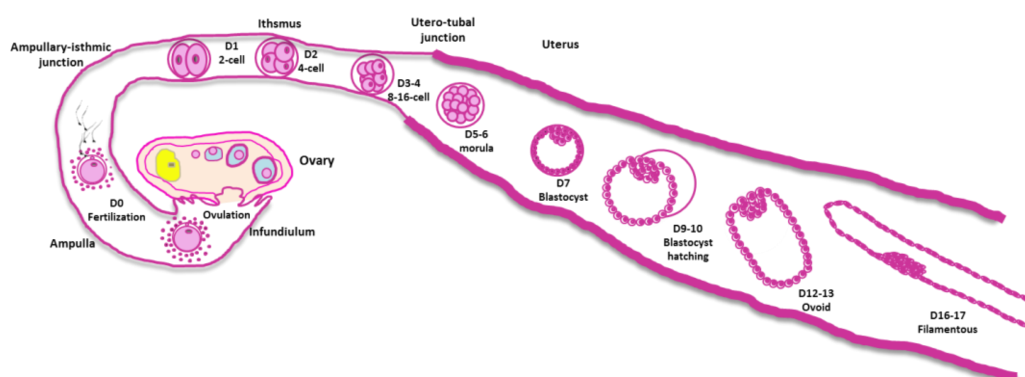

Figure 2: Schematic representation of the process of early embryo development *in vivo* in cattle. After the ovulation, the matured oocyte is fertilized (*D0*) at the ampullary-isthmic junction, while the first cleavage division takes place around 24-30h later in the isthmus (*D1*) followed by subsequent mitotic divisions up to 16-cell stage (*D4*). At this stage, the embryo passes into the uterus through the uterotubal junction and forms a morula (*D5-D6*) and then a blastocyst (*D7*). After hatching from the ZP (*D8*), the morphology of the embryos changes to ovoid (*D12-D13*), then to tubular and filamentous (*D16-D17*) before implantation begins D19 (Rizos *et al.* 2017)

2. ***In vitro* embryo production**

More than three decades have passed since the birth of the first calf by an IVF procedure (born in 1981) (Brackett *et al.* 1982) and many improvements have been made (Machaty *et al.* 2012). *In vitro* fertilization and embryo culture try to simulate as closely as possible the conditions that occur *in vivo* to provide high quality embryos capable of continued development and implantation, and resulting in viable births (Menezo *et al.* 1998). Moreover, the IVP allows us to enhance our understanding of early embryo development, above all, during the preimplantational stages. It is also used in other technologies, such as nuclear transfer and transgenesis (Gordon 2003). Furthermore, considering the many similarities existing between bovine and human embryonic development, it is suggested the use of bovine IVF as one of the models for human reproduction (Menezo and Herubel 2002). IVP allows to obtain embryos from: (i) oocytes derived from slaughtered heifers (Galli *et al.* 2003) or (ii) live donors by ultrasonography follicular aspiration (Ovum pick up - OPU) (van Wagtendonk-de Leeuw 2006). Besides, IVP offers the opportunity to recover and safe oocytes from high genetic value animals when they die, as well as to continue the existence of valuable endangered species (Wu and Zan 2012). The IVP is divided in three steps: *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC):

After the removal of fully grown mammalian oocytes from the ovarian follicles (>2-8 mm), IVM is performed by placing them in a suitable culture medium for 24 h in 38.5 ºC, 5% of

CO_2 and saturated humidity (Gordon 2003) to support the spontaneous resumption of meiosis and development of the oocyte to the MII and the complete of nuclear and cytoplasmic maturation. The oocytes can be cultured in different media classified as simple or complex. Tissue culture medium 199 (TCM-199) is the complex medium more extendedly used. Media are usually supplemented with macromolecules like those contained in fetal calf serum (FCS) or bovine serum albumin (BSA) (Gordon 2003). Also the supplementation of gonadotropins, steroids or growth factors improved the oocyte developmental competence (Lonergan and Fair 2008). Oocyte developmental competence, often defined as the ability of the oocyte to mature, be fertilized and develop to the blastocyst stage, has been associated with: (i) the size of the antral follicle from which it is recovered; (ii) the stage of the follicular wave; and (iii) the site of maturation - *in vivo* or *in vitro* [for review see (Lonergan and Fair 2016)]. Oocytes matured *in vivo* are of better quality than those matured *in vitro* and this is reflected in the number of embryos obtained subsequently (88% vs 35% respectively), irrespective of whether embryo culture occurred *in vivo* or *in vitro* (Rizos *et al.* 2002c). Also, oocytes originating from follicles bigger than 6 mm resulted in significantly more blastocysts than those from 2-6 mm follicles and those recovered prior to the LH surge (Rizos *et al.* 2002c). Recent evidence has shown that suboptimal conditions during oocyte IVM have an effect at the epigenetic level and on genomic imprinting (Anckaert and Fair 2015). *In vitro* models that mimic the *in vivo* situation of high-yielding dairy cows after calving, i.e. in which non esterified fatty acid (NEFA) concentrations are elevated, indicate that not only the oocyte developmental capacity is affected, but also that the phenotype of the resulting embryos is altered (Van Hoeck *et al.* 2011; 2013). Nevertheless, additional studies are required to investigate whether the expression and DNA methylation of imprinted genes in blastocysts, fetuses, and placental tissue derived from IVM oocytes are unaltered [for review see (Lonergan and Fair 2016)]

To mimic *in vivo* fertilization, sperm selection has to take place *in vitro* in a similar manner to that which occurs in the female reproductive tract. The selection of motile sperm is a prerequisite for successful IVF. Thus the sperm quality will be improved by enhancing progressive motility and morphological normal spermatozoa (Samardzija *et al.* 2006). Furthermore, the use of cryopreserved semen as the main source of sperm for IVF; where the proportion of fully functional sperm in a frozen–thawed sample is quite low (Holt 1997), increases the necessity for selection of motile sperm. Different methods to select motile sperm in bovine are used: swim-up (Parrish *et al.* 1986), Percoll® (Saeki *et al.* 1991) and Bovipure™. This last showed less toxicity than Percoll® but the same efficiency (Samardzija *et al.* 2006). After sperm selection, the concentration is adjusted normally to 10^6 sperm /ml and coincubated with the matured oocytes for 18 h, at 38.5 ºC, 5% CO_2 and saturated humidity. The media used is designed with a specific ionic balance for oocyte and sperm requirements [Tyrode's albumin

lactate pyruvate (TALP) or synthetic oviductal fluid (SOF) media] (Parrish 2014) and it usually contains heparin that capacitates the sperm and prepares it for the acrosome reaction to have a successful fertilization (Parrish 2014). Delays in fertilization or fertilization by a damaged spermatozoon could conceivably lead to oocyte aging or the formation of a defective embryo, respectively (Tarin *et al.* 2000). Any damage to the sperm after ejaculation can lead not only to a reduced fertilization rate, but also to the formation of embryos with reduced ability to develop to the blastocyst stage. This phenomenon was observed for sperm exposed to gossypol (Brocas *et al.* 1997), oxidative stress (Silva *et al.* 2007) and sorting for gender by flow cytometry (Wheeler *et al.* 2006; Wilson *et al.* 2006; Bermejo-Alvarez *et al.* 2008; Bermejo-Alvarez *et al.* 2010). In addition, the nature of the sire itself can affect cleavage and the ability of the resulting embryos to develop to the blastocyst stage and to establish pregnancy after transfer [for review see (Hansen *et al.* 2010)]. Studies in trout have shown that the oocyte is able to partially repair sperm with damaged DNA during the first cleavage; however when DNA repair is inhibited, damaged sperm is able to fertilize the oocyte but lead to embryo loss (Perez-Cerezales *et al.* 2010; Perez-Cerezales *et al.* 2011).

Embryo culture is the longest step during the process of IVP. The presumptive zygotes obtained after IVF are selected based on their morphological characteristics like homogeneity of the ZP, perivitelline space and cytoplasm and put into culture. The embryos can be cultured in defined or semi-defined media; co-cultured with oviductal, granulosa or Vero cells; or with CM. Today, the most used media is SOF that is constituted by chemically defined elements based on the biochemical composition of the OF and it is usually supplemented with 5% of FCS or BSA (Tervit *et al.* 1972; Holm *et al.* 1999). The embryos secrete factors that sustain their development and for this reason they grow better in groups than alone (Gardner *et al.* 1994). Thus, in most of the *in vitro* culture systems the embryos are cultured in droplets under paraffin oil. The incubation is performed at 38.5ºC in 5% CO_2, and 5% O_2 or not at maximum humidity. Usually, zygotes cultured *in vitro* until Day 8 or 9. Day 7 is the day which ET *in vivo* take place and is normally used *in vitro* for embryo cryopreservation and to measure the quality of the produced blastocysts by the use of invasive or non-invasive techniques.

3. *In vitro* conditions affecting embryo development and quality

Embryo culture is the longest step during the process of *in vitro* embryo production and the step during which the greatest reduction in development occurs, achieving only 30-40% of blastocyst rate (Rizos *et al.* 2008). **S**everal parameters seem to affect embryo development. The oocyte source and the environment of the ovarian follicle could influence the transcriptome of the matured oocyte and the subsequent cleavage stage of the embryo (Sirard 2012). Furthermore, the cleavage speed has been correlated with the subsequent blastocyst rates in

bovine (Lonergan *et al.* 2000) and humans (Salumets *et al.* 2003). It is known that the faster cleaved embryos have a higher chance to develop to the blastocyst stage (Lonergan *et al.* 1999). Related factors as oocyte, spermatozoa and culture conditions affect the timing of this event (Lechniak *et al.* 2008). Moreover, under *in vitro* conditions the dynamic of embryo development during compaction are related to the subsequent developmental stages (Gutiérrez-Adán *et al.* 2004). In IVP embryos, the compaction occurs in lesser degree that in the *in vivo* counterparts (Van Soom *et al.* 1997).

Embryo quality seems also to be affected by the suboptimal *in vitro* conditions, manifested by a darker morphology (Fair *et al.* 2001; Rizos *et al.* 2002a) (Figure 3), lower cryotolerance (Rizos *et al.* 2008), altered ICM/TE ratio (Plourde *et al.* 2012), altered gene expression patterns (Niemann and Wrenzycki 2000) and lower pregnancy rates of transferable embryos (Pontes *et al.* 2009) when compared to their *in vitro* counterparts. Also, a high demand for energy, manifested by two-fold higher rate of aerobic glycolysis was observed in *in vitro* produced embryos compared to *in vivo* embryos (Khurana and Niemann 2000). Among the factors that could affect the quality of *in vitro* produced blastocyst is the addition of FCS to the culture media. It accelerates embryonic developmental kinetics and increases the number of embryonic cells (Van Langendonckt *et al.* 1997). However, those embryos have a lower level of compaction at the morula stage (Thompson 1997) exhibit a greater accumulation of lipid droplets in the cytoplasm and a lower cryotolerance (Rizos *et al.* 2002a) and present alterations in gene expression (Rizos *et al.* 2003) compared to *in vivo* produced embryos. In addition, serum has been linked to the LOS (Farin *et al.* 2001; Lazzari *et al.* 2002) that causes the birth of large calves with musculoskeletal disorders, alterations in the development of the allantois and defects in vascularization and development of the placenta, showing a smaller area of maternal-fetal contact (Farin *et al.* 2001; Farin *et al.* 2006).

In vitro embryo culture has also been associated with epigenetic alterations in the embryo. During the period of epigenetic reprogramming embryo is especially vulnerable to *in vitro*-induced epigenetic defects (El Hajj and Haaf 2013). Imprinting disorders are more prevalent in gametes and embryos after ARTs than in their counterparts derived from *in vivo* production (Urrego *et al.* 2014). *In vitro* embryo culture has been associated with abnormal reprogramming of imprinted genes such as *SNPRN*, *IGF2* or *H19* in cattle (Nowak-Imialek *et al.* 2008; Curchoe *et al.* 2009; Suzuki *et al.* 2009) and mice (Khosla *et al.* 2001). These alterations to the epigenetic profile may have a direct effect on the subsequent embryo and fetal development.

In vitro manipulation of embryos has also been related with oxidative stress generation and the production of substances such as ammonia, oxygen radicals or growth factors that can

produce lipid peroxidation, membrane injury and structural damage, leading to decreased cryotolerance and apoptosis (Somfai *et al.* 2007) and an impaired development of mammalian embryos (Johnson and Nasr-Esfahani 1994).

4. Evaluation of embryo quality

The best method of embryo evaluation is the ability to establish and maintain a pregnancy after transfer. However, for practical and economic reasons, it is only ever possible to transfer a subset of embryos. Thereby a number of invasive and non-invasive methods are used in the laboratory to measure the quality of the *in vitro* produced embryos. An example of widely used non-invasive method is embryo cryotolerance by measuring the survival rate of the embryo after freezing/thawing (Rizos *et al.* 2002c; Moore *et al.* 2007). As invasive methods, it is used the differential staining of embryos, which provides the relation between the number of cells from the ICM and the TE (Thouas *et al.* 2001); and also the expression of the relative abundance of genes related with embryo quality (i.e. apoptosis, cell connections, antioxidant stress, metabolism, implantation etc)(Wrenzycki *et al.* 2005).

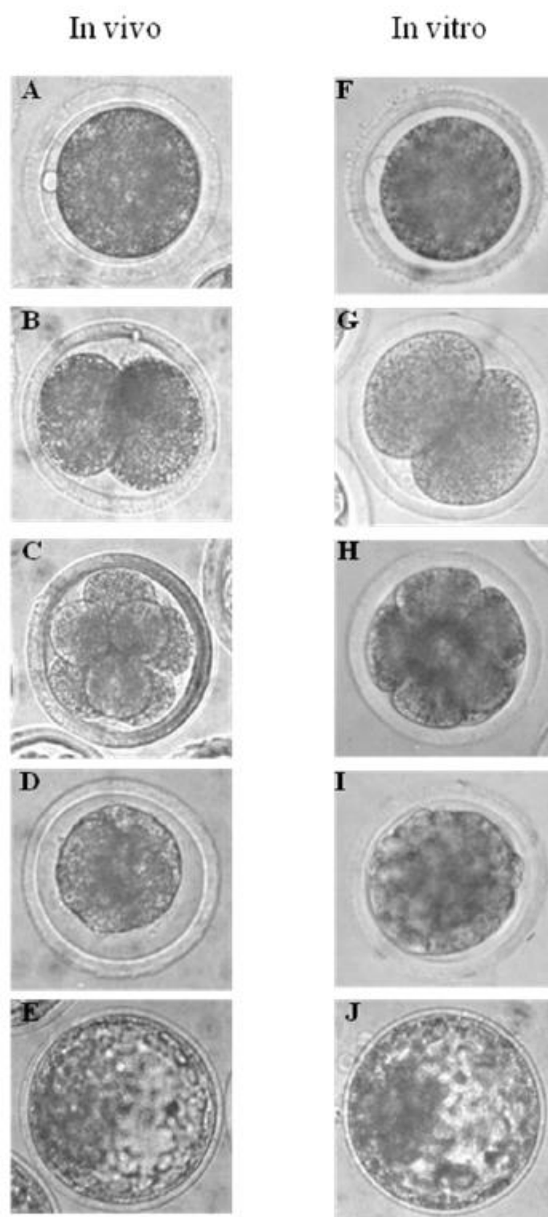

Figure 3: Morphology of bovine embryos produced *in vivo* (A-E) and *in vitro* (F-J). Images are representatives of matured oocytes (A and F), 2-cell embryo (B and G), 8 cell embryo (C and H).morulae (D and I) and blastocyst (E and J) (Rizos *et al.* 2002a)

5. The physiological environment of early embryo

The physiological environment in which early embryo development occurs has a considerable influence on its subsequent growth in the short and long term. In fact, embryo culture of *in vitro* produced bovine presumptive zygotes in cow, sheep or mice oviduct support the growth and improved the overall embryo quality, as measured by survival after cryopreservation (Rizos *et al.* 2002b; Rizos et al. 2007; Havlicek *et al*. 2010) Thus, studies on physiological mechanisms and interactions in the maternal reproductive tract (oviduct and uterus) are essential. Such studies will help advance our knowledge and will support the development of assisted reproductive technologies including *in vitro* embryo production that seek to mimic physiological conditions and generate good quality embryos. These advances

could help improve fertility treatments in humans and domestic animals to increase the efficiency of production and breeding schemes

5.1. Oviduct-anatomomorphogical characteristics

The oviducts (or fallopian tubes) were described for the first time by the 16th century Italian anatomist, Gabriele Falloppio (1523–1562). However, it was not until a century later when Regnier de Graaf (1641–1673) asserted that the human egg transits through the fallopian tubes. At this time, the fallopian tubes were viewed as "chimneys" enabling the smoke to rise from the matrix into the abdominal cavity (Alexandre 2001). The oviduct in bovine is a tubular structure, sustained by the mesosalpinx that connects the ovary with the uterine horn. It is divided in five morphological and functional parts: (i) the infundibulum the most proximal structure to the ovary and it is funnel shaped, (ii) the ampulla the wider part of the tubal structure, (iii) the ampullary-isthmic junction, (iv) the isthmus with the narrowest lumen in the oviduct and (v) the utero-tubal junction (Figure 4). The infundibulum receives the ovulated oocyte with dynamic movements, whereas the ciliated cells guide it to the lumen of the ampulla where the final events of oocyte maturation and fertilization take place (Talbot *et al.* 2003). The oviductal isthmus functions as a sperm reservoir where sperm adhere transiently to the epithelium and are released at the time of ovulation (Talevi and Gualtieri 2010). After fertilization in the ampullary-isthmic junction, the developing embryo passes through the isthmus, supported by ciliary activity and muscular contractions, until it reaches the uterus at about the 16-cell stage on Day 4 (Hackett *et al.* 1993)

Histologically, the oviduct in their external surface it is composed by a serosa layer (Tunica serosa), followed by the Tela subserosa, constituted by muscle fibers and blood vessels. The subsequent muscle layer (Tunica muscularis) is different in each oviduct segments, in the infundibulum and the ampulla it is thinner than in the isthmus. The inner part of the oviduct (Tunica mucosa) is composed by fibrous and cellular connective tissue and epithelial cell layer (Ellington 1991) (Figure 4).

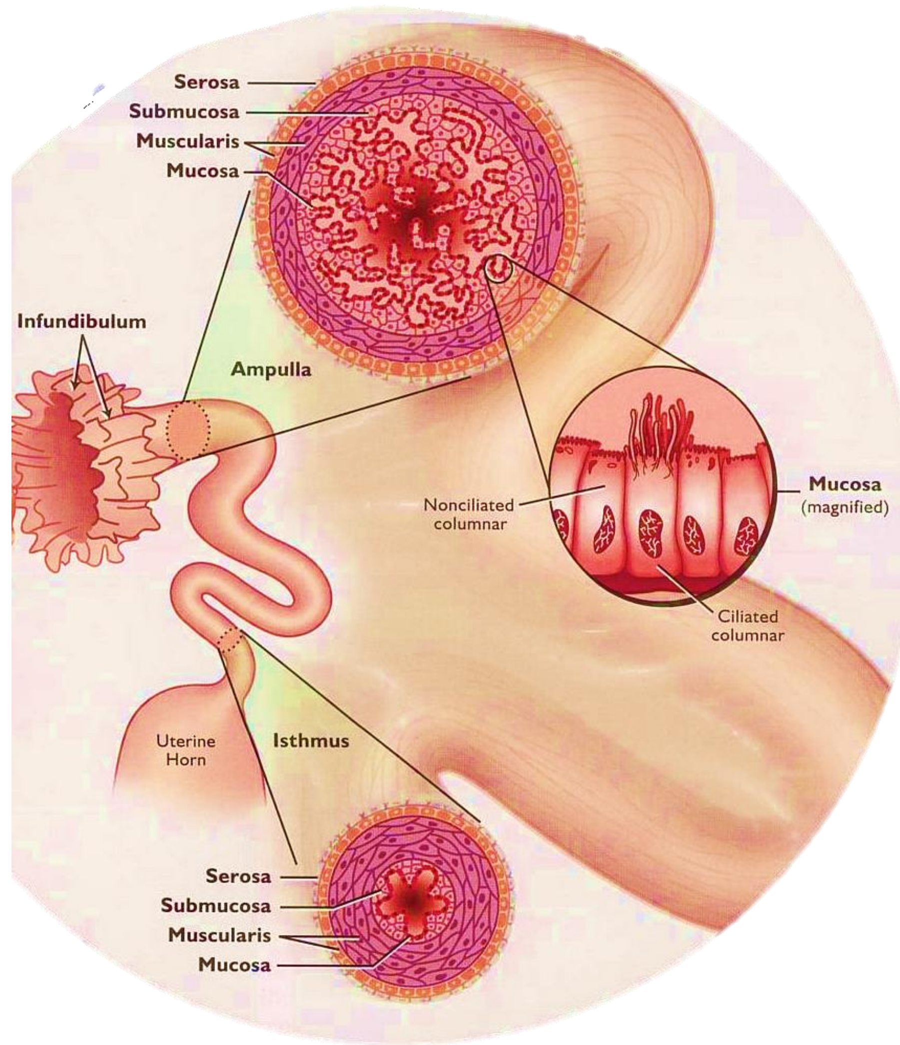

Figure 4: Oviduct components and histology (Senger 2005).

The oviductal epithelium is the intimate part of the oviduct. It is an active site of biosynthesis and secretion of amino acids, energy substances, growth factors and ions (Hugentobler *et al.* 2007; Leese *et al.* 2008), providing an optimal environment for early embryo development (Gandolfi 1995). It consists of ciliated and secretory simple columnar epithelial cells (Yániz *et al.* 2000) (Figure 5). During transport processes, the ciliary show a synchronized movement leading to a directed flow of fluids (Abe and Hoshi 1997). Secretory cells present microvilli on their apical side and secrete substances (oviduct-specific glycoprotein) and growth factors, usually by exocytosis (Abe 1996; Murray 1996; Murray 1997).

The mammalian oviduct undergoes significant endocrine-induced morphological, biochemical and physiological changes during the estrous cycle (Suuroia *et al.* 2002) and the processes happened in the oviduct are dependent on activities of the ciliated and secretory epithelia of the oviduct microenvironment, which are controlled by the ovarian steroids,

estradiol (E2) and P4 (Buhi 2002). In fact assessment of the oviductal transcriptome have elucidated a number of mechanisms regulated by the periovulatory endocrine milieu, i.e., proestrus and estrus increase in E2 and diestrus increase in P4 concentrations, potentially involved in oviductal function (Gonella-Diaza *et al.* 2015). Furthermore, It was observed that estrogen-epithelial receptor α is necessary to suppress oviductal protease activity, which is required for a successful fertilization and preimplantation embryo development (Winuthayanon *et al.* 2015). Recently it was also proposed that, sex-steroids act in an oviduct region-specific fashion and generate a periovulatory endocrine milieu that modulates morphological and functional features of the bovine oviduct which may support embryo survival and development (Gonella-Diaza *et al.* 2017).

The percentage of ciliated cells decreases in the infundibulum and the ampulla during the luteal phase compared with the follicular phase (Yániz *et al.* 2000). Interestingly, it has been suggested that cell morphology is modified in function of embryo development and cyclic changes (Suuroia *et al.* 2002). Thus, the height of ciliated cells decreases in the infundibulum and ampulla in the luteal phase and in the isthmus the height of secretory cells also diminishes (Abe *et al.* 1999). Furthermore, transcriptome approaches have identified different functional groups of genes involved in the regulation of the oviduct during the estrous cycle (Bauersachs *et al.* 2004). Moreover, the presence of gametes in the oviduct also alters the pattern of protein secretion of the epithelial cells (Georgiou *et al.* 2007).

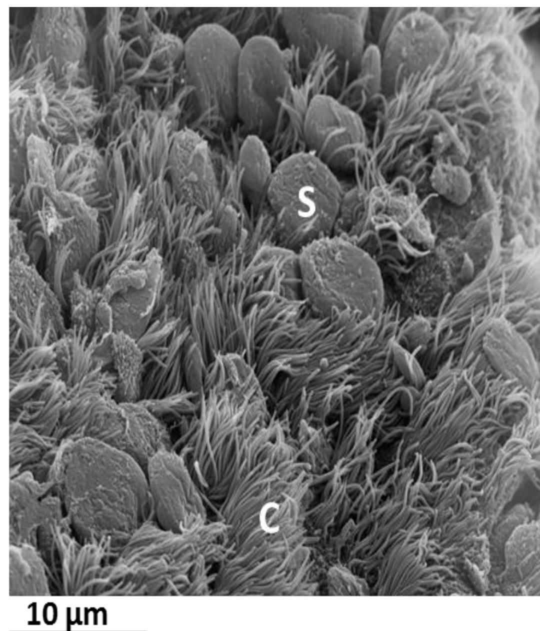

Figure 5: Scanning electron microscopic image of oviduct epithelium (Isthmus part). **C**: ciliated cells; **S**: secretory cells. Bar=10µm

The conditions of oviductal environment are reflected in the OF. The OF is generated by (i) transudation from plasma into the oviductal lumen together with (ii) the secretion of substances synthesized by the secretory cells (Menezo and Guerin 1997). It contains simple and complex carbohydrates, ions, lipids, phospholipids and proteins (Avilés *et al.* 2010) . Some of these components are energy substrates, such as lactate, pyruvate and glucose, as well as amino acids, the concentration of which differs between OF, UF and serum (Hugentobler *et al.* 2007; Hugentobler *et al.* 2008). In bovine, aspartate, glutamate, serine, glycine, tyrosine, phenylalanine, lysine and alanine are amino acids that were present in higher concentrations in OF than in blood plasma (Hugentobler *et al.* 2007). Identification and role of proteins present in the OF is currently the target of many studies because of the potential benefits for *in vitro* embryo culture. Proteomic analysis of OF has reported significant differences in protein content between the different phases of the estrous cycle (Seytanoglu *et al.* 2008). Among the proteins present in OF, Glycodelins, highly evolutionary conserved proteins, have been detected in the human oviduct at least in four isoforms (glycodelin S, A, F and C) based on the differences in glycosylation (Ghersevich *et al.* 2015). The use of recombinant glycodelin A was shown to inhibit capacitation of human and hamster sperm (Dutta *et al.* 2001). Oviductin, or oviduct-specific glycoprotein (OVGP1), is an estrus-associated protein that has been demonstrated to be highly conserved in all species studied (Avilés *et al.* 2010). Analyses of de novo synthesized and secreted proteins from the oviduct epithelia have identified OVPG1 as the major secretory product (Buhi 2002). In pigs and cows, OVGP1 participates in the functional modification of the ZP, which before fertilization makes it more resistant to enzymatic digestion and sperm penetration, contributing to the control of polyspermy. OVGP1 also promotes sperm capacitation while maintaining motility and viability (Coy *et al.* 2012b). Oviductin has been localized in the perivitelline space and the membrane of embryos from different species before implantation, potentially acting as a protective ‘shield’ around the early embryo (Ghersevich *et al.* 2015). Other proteins, such Bone morphogenetic protein 5 (BMP5) was detected in OF during the estrous cycle and it was suggested to act as an autocrine and/or paracrine regulator of the reproductive events that occur in the oviductal environment (Garcia *et al.* 2014). Furthermore, oviduct-derived embryotrophic factors such complement protein 3 (C3), and its derivatives, C3b and iC3b were found to be implicated in stimulating embryo development (Tse *et al.* 2008). Similar to all body fluids, OF also contains extracellular vesicles (EV) (Lopera-Vasquez *et al.* 2017a), which are important in intercellular communication playing a key role in the regulation of physiological and pathological processes (Revenfeld *et al.* 2014). The presence of EV in OF and their effect on early embryonic development may be of great importance and may provide information and new insights on early embryo-maternal communication and improve embryo quality in our current IVP systems. Indeed recent studies from our group reported that supplementation of *in vitro* culture media with EVs obtained from

OF (Lopera-Vasquez *et al.* 2017a) or BOEC *in vitro* cultures (Lopera-Vásquez *et al.* 2016) improve the development and quality of the produced embryos.

5.2. Utero-anatomomorphogical characteristics

The uterus is Y-shaped and consisting of a right and left horn, both of which are connected to their corresponding oviducts. The junction of these horns forms the body of the uterus. The uterus serves to transport sperm cells to the oviduct and provide nutrients and the environment for the developing fetus. At calving the muscular wall of the uterus takes on the responsibility for expulsion of the calf (McEntee 1990).

The uterus is composed by: the external layer called the serosa, a muscular layer or the myometrium and the internal layer or the endometrium. The surface of endometrium is characterized by having small, nonglandular protuberances; called caruncles (Figure 6). These regions are highly vascularized and will give rise to the maternal portion of the placenta if attachment of embryo occurs. The endometrium is also composed by spiral tubular structures, which extend to the myometrium called glands. Uterine glands are distributed uniformly between the caruncula and below them (McEntee 1990) (Figure 7). The secretory activity of the uterine glands changes depending of estrous cycle. During the estrous cycle the cells of the uterine endometrium produce prostaglandin F2α (PGF2α) (Senger 2005).

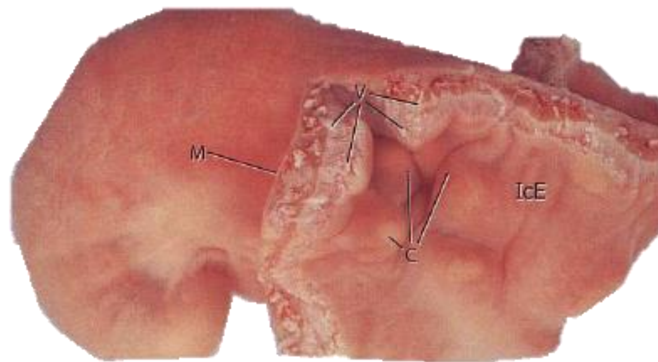

Figure 6: Excised uterine tissue. **C**: caruncles; **IcE**: intercaruncular endometrium; **M**: myometrium **V**: blood vessels (Senger 2005)

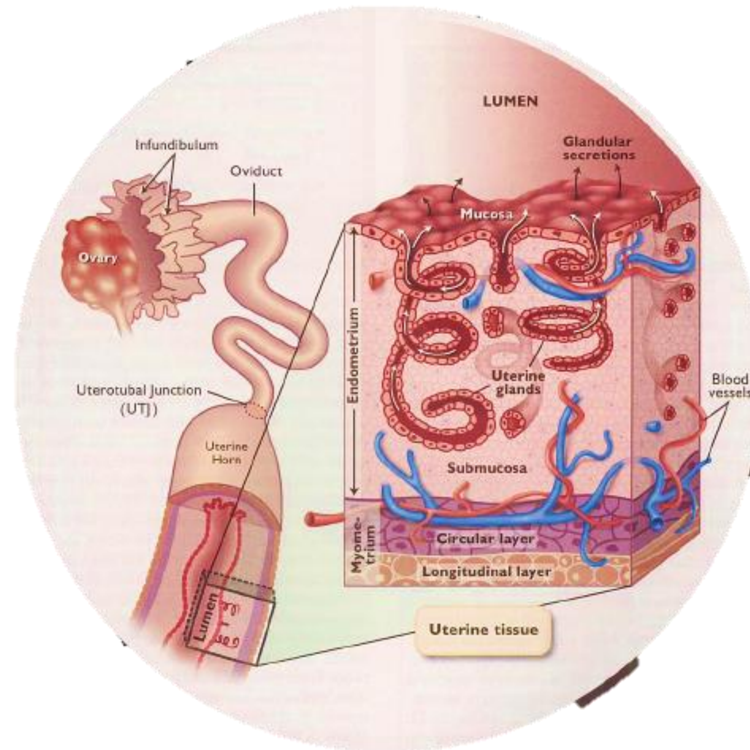

Figure 7: Schematic illustration of uterine tissue (Senger 2005)

The components of UF are derived from a number of sources: secretions from the luminal epithelium and glands, proteins selectively transudated from blood and likely contributions from tubal fluid; in a conception cycle, secretions from the developing blastocyst will also be present (Salamonsen *et al.* 2013). Uterine fluid and in particular the glandular secretions are critical for conceptus survival and implantation. In fact conceptus development was retarded and implantation failed to occur in ewes in which endometrial glandular development had been experimentally inhibited during early postnatal life (Gray *et al.* 2001b).The composition of UF is dependent on estrus cycle phases and interactions between the embryos and the genital tract. In cattle, systemic uterine changes are better understood than local molecular signaling between endometrial cells and embryos which are not sufficiently described at early stages, although they would surely lead to basic knowledge required to formulate media and develop *in vitro* culture systems (Muñoz *et al.* 2012). Local changes could be detectable and measured by sensitive proteomic techniques analyzing the UF. Muñoz *et al.* (2012) recorded an up-regulation of proteins that can favor or promote embryo development during preimplantational period such as hepatoma-derived growth factor (HDGF). Furthermore, in the same study, other proteins found in UF were implicated in embryo antioxidant protection such PRDX-1 and PRDX-2 belonging to peroxiredoxin family (Muñoz *et al.* 2012). In pregnant ewes it was reported a decrease of the complement factor B (CFB) which belongs to the C3 convertase complex, which might indicate that the protein is being used by the embryos to

generate iC3b (Koch *et al.* 2010). Furthermore, during conceptus elongation (i.e., Days 13 through 19) UF analysis revealed the presence of APOA1, ARSA, LCAT, NCDN as well as members of the PLIN family of proteins that may play roles in driving conceptus elongation in cattle (Forde *et al.* 2013).

6. Early embryo-maternal cross talk

Embryo-maternal interactions seem to be critical for establishment of a successful pregnancy. Evidences exist of reciprocal crosstalk between the developing conceptus and the uterine endometrium in early pregnancy. Indeed, posthatching conceptus elongation is entirely maternally driven, failing to occur *in vitro* (Brandao *et al.* 2004; Vejlsted *et al.* 2006) and also failing to occur *in vivo* in the absence of uterine glands (Allison Gray *et al.* 2000; Gray *et al.* 2002). In turn, the elongating conceptus secretes IFNT, which acts on the uterine luminal epithelium to suppress the transcription of estrogen receptor alpha and oxytocin receptor genes and thereby the release of luteolytic pulses of PGF2alpha, thus sustaining sufficient corpus luteum (CL)-derived P4 for pregnancy maintenance. Furthermore, the conceptus must elicit the appropriate transcriptional response from the endometrium to ensure survival (Heyman *et al.* 2002; Bauersachs *et al.* 2009). However, the evidence for reciprocal crosstalk during the transit of the early embryo through the oviduct is less clear. Moroever, the role of the oviduct in early embryo development has been underestimated since the fact that embryos can be produced *in vitro*, develop up to day 7 and then establish a pregnancy after ET. There is a poor understanding of cellular and molecular mechanisms that affect oviduct functions to impact embryo development and quality (Gonella-Diaza *et al.* 2017). Therefore, the oviduct is considered the starting point to search any signal between the embryo and the reproductive tract (Wolf *et al.* 2003).

6.1. Effect of the oviduct on embryo

The short-term culture of *in vitro*-produced bovine zygotes in the oviducts of cattle (Tesfaye *et al.* 2007; Gad *et al.* 2012), sheep (Lazzari *et al.* 2002; Lonergan *et al.* 2003a), or even mice (Rizos *et al.* 2007) has cleary shown the positive effect of the oviduct on embryo quality as measured in terms of morphology, gene expression, cryotolerance, and pregnancy rate after transfer. Many mechanisms could be implicated in such positive action. A coordinated action between cilia and oviductal smooth muscle contractions enable the embryo transport through the oviduct and the timely entrance of morula into the uterus. Such mechanisms are strictly coordinated by endocrine, paracrine and autocrine factors (Buhi *et al.* 1997; Buhi 2002).

Also, the composition of OF reflect the ability of the oviduct to adapt the environment to the different events from fertilization to early embryo development. Secretions present in the

OF affect oocyte, gamete interaction and early embryo development. Among those components playing a beneficial role on embryo development there is OVGP1 known for its critical role during fertilization, in sperm ZP-binding (Coy *et al.* 2008) and ZP hardening (Mondéjar *et al.* 2013). In bovine, the HA and the platelets activating factor (PAF) could be involved in very early embryo-maternal communication (Wolf *et al.* 2003). In fact the effect of HA in *in vitro* embryo culture has been reflected in increased blastocyst rates, cell number and gene expression paterns of bovine embryos (Stojkovic *et al.* 2002; Palasz *et al.* 2006). Studies in bovine oviduct *in vivo* reveal different expression patterns of IGF that may act directly on the embryo or via modulation of oviduct secretions and muscular activity to positively influence the success of early embryonic development (Pushpakumara *et al.* 2002). Furthermore, Garcia et al, (2014) demonstrated that different members of the BMP family are expressed in ex-vivo BOEC during the estrous cycle which suggest that these factors could act as reguator of the reproductive events that occur in the oviductal environment (García *et al.* 2014). Indeed, the addition of BMP5 to the embryo culture medium had a positive effect on the blastocyst rate and affected the relative expression of BMP target and pluripotency genes, suggesting that BMP5 could play an important role in the preimplantation development of bovine embryos (Garcia *et al.* 2015).

6.2. Oviduct response to the embryo

Some of the earliest evidence demonstrating that the embryo may have an effect on oviduct comes from investigations carried out in mares, hamsters and rats in the 1960s. In mares, it was found that nonfertilized oocytes were retained in the oviduct (Van Niekerk and Gerneke 1966) while the fertilized embryos that produced prostaglandin E2 were transported to the uterus (Weber *et al.* 1991). In hamsters, it was shown that embryos were transported to the uterus one day earlier than nonfertilized oocytes (Ortiz *et al.* 1986). In rats, when 1-cell embryos were transferred into the oviducts of recipients, 3 days later, they had reached the morula stage and remained in the oviduct; while when 4-cell embryos were transferred, 3 days later, a significant proportion of those embryos at the blastocyst stage were located in the uterus (Ortiz *et al.* 1989). More than 30 years have passed since those initial experiments; and the oviductal response to the presence of embryos has not been fully elucidated. Evidence *in vivo* in mice, by RT-qPCR showed changes in the oviduct gene expression depending on the presence or absence of embryos (Lee *et al.* 2002). Recently, new sequencing technologies (microarrays) have used to elucidate this question of a complex molecular dialogue between the maternal tract and embryo in pigs (Almiñana *et al.* 2012). Using these technologies Almiñana *et al*. (2012) showed that embryo-maternal communication exists at the very earliest stages of pregnancy, before that well-known embryonic signal of maternal recognition. In this communication, the embryo might play a role as a modulator of the immune system in the maternal tract, inducing the down-regulation of immune related genes to allow the refractory uterus to tolerate the

embryo and support its development (Almiñana *et al.* 2012). Presumably, any embryo-derived signals would be magnified in litter-bearing species compared with mono-ovulatory species which may explain why data demonstrating an effect of the embryo on the oviduct *in vivo* are scarce. In a recent study from our group, it was necessary to transfer multiple embryos (up to 50) into the oviduct of heifers to detect transcriptomic changes in the epithelia cells related with complement system, inflammation, or major histocompatibility complex, suggesting that 8 cell embryo could be able to avoid the maternal immune response by decreasing inflammation, antigen presentation, and action of the complement system. While when a single 8 cell embryo was present in the oviduct (pregnant vs cyclic heifers), no differences were found, suggesting a local effect of the embryo (Maillo *et al.* 2015). In the same line, Smits *et al.* (2016) reported also a local influence of the embryo on the transcriptome of the equine oviduct epithelium (Smits *et al.* 2016). In the studies mentioned above, in cattle, horses and pigs, the presence of an embryo induces subtle changes in the oviductal expression of genes related to immune function. This decrease in the reactivity of the immune system is not that surprising given the semiallogenic nature of the embryo/foetus. Without the appropriate control of the maternal immune system, the embryo will be rejected (Tolosa *et al.* 2012).

7. *In vitro* models to study embryo-maternal interaction

Analysis of local interactions between early embryos and genital tract *in vivo* still remains as a challenge because of intrinsic technical difficulties in exploring the environment surrounding the embryo. The use of *in vitro* models can provide evidence of physiological embryo-maternal interactions and mechanisms that are difficult to study *in vivo*. The advantages of the oviductal environment have been demonstrated in different models, through the substances isolated and the effect of environment per se. Indeed many physiological aspects have been clarified using *in vitro* models such bovine oviduct epithelial cells (BOEC) to study sperm oviduct-binding (Suarez 2008; Miller 2015) and sperm selection mechanisms (Ellington *et al.* 1999; Talevi and Gualtieri 2004) or also OF and its proteins to study fertilization and embryo development (Coy and Yanagimachi 2015). However, many others are still unknown (Hunter 2012). Van Soom et al. (2010) pointed out, when choosing an *in vitro* model, the aim of the experiment is an important consideration (Van Soom *et al.* 2010).

7.1. The use of bovine oviduct epithelial cells (BOEC)

BOEC are considered as the most suitable *in vitro* model to study early embryonic events and embryo-maternal interactions approaching to mimic phypsiological conditions (Rottmayer *et al.* 2006; Ulbrich *et al.* 2010). BOEC are usually obtained from oviducts of slaughtered heifers or cows. They could be isolated by enzymatic procedures using trypsin

and/or pancreatin (McNutt-Scott and Harris 1998) or collagenase (Bosch *et al.* 2001; Mishra *et al.* 2003) or by mechanical procedures (Gualtieri and Talevi 2000; De Pauw *et al.* 2002; Ulbrich *et al.* 2003). Many systems exist for *in vitro* culture of BOEC which will be explained below.

7.1.1. BOEC in standard culture condition (suspension or monolayer)

Standard conditions consist of seeding of BOEC in a cluture dish (Walter 1995). They could be grown on suspension or monolayer. Rottmayer *et al* (2006) proposed a short term (24 hours) epithelial cell suspension culture as a suitable system for holistic and specific analyses of early embryo-oviduct interactions. After 24 hours, the epithelial cell suspension maintained morphological characteristics and gene markers present in the cell in vivo such as OVGP1, estrogen, and progesterone receptors (Rottmayer *et al.* 2006). However in suspension condition BOEC do not attach and mitosis will not occur (Walter 1995). In monolayer cells are grown in adhesion to the culture dish. Beneficial effects of BOEC on co-cultured embryos have been used at the early days of IVP to overcome the developmental block at the 8- to 16-cell stage in ruminants (Gandolfi and Moor 1987; Ellington 1991). The positive effects of the cells on embryo development are attributed to their ability to modify the surrounding environment. Thus, BOEC secrete embryotrophic growth factors (Nancarrow and Hill 1994; Tse *et al.* 2008) while at the same time decrease oxygen levels which may prevent the formation of deleterious ROS (Thompson *et al.* 1990), remove toxic substances such as ammonia (Nancarrow and Hill 1994) and decrease glucose and ion levels (Edwards *et al.* 1997) that could have detrimental effects on the embryos. Furthermore, co-culture of embryos with BOEC improved the quality of the embryos produced in terms of cell number and cryosurvival (Schmaltz-Panneau *et al.* 2015). Recently, Cordova et al.(2014) used estrous-metoestrus (Day 0-3) BOEC for early (Day 1-4) or late (Day 4-7) embryo co-culture showing that the presence of the cells during the four first days of development, which correspond to the presence of embryos in the oviduct *in vivo*, accelerate the kinetics of blastocyst development and induce changes of genes involved in epigenetic control (Cordova *et al.* 2014).

Althouh a concomitant loss of important morphological characteristics of *in vitro* culture of BOEC in monolayer (Rottmayer *et al.* 2006) including reduction of cell height, loss of cilia, and loss of secretory granules and bulbous protrusions (Thibodeaux *et al.* 1992; Walter 1995), the use of co-culture systems offer the opportunity to detect essential and functional candidate genes in embryo-maternal dialogue related with antiviral and immune response (Schmaltz-Panneau *et al.* 2014) that under *in vivo* conditions are difficult to study.

Furthermore, the co-culture is associated with methodological complexity, lack of reproducibility and biosanitary risk (Guerin *et al.* 1997). But alternative to reduce the variability

in such systems could be the use of cell lines that maintain primary culture attributes (Ulbrich *et al.* 2010) or even BOEC conditioned media (BOEC-CM), which pose several advantages over the co-culture, such as the absence of foreign cells and the presence of embryotrophic factors (Ramos-Ibeas *et al.* 2014). Recently, Lopera-Vásquez *et al.* (2016) reported that CM, produced from extended culture of BOEC monolayer had a positive efffect on blastocyst quality when they were used during IVC (Lopera-Vásquez *et al.* 2016)

7.1.2. BOEC in polarized system

The BOEC polarized system consists of growing the cells on inserts to allow media access from basolateral and apical side, to maintain the polarized asymmetrical structure of the oviductal epithelial cells. It has been shown that this system preserves detailed morphological features of the porcine oviduct and oviduct-specific markers (Miessen *et al.* 2011). BOEC cultured in polarized system have been used as a model to elucidate the mechanism and physiology employed by the oviduct to face situations of metabolic stress produced by elevated non-esterified fatty acids (Jordaens *et al.* 2015; Jordaens *et al.* 2017). Another category of polraized culture is the air-liquid interface system that allows the access to medium from only basolateral side and the formation of oviduct fluid surrogate (OFC) in the apical period. Epithelial cells derived from human, porcine or bovine oviduct maintained polarity and *in vivo*–like morphology when they were cultured for long term in polarized air-liquid interface system (Levanon *et al.* 2010; Chen *et al.* 2013; Chen *et al.* 2017). Chen *et al,* (2017) reported that air-liquid system supports embryo development *in vitro* in the OFC without culture medium supply, in procine, mouse and bovine species. However, the obtained blastocyst rates could not yet match the outcome of optimized standard IVP procedures, suggesting further improvement of the model by: a) simulation of the hormonal changes taking place during the periconceptional period and b) development of a sequential culture system using oviductal as well as uterine epithelial cell (Chen *et al.* 2017)

7.1.3. BOEC in three-dimensional system

Using the three-dimensional (3D) printing technology in combination with microfluidics for assisted reproduction allow the creation of "oviduct-on-a-chip" with a U-shaped porous membrane allowing BOEC polarization that could be maintained during long-term mimicking tissue- and organ-specific micro-architecture (Ferraz *et al.* 2017a).It was recently demonstrated that specific tissue morphology and functions can be preserved better in customized 3D culture systems than in conventional 2-dimentional systems (Gualtieri *et al.* 2012; Costello *et al.* 2014). Ferraz *et al*. (2017b) reported that culturing BOEC in 3D system improved bovine embryo production by allowing proper sperm and oocyte interactions,

fertilization and completely abolished polyspermic and parthenogenic activation of oocytes in the absence of added sperm activating factors (Ferraz *et al.* 2017b). However, they observed that bovine embryo production is very sensitive to toxins released from 3-D printed acrylate chambers while a functional BOEC barrier partially protects the early embryo from this toxic effect. Therefore, future studies should identify the component(s) responsible for embryo toxicity, while the data emphasize that care is needed in selecting and testing materials for 3-D printing technologies before applying them to mimic *in vitro* maternal oviductal environment (Ferraz *et al.* 2017a).

7.2. The use of oviductal fluid

Oviductal fluid has also been used during *in vitro* embryo culture in an attempt to mimic the *in vivo* environment. A short exposure of matured porcine oocytes to bovine OF for 30 min before fertilization increases the blastocyst rate and quality in terms of morphology, cell number and gene expression patterns of apoptotic and developmentally related genes (Lloyd *et al.* 2009). Exposure of matured bovine oocytes to bovine OF before fertilization did not affect embryo development but altered the expression of gene transcripts such as G6PD and SOD32 (Cebrian-Serrano *et al.* 2013). Coy and Yanagimachi 2015 affirmed that the inclusion of reproductive fluids in human and animal-assisted reproductive technologies may improve the periconceptional environment for *in vitro* derived embryos, and improve the efficiency of the current IVP systems (Coy and Yanagimachi 2015). Barrera et al (2013) demonstrated that the use of 10% OF supplementation to embryo culture media modulates the expression of genes related with epigenetics in the 4-cell stage embryo (Barrera *et al.* 2013). Moreover, in a recent study trying to identify which embryonic stage is more sensitive to the OF on DNA methylation: before (from the 1-cell to 8-cell stage) during the main phase of the EGA (from 8-cell to 16-cell stage) or during the whole period in which the embryo is physiologically in the oviduct (from the 1-cell to 16-cell stage), we evidenced changes on methylation marks in CpG sites within certain genomic regions (*MTERF2*) and repetitive elements (LINE 1) in bovine blastocyst after being cultured with OF from 1-cell to 16 stage (Barrera *et al.* 2017). It is worth to mention that until recently no evidence of OF supplementation in *in vitro* embryo culture media exists. Thus, a study from our group attempt to mimic *in vivo* conditions of embryo development, by studying the effect of bovine OF supplementation in *in vitro* embryo culture without serum, on the developmental competence and quality of produced blastocysts. Our findings indicate a positive effect of OF at low concentrations (<5%) as a replacement for serum during embryo culture with an increase in developmental rates and better quality embryos in terms of survival after vitrification and warming, cell number and gene expression patterns (Lopera-Vasquez *et al.* 2017b). In this study, OF was used during the entire period of *in vitro*

embryo culture. However, if *in vivo* embryo-maternal environment was to be mimicked, an alternating use of OF and UF must be applied as the embryo until day 4 develops into the oviduct and then passes into the uterus.

JUSTIFICATION AND OBJECTIVES

During the pre-implantation period, the embryo is free-living and moves continuously through a specific tubal micro-environment before implantation. Bovine embryo spends the first 4 days in the oviduct where important morphological and metabolic changes occur, including the first mitotic division and EGA (Duranthon *et al.* 2008a). Then, the embryo enters the uterus and remains free floating in the UF until the initiation of implantation at approximately Days 19–22 (Bazer *et al.* 2009). These crucial events require an effective embryo–maternal dialogue that involves fluid secretions and cellular interactions (Leese *et al*. 2008).

Embryo development until blastocyst stage has been successfully developed *in vitro* through IVP technology. However IVP is a multifactorial process; influenced by extrinsic and intrinsic factors. The embryo development and quality is mainly affected by the origin of the oocyte and by the post-fertilization culture environment (Lonergan and Fair 2008; Rizos *et al.* 2008). The culture environment affects the viability of the early embryo, its ability to establish a pregnancy and the characteristics of the newborn calf. This is due to culture media composition which varies from chemically defined to semi-define normally supplemented with serum, proteins and macromolecules (Rizos *et al.* 2003). Therefore, it is important to understand the underlying regulatory mechanisms controlling embryo development (Avilés *et al.* 2015). Furthermore, mimicking the maternal environment during the preimplantation period of embryo development should help to design new strategies to promote pregnancy rate and apply this knowledge to improve IVP. Some molecules identified OF and UF (Aguilar and Reyley 2005; Aviles *et al.* 2010) have been used *in vitro* to improve embryo production. However, to date, a limited number of studies exists concerning OF or UF supplementation during IVC. In a recent study performed by our group, it was demonstrated that low concentrations of OF in serum-free embryo culture medium had a positive effect on embryo development and improved the quality of the resulting blastocysts in terms of cryotolerance, number of TE and the expression of important development-related genes (Lopera-Vasquez *et al.* 2017b).

To understand more precisely how the embryo develops physiologically, it is necessary to ascertain the crucial role of the oviductal environment on development in the short and long term (Maillo *et al.* 2016) and whether the embryo itself is able to modulate changes in the oviduct. The challenge today is to develop *in vitro* models for studying mechanisms that are difficult to study *in vivo* and to provide evidence of physiological embryo-maternal interactions. BOEC the intimal structure of the oviduct have been largely used *in vitro* in different culture systems. They might provide a good starting point with which to study putative embryo or oviduct derived signals. Indeed, BOEC *in vitro* has allowed the study of mechanisms involved in sperm storage (Abe and Hoshi 1997) and the embryotrophic effects on early embryo development and quality (Cordova *et al.* 2014), however few studies exist about the possible

effect of embryos on BOEC transcriptome. Schmaltz-Panneau *et al* (2015) showed clearly the effect of blastocysts on a monolayer of BOEC, in co-culture from Day 1 to 7, by altered several gene transcripts (Schmaltz-Panneau *et al.* 2015). Nevertheless, in order to mimic the oviduct embryo interaction, it is necessary to develop systems where the effect of the first stages of embryo development, when embryo still in the oviduct, could be studied.

It is known that epithelial cells lining the lumen of the mammalian oviduct can synthesize and secrete a wide range of proteins, including diverse growth factors and cytokines that can mediate a local interaction with the embryo through different signaling pathways (Buhi 2002; Aviles *et al.* 2010). Among the candidate signaling pathways that could actively participate in this local embryo-maternal cross talk in the oviduct, we focused our attention on the signaling mediated by bone morphogenetic proteins (BMPs). BMPs are a subfamily of growth factors that belongs to the TGF-β superfamily (Bragdon *et al.* 2011). Previous studies have revealed that different members of the BMP family are expressed in the epithelial cells of the bovine oviduct in an anatomically and temporally regulated fashion during the estrous cycle (García *et al.* 2014). Furthermore, mRNA expression of BMP receptors is greater in early stage embryos (from 2-cell to 8-cell stage), suggesting that the embryo could be target of BMPs during its transit through the oviduct (Garcia *et al.* 2015). Moreover, in a recent study by RNA sequencing, Maillo *et al* (2015) observed that the presence of multiple embryos induces changes in the expression levels of several genes associated with the BMP signaling pathway (Maillo *et al.* 2015). However, until now the participation of this signaling pathway in an initial cross talk between the embryo and the oviduct during the preimplantation period has not been explored in depth.

Therefore, the main objective of this thesis was to mimic the maternal environment during early preimplantation period, and optimize the *in vitro* culture systems for maximize embryo development and produce embryos of high quality and understand the mechanisms involved in the embryo-maternal communication in cattle.

To accomplish this general objective a number of specific objectives have been addressed in three experimental chapters:

Chapter 1: The objective was to evaluate the effects of both OF and UF supplementation during IVC on the developmental capacity of bovine zygotes to reach blastocyst stage, and on the quality of the produced blastocysts in terms of:

(i) Cryotolerance
(ii) Gene expression analysis
(iii) Intracellular ROS accumulation

Chapter 2: In order to *in vitro* test the hypothesis of a possible local interaction between early embryo and BOEC; we established an *in vitro* co-culture system allowing a local and temporal interaction between early bovine embryos and isthmus epithelial cells. The specific objectives were:

(i) To evaluate the transcriptome response of BOEC to early embryo and to elucidate whether this effect is local, due to a contact-depending signaling or the result of the BOEC interaction with embryo secretions and gene expression analysis

(ii) To assess the metabolic changes of BOEC that might result from BOEC-embryo interaction.

Chapter 3: In order to evaluate if the BMP signaling pathway represents an active cross talk between the embryo and the oviduct we used the same *in vitro* co-culture system as in the 2nd chapter. The objective was to evaluate whether early embryo-oviduct interaction induces changes in the gene expression levels of BMP signaling components both in the oviductal cells and in the preimplantation embryos.

Chapter 1

Bovine oviductal and uterine fluid support *in vitro* embryo development

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Bovine oviductal and uterine fluid support *in vitro* embryo development

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Abstract. In order to mimic the maternal oviductal environment, we evaluated the effect of oviductal fluid (OF) and/or uterine fluid (UF) supplementation on *in vitro* embryo development and quality. *In vitro*-produced zygotes were cultured with 1.25% OF from Day 1 to Day 4 after insemination (OF group), 1.25% OF from Day 1 to Day 4 followed by 1.25% UF from Day 4 to Day 9 (OF+UF group) or 1.25% UF only from Day 4 to Day 9 (UF group). Control groups were cultured in the presence of synthetic oviduct fluid (SOF) supplemented with 3 mg mL^{-1} bovine serum albumin (BSA) or 5% fetal calf serum (FCS). Supplementation of the culture medium with OF and/or UF (both at 1.25%) supported embryo development (Day 9 blastocyst rate 28.2–30.6%). At 72 h after vitrification–warming, the survival of blastocysts from the OF and OF+UF groups was similar to that of blastocysts in the SOF+BSA group ($61.0 \pm 5.7\%$ and $62.8 \pm 6.4\%$ vs $64.8 \pm 6.4\%$ respectively), but significantly higher than that of blastocysts from the SOF+FCS group ($31.6 \pm 4.9\%$; $P < 0.001$). Blastocysts from the OF group exhibited upregulation of epigenetic genes (i.e. DNA methyltransferase 3α (*DNMT3A*) and insulin-like growth factor 2 receptor (*IGF2R*)), compared with expression in the SOF+FCS group ($P < 0.05$). Whereas those from OF+UF and UF groups exhibited downregulation of oxidative stress genes compared to SOF+BSA and OF groups for glutathione peroxidase (*GPX1*) and to SOF+FCS, SOF+BSA and OF groups for chloride intracellular channel 1 (*CLIC1*) ($P < 0.05$). In addition, accumulation of reactive oxygen species was lower in blastocysts from the OF, OF+UF and UF groups. In conclusion, the use of low concentrations of OF and UF in *in vitro* serum-free culture supports embryo development, with OF providing a better control of embryo methylation, whereas UF may have antioxidant activity.

Additional keywords: culture medium, gene expression, *in vitro* fertilisation.

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Introduction

Many advances have been achieved in recent decades in *in vitro* embryo production (IVP). The technique has become a routine research tool in many scientific laboratories, as well as in the embryo transfer (ET) industry, mainly in the bovine. However, the quality of IVP blastocysts continues to lag behind that of blastocysts obtained *in vivo* based on their morphology, metabolism, gene expression and cryotolerance, which may have a knock-on effect further along the developmental axis (Rizos *et al.* 2008).

It has been well demonstrated that modification of the *in vitro* post-fertilisation culture environment can improve blastocyst quality (Rizos *et al.* 2008). Some supplements, such as fetal calf serum (FCS) or bovine serum albumin (BSA), are commonly used in culture media to improve the suboptimal conditions (Bavister 1995). However, a paradoxical effect is evident with serum, because although its presence accelerates embryo development and increases the number of blastocysts produced, the quality is reduced (Rizos *et al.* 2003). Most embryos grown under serum-free conditions have a higher abundance of mRNAs of developmentally marker genes, such as connexin 43, desmocollins I, II and III, glucose transporter-l, poly(A) polymerase and trophoblast protein, than those cultured in serum-enriched medium (Niemann and Wrenzycki 2000). Indeed, serum decreases the expression of genes associated with metabolism, but enhances the expression of those related to DNA repair (Heras *et al.* 2016), and has been associated with fetal overgrowth at birth, known as 'large offspring syndrome' (Lazzari *et al.* 2002). Furthermore, embryos produced under serum-free conditions show aberrations in genes involved in lipid metabolism, indicating that the serum-free conditions require further optimisation (Heras *et al.* 2016).

During preimplantation development, the bovine embryo spends the first 4 days in the oviduct. During this period, important morphological and metabolic changes occur, including the first mitotic division and embryonic genome activation

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(EGA; Duranthon *et al.* 2008). Then, the embryo enters the uterus and remains free floating in the uterine fluid (UF) until the initiation of implantation at approximately Days 19–22 (Bazer *et al.* 2009). During its prolonged preimplantation phase, the growing conceptus is solely dependent on the nutrients secreted from the oviduct and uterine epithelial cells, as well as serum transduction (Hugentobler *et al.* 2007). Therefore, several strategies have been used to improve *in vitro* culture (IVC) of embryos by mimicking the physiological conditions that exist *in vivo*. Embryos benefit from coculture with oviduct epithelial cells, because the developmental block at the 8- to 16-cell stage is overcome (Gandolfi and Moor 1987), embryo development and quality improve (Cordova *et al.* 2014) and the pregnancy rate after transfer increases (Galli *et al.* 2003). Nevertheless, coculture with cells has several problems, such as methodological complexity, lack of repeatability and biosanitary risks (Cebrian-Serrano *et al.* 2013).

Oviductal fluid (OF) and UF contain carbohydrates, ions, lipids, phospholipids and proteins (Aguilar and Reyley 2005; Avilés *et al.* 2010), as well as other unknown components. Some molecules identified have been used *in vitro* to improve embryo production. For example, the use of physiological concentrations of essential and non-essential amino acids in embryo culture media reduces the incidence of the 8- to 16-cell stage block in bovine embryos, promotes blastocyst formation and hatching (Takahashi and First 1992; Rosenkrans and First 1994; Steeves and Gardner 1999) and improves embryo quality (Santana *et al.* 2014). Oviductin, a specific glycoprotein present in the oviduct, is involved in *in vitro* sperm capacitation, sperm–oocyte binding, oocyte penetration, zona pellucida hardening, polyspermy reduction and embryo development (Killian 2004; Coy *et al.* 2008; Mondéjar *et al.* 2013). Furthermore, the addition of plasminogen, naturally present in OF, to IVF medium reduces the number of spermatozoa bound to the zona pellucida and entering the oocyte, thus increasing the percentage of monospermy in bovine and pig oocytes (Mondéjar *et al.* 2012). In addition, extracellular vesicles (EVs) are implicated in a dynamic mutual paracrine communication in the organism, including the embryo–maternal environment (Saadeldin *et al.* 2015). We observed that EVs isolated from conditioned medium following extended culture of bovine oviductal epithelial cells (Lopera-Vásquez *et al.* 2016) or from *in vivo*-derived OF (Lopera-Vasquez *et al.* 2017*a*) improve embryo quality during IVC. Moreover, growth factors detected in the UF, including granulocyte–macrophage colony-stimulating factor (GMCSF; or colony-stimulating factor (CSF) 2), platelet-activating factor (PAF) and hepatoma-derived growth factor (HDGF), enhance *in vitro* blastocyst development and increase cell numbers (Gopichandran and Leese 2006; Loureiro *et al.* 2009; Gómez *et al.* 2014). CSF2 and other factors, such as insulin-like growth factor (IFG) 1 and hyaluronan, have been shown to improve the survival-to-term rate following embryo transfer (Block *et al.* 2011).

Therefore, it is clear that IVC requires an environment as similar as possible to oviductal and uterine conditions during the preimplantation phase in order to maximise embryo development and quality. Lloyd *et al.* (2009) observed that short exposure of porcine oocytes to bovine OF before fertilisation improved the cleavage and blastocyst rates, as well as the quality of the blastocysts obtained. In contrast, in bovine oocytes, short exposure to OF did not affect the cleavage or blastocyst rates, although some significant changes in the blastocyst transcriptome were detected (Cebrian-Serrano *et al.* 2013). However, to date, there is a limited number of studies regarding OF or UF supplementation during IVC. In a recent study performed by our group, it was clearly demonstrated that low concentrations of OF in serum-free embryo culture medium had a positive effect on embryo development and improved the quality of the resulting blastocysts in terms of cryotolerance, number of trophectoderm cells and the expression of important development-related genes (Lopera-Vasquez *et al.* 2017*b*).

To our knowledge, there is no evidence of any developmental consequences of bovine UF supplementation during post-fertilisation embryo culture *in vitro*. Thus, the aim of the present study was to evaluate the effects of both OF and UF supplementation during IVC on bovine embryo development and quality.

Materials and methods

Unless stated otherwise, all chemicals were purchased from Sigma Aldrich Quimica.

Oocyte collection and IVM

Immature cumulus–oocyte complexes (COCs) were obtained by aspirating follicles (diameter 2–8 mm) from ovaries of mature heifers collected at a local slaughterhouse. Groups of 50 COCs were selected and cultured in 500 µL maturation medium in four-well dishes (Nunc) for 24 h at 38.5°C under an atmosphere of 5% CO_2 in air, with maximum humidity. The maturation medium consisted of TCM 199 supplemented with 10% (v/v) FCS and 10 ng mL^{-1} epidermal growth factor.

Sperm preparation and IVF

IVF was performed as described previously (Lopera-Vasquez *et al.* 2017*b*). Briefly frozen semen straws (0.25 mL) from a single Asturian Valley bull were treated with Bovipure (Nidacon Laboratories). The final concentration of spermatozoa was adjusted to 1×10^6 spermatozoa mL^{-1}. Gametes were co-incubated for 18–22 h in 500 µL fertilisation media (Tyrode's medium (Parrish 2014) with 25 mM bicarbonate, 22 mM sodium lactate, 1 mM sodium pyruvate and 6 mg mL^{-1} fatty acid-free BSA supplemented with 10 µg mL^{-1} heparin sodium salt; Calbiochem) in a four-well dish in groups of 50 COCs per well under an atmosphere of 5% CO_2 in air, with maximum humidity at 38.5°C.

In vitro *culture of presumptive zygotes*

At 18–20 h post-insemination (h.p.i.), presumptive zygotes were denuded of cumulus cells by vortexing for 3 min and were then cultured in groups of 25 in 25-µL droplets of culture medium SOF (Holm *et al.* 1999) with 4.2 mM sodium lactate, 0.73 mM sodium pyruvate, 30 µL mL^{-1} BME amino acids, 10 µL mL^{-1} minimum essential medium (MEM) amino acids and 1 µg mL^{-1} phenol red, covered with mineral oil at 38.5°C under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. Depending on the experiment (see below), the SOF was supplemented with OF

and/or UF. The control groups were cultured in SOF supplemented with 3 mg mL^{-1} BSA (SOF+BSA) or SOF supplemented with 5% FCS.

Collection of OF and UF

Oviducts and uteri from slaughtered heifers were selected according to the stage of the corpus luteum, and transported to the laboratory on ice. Only oviducts corresponding to Stage I (from Day 1 to Day 4 of the oestrous cycle) and Stage II uteri (from Day 5 to Day 10; Ireland *et al.* 1980) ipsilateral to the corpus luteum were used. OF was collected from the oviduct ipsilateral to the corpus luteum according to the protocol of Carrasco *et al.* (2008) with some modifications. Briefly, each oviduct was trimmed free of associated tissues and washed in phosphate-buffered saline (PBS) without BSA. Under a laminar hood, OF was collected by gentle squeezing from the isthmus to ampulla. The OF from six oviducts was pooled and centrifuged twice at 7000*g* for 10 min at 4°C to remove cellular debris. The resulting supernatant was aliquoted and stored in 5-μL aliquots at −80°C. UF was also collected from a pool of six uteri by squeezing from the internal opening of the cervix towards the uterotubal junction. This fluid was then centrifuged twice at 7000*g* for 10 min at 4°C, and the resulting supernatant was aliquoted and stored in 5-μL aliquots at −80°C.

Assessment of embryo development and quality

Embryo development

Cleavage rate was recorded at Day 2 (48 h.p.i.) and cumulative blastocyst yield was recorded on Days 7, 8 and 9 after insemination.

Embryo quality

Blastocyst vitrification. The ability of the blastocyst to withstand cryopreservation was used as an indicator of quality. Day 7–8 blastocysts were vitrified in holding medium (HM), consisting of TCM 199 supplemented with 20% (v/v) FCS, and cryoprotectants as described previously (Rizos *et al.* 2002) in a two-step protocol using the Cryoloop device (Hampton Research). In the first step, HM was supplemented with 7.5% ethylene glycol and 7.5% dimethyl sulfoxide; in the second step, HM was supplemented with 16.5% ethylene glycol, 16.5% dimethyl sulfoxide and 0.5 M sucrose. Blastocysts were warmed in two steps in HM with 0.25 and 0.15 M sucrose and then cultured in 25-μL droplets of SOF with 5% FCS. Survival was defined as re-expansion of the blastocoel and its maintenance for 72 h after warming.

Gene expression analysis. Gene expression analysis was performed using 4 groups of 10 Day 7–8 blastocysts per treatment: SOF+FCS, SOF+BSA, OF+UF, OF and UF. Poly (A) RNA was extracted using the Dynabeads mRNA Direct Extraction Kit (Ambion; Thermo Fisher Scientific) as described previously (Bermejo-Alvarez *et al.* 2008). Immediately after poly(A) RNA extraction, reverse transcription (RT) was performed using a MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit according to the manufacturer's instructions (Epicentre Technologies) using poly(T) random primers and Moloney murine leukaemia virus (MMLV) high-performance reverse transcriptase enzyme in a total volume of 40 μL to prime the RT reaction and to produce cDNA. Tubes were heated to 70°C for 5 min to denature the secondary RNA structure and the RT mix was then completed by adding 50 units reverse transcriptase. Samples were incubated at 25°C for 10 min to help the annealing of random primers, followed by incubation at 37°C for 60 min to allow the RT of RNA and finally at 85°C for 5 min to denature the enzyme.

All mRNA transcripts were quantified in duplicate using a Rotorgene 6000 Real Time Cycler (Corbett Research). RT–quantitative polymerase chain reaction (qPCR) was performed by adding 2-μL aliquot of each cDNA sample (~60 ng μL^{-1}) to the PCR mix containing the specific primers to amplify transcripts for histone (H2A histone family member Z (*H2AFZ*)), lysine (K)-specific demethylase 1A (*KDM1A*), aquaporin-3 (*AQP3*), DNA (cytosine-5-)-methyltransferase 3α (*DNMT3A*), insulin-like growth factor 2 receptor (*IGF2R*), chloride intracellular channel 1 (*CLIC1*), glutathione peroxidase 1 (*GPX1*) and placenta-specific 8 (*PLAC8*). All primers were designed using Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/, accessed July 2017) to span exon–exon boundaries when possible. Primer sequences and the approximate sizes of the amplified fragments of all transcripts are given in Table 1. For quantification, RT-qPCR was performed as described previously (Bermejo-Alvarez *et al.* 2010). The PCR conditions were tested to achieve efficiencies close to 1. The comparative quantification cycle (Cq) method was used to quantify expression levels (Schmittgen and Livak 2008). Values were normalised to the endogenous control *H2AFZ*. Fluorescence was acquired in each cycle to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background for each sample. Within this region of the amplification curve, a difference of one cycle is equivalent to a doubling of the amplified PCR product. According to the comparative Cq method, the ΔCq value for each sample was determined by subtracting its *H2AFZ* Cq value from the gene Cq value of the same sample. The calculation of ΔΔCq involved using the highest treatment ΔCq value (i.e. the treatment with the lowest target expression) as an arbitrary constant to subtract from all other ΔCq sample values. Fold-changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta Cq}$.

Labelling of reactive oxygen species

Intracellular reactive oxygen species (ROS) were labelled by chloromethyl-2′-7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; Invitrogen). On Days 7–8, 10 blastocysts per group were treated with 10 μM CM-H_2DCFDA added to the culture medium and incubated in the dark at 38°C under 5% CO_2 for 30 min, then washed twice with PBS. Embryos were visualised under a confocal microscope (Leica TCS SP2) after excitation at 492 nm and emission at 517 nm. The digital images were processed and analysed using ImageJ (National Institutes of Health) to determine the relative fluorescence of ROS intensity in each embryo. After selection using the freehand selection tool, each embryo was measured to determine its area and its integrated density (IntDen), which corresponds to pixel intensity. In addition, the background

Table 1. Details of primers used for reverse transcription–quantitative polymerase chain reaction
H2AFZ, H2A histone family member Z; *AQP3*, aquaporin 3; *KDM1A*, lysine demethylase 1A; *DNMT3A*, DNA methyltransferase 3α; *IGF2R*, insulin-like growth factor 2 receptor; *CLIC1*, chloride intracellular channel 1; *GPX1*, glutathione peroxidase 1; *PLAC8*, placenta-specific 8

Gene	Primer sequence (5′–3′)	Fragment size (bp)	GenBank Accession no.
H2AFZ	Forward: AGGACGACTAGCCATGGACGTGTG	209	NM_174809
	Reverse: CCACCACCAGCAATTGTAGCCTTG		
AQP3	Forward: CGGTGGTTTCCTCACCATCA	299	NM_001079794.1
	Reverse: CAGAGGGGTAGGTGGCAAAG		
KDM1A	Forward: TTGGCCAACCTCTCAGAAGAC	147	XM_002685717.3
	Reverse: CTCCACACCAGATGGTTCCTC		
DNMT3A	Forward: CTGGTGCTGAAGGACTTGGGC	318	AY271299
	Reverse: CAGAAGAAGGGGCGGTCATC		
IGF2R	Forward: GCTGCGGTGTGCCAAGTGAAAAAG	201	NM_174352.2
	Reverse: AGCCCCTCTGCCGTTGTTACCT		
CLIC1	Forward: CCATTCCGGATGTGTTTCGTGG	196	NM_001015608.1
	Reverse: GAAACCACCCAGGGCCTTTGTG		
GPX1	Forward: GCAACCAGTTTGGGCATCA	116	NM_174076.3
	Reverse: CTCGCACTTTTCGAAGAGCATA		
PLAC8	Forward: CGGTGTTCCAGAGGTTTTTCC	163	NM_001025325.2
	Reverse: AAGATGCCAGTCTGCCAGTCA		

fluorescence of an area outside the embryo was measured. The relative ROS fluorescence intensity in each embryo was determined using the following formula:

$$\text{Relative ROS fluorescence} = \text{IntDen} - (\text{area of selected embryo} \times \text{mean fluorescence of background readings})$$

Relative ROS fluorescence intensity is expressed in arbitrary units (a.u.).

Experimental design

Based on a previous study (Lopera-Vasquez *et al.* 2017*b*), the concentration of OF used in the present study was 1.25%. However, because there are no data in the literature regarding the use of UF during IVC, it was necessary to perform preliminary experiments to determine the optimum concentration of UF for supplementation of embryo culture media. Therefore, bovine zygotes were cultured in SOF supplemented with 1.25% OF from Day 1 until Day 4 after insemination and then cultured in SOF supplemented either with 5%, 2.5% or 1.25% of UF until Day 9 after insemination ($n = 4$ replicates). Based on the results of this preliminary experiment, at 20 h.p.i., presumptive zygotes were distributed into five experimental groups (Fig. 1): (1) SOF supplemented with FCS at 5% (SOF+FCS; $n = 400$) used as a laboratory control; (2) SOF supplemented with 3 mg mL^{-1} BSA (SOF+BSA; $n = 430$) used as an experimental control; (3) SOF supplemented with 1.25% OF until Day 4, then with 1.25% UF until Day 9 (OF+UF; $n = 460$); (4) SOF supplemented with 1.25% OF until Day 4, then with SOF+BSA until Day 9 (OF; $n = 479$); (5) SOF+BSA until Day 4, followed by supplementation of SOF with 1.25% UF until Day 9 (UF; $n = 486$). Eight replicates were performed for each group.

To assess embryo quality, a representative number of Day 7–8 blastocysts from each group were vitrified–warmed ($n \approx 55$ per group) and survival rate was recorded every 24 h up to 72 h after warming. In addition, Day 7–8 blastocysts were frozen in liquid nitrogen ($n = 40$ per group) in groups of 10 and stored at −80°C for gene expression analysis. Furthermore, 10 Day 7–8 blastocysts per group were used to assess embryo ROS levels.

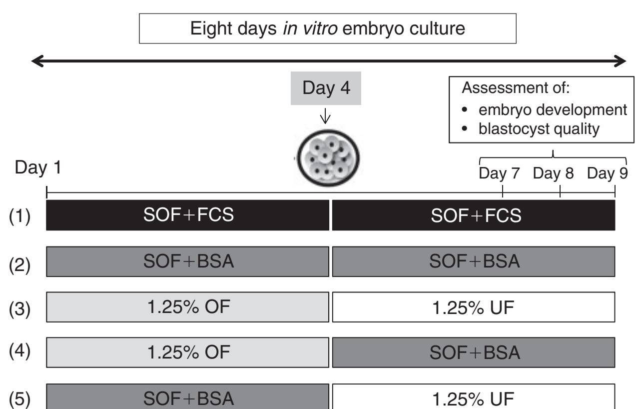

Fig. 1. Experimental design. *In vitro*-produced presumptive zygotes were cultured in five experimental groups: (1) synthetic oviductal fluid (SOF) supplemented with 5% fetal calf serum ($n = 400$); (2) SOF supplemented with 3 mg mL^{-1} bovine serum albumin (BSA; $n = 430$); (3) SOF supplemented with 1.25% oviductal fluid (OF) until Day 4 and then with 1.25% uterine fluid (UF) until Day 9 ($n = 460$); (4) SOF supplemented with 1.25% OF until Day 4, then with SOF+BSA until Day 9 ($n = 479$); and (5) SOF+BSA until Day 4, then SOF supplemented with 1.25% UF until Day 9 ($n = 486$). A total of eight replicates was performed.

Statistical analysis

Statistical analysis was performed using SigmaStat (Jandel Scientific). Cleavage and blastocyst rates, the proportion of blastocysts surviving cryopreservation, relative mRNA abundance

Table 2. Effects of *in vitro* embryo culture with 1.25% bovine oviductal fluid (OF; Days 1–4) and 1.25%, 2.5% or 5% bovine uterine fluid (UF; Days 4–9) on development

Data are the mean ± s.e.m. Within columns, values with different superscript letters differ significantly ($P < 0.01$). SOF+BSA, blastocysts cultured in the presence of synthetic oviductal fluid (SOF) and 3 mg mL^{-1} bovine serum albumin (experimental control); SOF+FCS, blastocysts cultured in the presence of SOF and 5% fetal calf serum (laboratory control)

	Total no. presumptive zygotes in culture	Cleavage rate		Blastocyst yield					
		n	%	*n*	Day 7 (%)	*n*	Day 8 (%)	*n*	Day 9 (%)
SOF+FCS	208	179	86.7 ± 2.7	46	22.2 ± 4.5[a]	54	26.2 ± 4.2[a]	62	30.0 ± 3.8[a]
SOF+BSA	223	187	84.2 ± 3.3	26	11.7 ± 0.6[ab]	41	18.0 ± 1.1[a]	47	20.9 ± 0.4[a]
OF+1.25% UF	253	211	83.5 ± 1.2	51	20.1 ± 4.6[a]	70	28.2 ± 5.8 [a]	81	32.1 ± 4.7[a]
OF+2.5% UF	269	220	82.4 ± 2.5	36	13.2 ± 1.0[ab]	66	24.9 ± 3.1[a]	72	28.0 ± 4.5[a]
OF+5% UF	223	183	82.0 ± 0.7	1	3.0 ± 3.0[b]	13	6.3 ± 2.1[b]	18	8.9 ± 2.6[b]

Table 3. Effects of *in vitro* embryo culture with 1.25% bovine oviductal fluid (OF; Days 1–4) and/or 1.25% bovine uterine fluid (UF; Days 4–9) on development

Data are the mean ± s.e.m. Within columns, values with different superscript letters differ significantly ($P < 0.05$). SOF+BSA, blastocysts cultured in the presence of synthetic oviductal fluid (SOF) and 3 mg mL^{-1} bovine serum albumin (experimental control); SOF+FCS, blastocysts cultured in the presence of SOF and 5% fetal calf serum (laboratory control); OF+UF, blastocysts cultured in the presence of SOF supplemented with 1.25% OF until Day 4 and then with 1.25% UF until Day 9; OF, blastocysts cultured in the presence of SOF supplemented with 1.25% OF until Day 4; UF, blastocysts cultured in the presence of SOF supplemented with 1.25% UF from Day 4 to Day 9

	Total no. presumptive zygotes in culture	Cleavage rate		Blastocyst yield					
		n	%	*n*	Day 7 (%)	*n*	Day 8 (%)	*n*	Day 9 (%)
SOF+FCS	400	330	83.5 ± 1.7	100	25.6 ± 2.0[a]	111	28.1 ± 2.4	115	29.0 ± 1.9
SOF+BSA	430	380	88.6 ± 1.9	75	17.8 ± 3.1[b]	119	27.2 ± 3.2	129	30.6 ± 2.9
OF+UF	460	399	86.8 ± 1.5	96	21.3 ± 1.8[ab]	139	29.5 ± 2.3	143	30.2 ± 2.5
OF	479	410	85.5 ± 1.5	87	18.6 ± 1.5[b]	133	28.1 ± 1.5	142	29.5 ± 1.4
UF	486	404	83.0 ± 1.4	98	21.5 ± 2.8[ab]	125	27.2 ± 3.1	131	28.2 ± 2.9

and ROS measurements were normally distributed with homogeneous variance, so one-way analysis of variance (ANOVA) with arcsine data transformation was performed to evaluate the significance of differences between groups. When the normality and homogeneity tests failed, Tukey's test was used to determine the significance of differences between groups. Values were considered significantly different at $P < 0.05$. Unless otherwise indicated, data are presented as the mean ± s.e.m.

Results

Effects of different concentrations of UF on embryo development in vitro

The blastocyst yield on Day 9 was markedly reduced when 5% UF was used (8.9% ± 2.6), whereas supplementation of the culture medium with 2.5% or 1.25% UF resulted in similar blastocyst yields on Day 9 to those seen in the two control (SOF+FCS and SOF+BSA) groups (i.e. 28.0 ± 4.5% and 32.1 ± 4.7% vs 30.0 ± 3.8% and 20.9 ± 0.4% respectively; Table 2).

Effects of sequential embryo culture with 1.25% OF (Day 1–4) and 1.25% UF (Day 4–9) on embryo development and quality

Embryo development

No differences were observed in cleavage rate between the different experimental groups, which ranged from 83.0 ± 1.4% to 88.6 ± 1.9% (Table 3). However, the blastocyst yield on Day 7 was higher in the SOF+FCS group than in the SOF+BSA and OF groups, but similar to that in the OF+UF and UF groups. On Days 8 and 9, there were no differences observed in blastocyst yield between the groups, which ranged from 27.2 ± 3.2% to 29.5 ± 2.3% on Day 8 and from 28.2 ± 2.9% to 30.6 ± 2.9% on Day 9 (Table 3).

Vitrification–warming

In terms of blastocyst cryotolerance, during the first 24 h after warming, there were no differences in survival between groups, which ranged from 59.0 ± 6.0% to 74.2 ± 6.4% (Table 4). However, 48 h after warming, the survival rates of blastocysts obtained from sequential culture with OF+UF and from culture with OF only during the first 4 days were similar to those of the SOF+BSA group (62.8 ± 6.4% and 61.0 ± 5.7% vs 73.3 ± 6.7% respectively) and significantly higher than those of the SOF+FCS group (41.1 ± 6.4%; $P < 0.001$). Blastocyst rates following culture with UF only during the last 3–4 days were significantly lower than those following culture with SOF+BSA (53.3 ± 4.3% vs 73.3 ± 6.7% respectively; $P < 0.001$; Table 4). At 72 h after warming, those differences were even more marked ($P < 0.001$), particularly after the decrease in the survival rate in the SOF+FCS (31.6 ± 4.9%) and SOF+BSA (64.8 ± 6.4%) groups, whereas survival rates at 72 h in the OF+UF, OF and UF groups were maintained at levels seen at 48 h in the same groups (Table 4).

Table 4. Survival rates after vitrification–warming of Day 7 blastocysts cultured with 1.25% bovine oviductal fluid (OF; Days 1–4) and/or 1.25% bovine uterine fluid (UF; Days 4–9)

Data are the mean ± s.e.m. Within columns, values with different superscript letters differ significantly ($P < 0.001$). SOF+BSA, blastocysts cultured in the presence of synthetic oviductal fluid (SOF) and 3 mg mL^{-1} bovine serum albumin (experimental control); SOF+FCS, blastocysts cultured in the presence of SOF and 5% fetal calf serum (laboratory control); OF+UF, blastocysts cultured in the presence of SOF supplemented with 1.25% OF until Day 4 and then with 1.25% UF until Day 9; OF, blastocysts cultured in the presence of SOF supplemented with 1.25% OF until Day 4; UF, blastocysts cultured in the presence of SOF supplemented with 1.25% UF from Day 4 to Day 9

	Total no. presumptive zygotes in culture	Blastocyst survival rate after vitrification-warming							
		n	4 h (%)	*n*	24 h (%)	*n*	48 h (%)	*n*	72 h (%)
SOF+FCS	54	35	66.5 ± 6.9	32	59.0 ± 6.0	24	41.1 ± 6.4^{a}	19	31.6 ± 4.9^{a}
SOF+BSA	54	47	86.2 ± 6.3	41	74.2 ± 6.4	40	73.3 ± 6.7^{b}	36	64.8 ± 6.4^{b}
OF+UF	56	50	87.1 ± 7.2	39	70.2 ± 6.5	34	62.8 ± 6.4^{bc}	34	62.8 ± 6.4^{bc}
OF	53	45	83.2 ± 5.6	39	70.7 ± 4.1	33	61.0 ± 5.7^{bc}	33	61.0 ± 5.6^{bc}
UF	59	51	84.7 ± 6.4	45	69.5 ± 8.9	33	53.3 ± 4.3^{ac}	33	53.3 ± 4.3^{ac}

Gene expression

In relation to blastocyst gene expression, expression of the epigenetic control genes *DNMT3A* and *IGF2R* was upregulated in the OF compared with SOF+FCS group. Moreover, expression of oxidative stress regulation genes was downregulated in the UF and OF+UF groups compared to SOF+BSA and OF groups for *GPX1* and to SOF+FCS, SOF+BSA and OF groups for *CLIC1* ($P < 0.05$). No differences in expression were observed for the remaining transcripts studied (*KDM1A*, *PLAC8* and *AQP3*; Fig. 2).

Detection of ROS

Relative ROS fluorescence intensity (green) was significantly lower in blastocysts from the OF+UF, OF and UF groups compared with blastocysts from the SOF+FCS group ($P < 0.001$; Fig. 3). In addition, ROS levels in blastocysts from the OF+UF group were significantly lower than in blastocysts from the SOF+BSA group ($P < 0.001$), but did not differ to those in blastocysts from the OF and UF groups (Fig. 3).

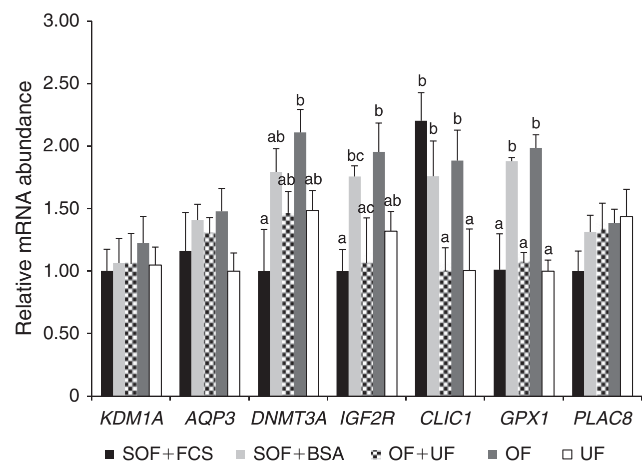

Fig. 2. Relative mRNA expression (normalised against that of the endogenous control H2A histone family member Z (*H2AFZ*) gene) of genes related to methylation (lysine (K)-specific demethylase 1A (*KDM1A*)), water channels (aquaporin-3 (*AQP3*)), epigenetic control (DNA (cytosine-5-)-methyltransferase 3α (*DNMT3A*) and insulin-like growth factor 2 receptor (*IGF2R*)), regulation of oxidative stress (chloride intracellular channel 1 (*CLIC1*) and glutathione peroxidase 1 (*GPX1*)) and implantation (placenta-specific 8 (*PLAC8*)) in *in vitro* bovine blastocysts (from Day 7 and 8 after insemination) cultured with or without a low concentration (1.25%) of oviductal fluid (OF) and/or uterine fluid (UF). SOF+BSA, blastocysts cultured in the presence of synthetic oviductal fluid (SOF) and 3 mg mL^{-1} bovine serum albumin (experimental control); SOF+FCS, blastocysts cultured in the presence of SOF and 5% fetal calf serum (laboratory control); OF+UF, blastocysts cultured in the presence of SOF supplemented with 1.25% oviductal fluid (OF) until Day 4 and then with 1.25% uterine fluid (UF) until Day 9; OF, blastocysts cultured in the presence of SOF supplemented with 1.25% OF until Day 4; UF, blastocysts cultured in the presence of SOF supplemented with 1.25% UF from Day 4 to Day 9. Data are the mean ± s.e.m. Different letters above columns indicate significant differences in gene expression among the experimental groups ($P < 0.05$).

Discussion

In an attempt to optimise the bovine embryo IVP system, we evaluated the effects of sequential culture following supplementation of the post-fertilisation culture medium with OF and UF on bovine embryo development and quality. The main finding of the present study was that a low concentration of OF and/or UF supports embryo development and improves embryo quality. Indeed, OF provided control of embryo methylation, whereas UF exhibited antioxidant activity. Thus, the use of low concentrations of OF and UF *in vitro* may represent a good alternative to FCS for IVC.

Early embryonic development is one of the most critical and complex periods of mammalian development. Despite the successful improvement of IVP in cattle, evidence demonstrates a clear effect of the post-fertilisation culture environment on blastocyst quality, measured in terms of cryotolerance and the relative abundance of several developmentally important genes (Enright *et al.* 2000; Niemann and Wrenzycki 2000; Rizos *et al.* 2008). Furthermore, ontological classification of differentially expressed genes highlighted a marked contrast in the pattern of gene expression related to lipid metabolism and oxidative stress response when comparing blastocysts generated *in vivo* with those generated *in vitro* (Gad *et al.* 2012).

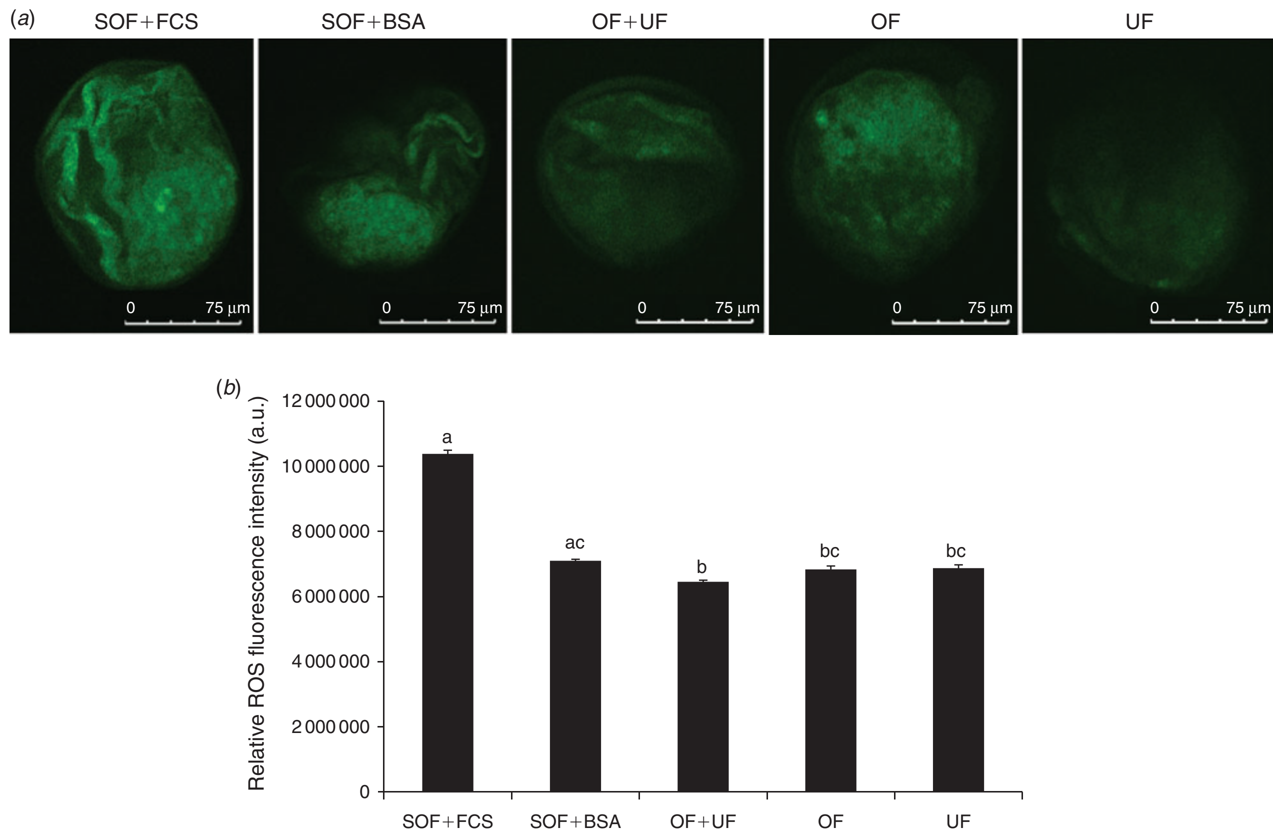

Fig. 3. (*a*) Representative fluorescent images of reactive oxygen species (ROS; green) detection with chloromethyl-2′-7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) in Day 7 blastocysts from the different experimental groups. (*b*) Quantification of relative ROS fluorescence intensity. SOF+BSA, blastocysts cultured in the presence of synthetic oviductal fluid (SOF) and 3 mg mL^{-1} bovine serum albumin (experimental control); SOF+FCS, blastocysts cultured in the presence of SOF and 5% fetal calf serum (laboratory control); OF+UF, blastocysts cultured in the presence of SOF supplemented with 1.25% oviductal fluid (OF) until Day 4 and then with 1.25% uterine fluid (UF) until Day 9; OF, blastocysts cultured in the presence of SOF supplemented with 1.25% OF until Day 4; UF, blastocysts cultured in the presence of SOF supplemented with 1.25% UF from Day 4 to Day 9. Data are the mean ± s.e.m. Different letters above columns indicate significant differences between groups ($P < 0.001$).

In the present study, sequential IVC with OF (1.25%; Days 1–4) and/or UF (1.25%; Days 4–9) supports embryo development. A similar effect was also observed with OF when it was used during the entire period of IVC (Lopera-Vasquez *et al.* 2017*b*). However, the results of the present study suggest that alternating OF with UF in IVC may mimic more adequately the physiological conditions of the reproductive tract, where the early embryo normally develops to the blastocyst stage. It is important to mention that during this time major developmental events occur in the embryo, such as first cleavage, EGA, morula compaction, blastocyst formation and hatching. All these events appear to be sensitive to culture conditions, particularly EGA (Doherty *et al.* 2000).

In agreement with the present study, a positive effect of OF has been reported in other species, such as porcine, increasing zona pellucida hardening, reducing the incidence of polyspermy without decreasing the penetration rate and improving embryo quality (Cebrian-Serrano *et al.* 2013; Ballester *et al.* 2014; Batista *et al.* 2016). These effects could be attributed to several oviduct factors, such as oviductal glycoprotein 1 (OVGP1), which is the most studied protein in the OF (Soleilhavoup *et al.* 2016). It seems that OVGP1 is responsible for zona pellucida changes in the oviduct before fertilisation, affecting sperm binding and contributing to the regulation of polyspermy (Coy *et al.* 2008). OVGP1 is also responsible for overcoming the 2-cell embryo block in rabbits (Yong *et al.* 2002). Moreover, it has been shown that OVGP1 increases embryo development *in vitro* in porcine (McCauley *et al.* 2003) and ovine (Pradeep *et al.* 2011). In addition, other proteins have been described in the OF, including plasminogen, heat shock protein family members, protein disulfide isomerase family A member 4, elongation factor 2, phosphoglycerate kinase 1, threonine-tRNA ligase and ezrin (Mondéjar *et al.* 2012, 2013).

Studies concerning the effects of UF during IVC are lacking. To our knowledge, the present study is the first that has included bovine UF during IVC to evaluate its effects on embryo development and quality. It is known that 5–9 days following ovulation the endometrium becomes receptive to allow conceptus elongation before implantation. At this time, the uterus becomes highly secretory, producing and secreting a wide array of factors into the uterine lumen. Uterine glands and their secretions play a crucial role in establishment of mammalian

pregnancy (Filant and Spencer 2014). Furthermore, the infertility in the uterine gland-knockout ewe model appears to result from a lack of endometrial glands and, by extension, of their secretions that are required to support the growth and development of peri-implantation conceptuses (Gray *et al.* 2000). Moreover, several cytokines and growth factors found in human UF are involved in embryo development and implantation, as well as regulating the immune environment in the uterus (Bhusane *et al.* 2016). In addition, *in vitro* supplementation of uterine proteins such as HDGF (Gómez *et al.* 2014) and vascular endothelial growth factor (Binder *et al.* 2014) have a positive effect on embryo development, blastocyst cell numbers, embryo implantation rates and limb development.

Similar to previous observations (Lopera-Vasquez *et al.* 2017*b*), herein we demonstrated that concentrations of OF and UF >2.5% have a negative effect on embryo development, which confirms the dynamic, well-synchronised and a continuous renewal of OF and UF *in vivo* (Buhi 2002) in order to provide an adequate environment for preimplantation embryo development in the oviduct and uterine horn, whereas under *in vitro* conditions OF and UF are degraded, with negative effects on the embryo. Thus, supplementation of post-fertilisation media with low concentrations of OF and UF tends to diminish the detrimental effects, supporting embryo development and improving embryo quality

The ability of the embryo to survive cryopreservation has been extensively used as an indicator of embryo quality and viability (Rizos *et al.* 2002, 2003; Moore *et al.* 2007). IVC conditions affect the ability of the embryos produced to develop and survive after cryopreservation (Rizos *et al.* 2008). In the present study, the use of OF during the first 4 days of embryo culture and sequential culture with OF and UF improved embryo cryotolerance by doubling the blastocyst survival rate compared with embryos cultured with serum (61–65% vs 31% respectively; Table 4). This result confirms the role of physiological fluids in determining embryo quality and highlights the importance of embryo exposure to the maternal environment (Tesfaye *et al.* 2007; Lazzari *et al.* 2010; Rizos *et al.* 2010).

The importance of DNA methylation during embryo development is well established (Auclair *et al.* 2014). Epidemiological studies have reported a high incidence of disorders involving imprinted genes among children conceived using assisted reproductive technology (ART), suggesting that ART procedures may disrupt imprinted gene methylation patterns (Turan *et al.* 2010). In the present study, gene expression analysis revealed that OF in the culture medium increased the expression of epigenetic marker genes (*DNMT3A* and *IGF2R*) compared with culture medium supplemented with serum. A similar result was observed in our previous study when the same concentration of OF was used during the entire embryo culture period (Lopera-Vasquez *et al.* 2017*b*). Moreover, another recent study from our group suggests that *in vitro* embryos exhibit a temporal sensitivity to OF at early embryonic stages, which is reflected in DNA methylation changes of specific genomic regions at the blastocyst stage (Barrera *et al.* 2017). In accordance with our results, a high level of *DNMT3A* expression was observed in the bovine blastocyst at Day 7, which agrees with the period of greatest imprint instability, followed by a significant decrease in transcript abundance at later stages of development (Days 16 and 19), in tandem with DNA methylation imprint stabilisation (O'Doherty *et al.* 2015). In addition, loss of methylation in the differentially methylated region of *IGF2R* was associated with an increase in *IGF2R* transcription and fetal overgrowth in sheep embryos cultured *in vitro* (Young *et al.* 2001).

The metabolism of molecular oxygen is important during embryo development (Thompson *et al.* 1996). This metabolism is usually related to production of ROS, such as H_2O_2, even under basal conditions (Guérin *et al.* 2001). However, an imbalance in intracellular ROS levels leads to oxidative stress (Balaban *et al.* 2005). *In vitro* production and manipulation of embryos increase ROS generation and decrease antioxidant defences. ROS have also been related to impaired development of mammalian embryos (Johnson and Nasr-Esfahani 1994). In the present study, both *CLIC1*, considered a sensor and effector during oxidative stress (Averaimo *et al.* 2010), and *GPX1*, which is implicated in ROS clearance (Cao *et al.* 2016), were significantly downregulated in embryos obtained from the OF+UF and UF groups compared with the other groups. This represents a possible antioxidant activity derived primarily from UF. In relation to possible activity in the uterus, a previous study suggested that superoxide dismutase, an important antioxidant, plays a significant role in the proliferation and differentiation of the endometrium, implantation and maintenance of human pregnancy (Sugino *et al.* 1996). In another study, a dynamic change in the antioxidative pathways at the endometrial–extra-embryonic membrane interface during the transition from implantation to postimplantation development was described in sheep (Al-Gubory and Garrel 2012). CM-H_2DCFDA, used in the present study to detect ROS accumulation, is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidised by ROS into 2′,7′-dichlorofluorescein (DCF), and becomes fluorescent (Nasr-Esfahani *et al.* 1990; Kalyanaraman *et al.* 2012). The intracellular redox chemistry of CM-H_2DCFDA is complex and it has several limitations and artefacts for the measurement of intracellular H_2O_2 and other ROS. Therefore, it is essential to keep these limitations in mind for proper interpretation of the results obtained in the CM-H_2DCFDA assay (Kalyanaraman *et al.* 2012). However, in our case, reduced ROS detection by CM-H_2DCFDA in blastocysts produced in the UF or OF+UF groups was supported by the decreased abundance of *CLIC1* and *GPX1* mRNA in these embryos.

In conclusion, the present study indicates that the use of low concentrations of OF and UF in serum-free culture medium during IVM supports early embryo development and improves blastocyst quality by increasing their cryotolerance, altering the abundance of developmentally related genes and providing better antioxidant activity. This evidence may provide a step towards the elucidation of relevant proteins for embryo development present in the physiological environment of the reproductive tract (OF and UF) to improve ART in mammals.

Conflicts of interest

The authors declare no conflicts of interest.

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Chapter 2

Gene expression and metabolic response of bovine oviduct epithelial cells to early embryo *in vitro*

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Abstract

During its journey through the oviduct, bovine embryo may elicit a transcriptomic and metabolic response on the direct or indirect contact with oviduct epithelial cells (BOEC). In order to study this response, an *in vitro* model was established allowing a local interaction during 48h between BOEC monolayer and early embryos (2- or 8-cell stage) or their respective conditioned media (CM). The gene response of BOEC to early embryo was assessed by analyzing the transcript levels of *SMAD6, TDGF1, ROCK1, ROCK2, SOCS3, PRELP* and *AGR3* selected from *in vivo* studies and *GPX4, NFE2L2, SCN9A, EPSTI1, IGFBP3* selected form *in vitro* studies. Moreover, metabolic analyses were performed on the media obtained from the co-culture. Gene expression analysis revealed that BOEC response to 2-cell embryos or their CM was different from the one observed to 8-cell embryo or their CM. Eight-cell embryos or their CM affect *SMAD6, ROCK1, ROCK2, SOCS3* and *PRELP* transcripts in the same manner as 8 cell embryo affected the *in vivo* expression in the oviduct. However, energy substrates and amino acid analysis revealed that BOEC metabolism was not affected by presence of early embryos or by their CM. Interestingly embryo metabolism before embryo genome activation (EGA) seems to be independent of exogenous source of energy. In conclusion, this study confirms that early embryo alters the gene expression of BOEC depending on the phase of EGA. Moreover, embryo affects BOEC via a direct contact or via its secretions. However transcriptomic response of BOEC to embryo did not obligatory unchain a metabolic response.

Introduction:

The mammalian oviduct is the venue of important events leading to the establishment of pregnancy. The cattle embryo spends four days in the oviduct (Hackett *et al.* 1993). During its journey through it, the embryo undergoes important morphological and transcriptional changes such as first mitotic cell division and embryonic genome activation (EGA) (Memili and First 2000). Many convincing evidences suggested its positive influence on the quality of the early embryo, e.g., short-term culture of *in vitro*-produced bovine zygotes in the oviducts of cattle (Tesfaye *et al.* 2007; Gad *et al.* 2012), sheep (Enright *et al.* 2000) or even mice (Rizos *et al.*, 2007) shown an improvement of embryo quality as measured in terms of morphology, gene expression, cryotolerance and pregnancy rate after transfer. Also, the metabolic environment of the oviduct affects embryo development. In fact, the oviductal environment in metabolically stressed lactating dairy cattle was less supportive for blastocyst formation compared to heifers (Rizos *et al.* 2010a) and non-lacting cows (Maillo *et al.* 2012).

Moreover, it is necessary to examine whether the embryo itself is able to modulate changes in the oviduct and consequently induce similar effects. Some studies have reported morphological, proteomic and transcriptomic cycle-dependent changes in the oviduct which may be related to the regulation of embryonic development. For example, Bauersachs *et al.* (2003) noticed that most of bovine oviduct genes up-regulated in the luteal phase were involved in regulating transcription and cell proliferation, while the majority of those up-regulated during the follicular period were involved in protein secretion (Bauersachs *et al.* 2003). Furthermore, in mice, the presence of embryos in the oviduct upregulated the expression of specific genes such as thymosin beta-4, ribosomal protein L41 and nonmuscle myosin light chain 3 (Lee *et al.* 2002). In pigs, Almiñana *et al*. (2012) found that the presence of embryos downregulated the expression of genes related with the immune system. Furthermore, transcriptomes like transforming growth factor-alfa, transforming growth factor-beta-binding protein 2 and astral natriuretic factor receptor-like were up regulated in the presence of 4 cell porcine embryo (Chang *et al.* 2000). Thus it is proposed that such embryo-signal would be magnified in litter bearing species due to the presence of multiple embryos in the oviduct (Maillo *et al.* 2015). In bovine, a mono-ovulatory species, an in *vivo* study failed to detect transcriptomic response of the oviduct to the presence of a single embryo, while a response was observed after a multiple ET (Maillo *et al.* 2015). Similar results were observed in equine (Smits *et al.* 2016). Those results suggest a possible dilution of BOEC transcriptome response due to the very local embryo effect. Although, *in vivo* studies remain challenging, due to the specific equipment and techniques required, and the difficulty to interpret the results considering the complexity of the whole organism (Velazquez *et al.* 2010).

Holistic transcriptome and proteome analyses, as well as specific experiments targeting embryo–maternal interactions in the oviduct, require sufficient numbers of well-defined cells and embryos in a standardized experimental environment. Therefore, adequate *in vitro* model would be an alternative to understand the complex interactions between the embryo and the maternal environment. Bovine oviduct epithelial cells (BOEC) representing the intimate part of the oviduct have been cultured *in vitro* in many systems; as monolayers, in perfusion chambers, in suspension, in polarized or in three- dimensional (3D) systems (Maillo *et al.* 2016b) and have been employed by several investigators to study the oviduct environment. Recently, BOEC cultured in polarized system have been used to elucidate the mechanism and physiology employed to face situations of metabolic stress produced by elevated non-esterified fatty acids (Jordaens *et al.* 2015; Jordaens *et al.* 2017). Also, BOEC have been cultured in 3D system to study polyspermic fertilization and parthenogenic activation (Ferraz *et al.* 2017b). Rottmayer *et al.* (2006) proposed a short-term (24 hours) epithelial cell suspension culture as a suitable system for holistic and specific analyses of early embryo-oviduct interactions (Rottmayer *et al.* 2006). While, Schmaltz-Panneau *et al.* (2014) identified differentially expressed genes related to immune system and interferon signaling pathway, by co-culturing BOEC monolayer of 13 days with early embryos for 8 days (Schmaltz-Panneau *et al.* 2014). Those *in vitro* systems preserve differently BOEC features and characteristics. In fact, it was reported that systems grown in monolayers dedifferentiate with a concomitant loss of important morphological characteristics. These include reduction of cell height, loss of cilia, and loss of secretory granules and bulbous protrusions (Thibodeaux *et al.* 1992; Walter 1995). Furthermore, in a direct comparison between BOEC grown as a monolayer or in suspension culture, only suspended cells maintained cilia and secretory activity. In other words, monolayers are less able to mimic the oviduct environment *in vivo* but they might provide a good starting point with which to study putative embryo or oviduct derived signals. For instance, in a recent study carried out by our group using BOEC monolayer model, we reported that bovine embryo-oviduct interaction *in vitro* reveals an early cross talk mediated by Bone morphogenetic protein (BMP) signaling (Garcia *et al.* 2017).

In order to test *in vitro* the hypothesis of a possible effect of early embryo on BOEC, we established an *in vitro* co-culture system allowing both local and temporal interactions between early bovine embryos and isthmus epithelial cells. The objectives were: (i) to assess the transcriptome response of BOEC to early embryo and whether it is due to a contact-depending signal or the result of BOEC interaction with embryo secretions; and (ii) to examine the metabolism changes of BOEC under a possible effect of early embryos.

Materials and methods

Chemicals

All reagents were purchased from Sigma Chemical Química S.A Company (Madrid, Spain) unless otherwise stated**.**

Bovine oviduct epithelial cells isolation and *in vitro* culture

Bovine oviducts corresponding to early luteal phase (0-5 days of estrous cycle) were obtained from local slaughterhouse based on corpus luteum morphology (Ireland et al., 1980) and transported to the laboratory on ice. Once in the laboratory, each oviduct was carefully trimmed from surrounding tissues, then ligated, and washed three times in sterile PBS without Ca^{2+}/Mg^{2+}, supplemented with 0,5% of gentamycin. The infundibulum and the ampulla were removed and mucosa was collected from isthmus part by squeezing using cover slip, and then centrifuged twice with PBS at 280 g for 7 min. The resulted pellet was diluted in 1ml of culture medium (TCM 199 supplemented with 10% fetal calf serum (FCS), 2.5% of gentamycin and 1% of amphotericin) and pipetted 10 times with a 25G needle to individualize the cells. After count with hemocytometer, cells were diluted with culture medium and the final concentration was adjusted to 2×10^6 cells/ml and cultured in four well plates at 38.5 ºC, 20% O_2 and saturated humidity until confluence. Half of the media was renewed every 48 h. Twenty four hours before starting the co-culture with the embryos and their CM, the cell culture medium was changed to SOF with 5% FCS.

Immunocytochemistry

The epithelial origin of the isolated BOECs was confirmed by subjecting the cells to immunocytochemical staining for the expression of cytokeratin using antibodies antibovine-pancadherin (C1821), anti-bovine-pancytokeratin (C2931) and anti-bovine-vimentin (V2258) (Lopera-Vásquez *et al.* 2016). BOEC were cultured as monolayer on coverslips coated with 5% gelatin and fixed for 7 Days. The monolayer was fixed with 4% paraformaldehyde (Panreac) for 30 min. Then, samples were permeabilized with Triton x-100 0.5% for 5 min and they were then washed and stained with the appropriate primary and secondary antibodies. Then samples were mounted with Hoechst 33342-containg Prolong (Invitrogen) and visualized under an epifluorescence microscope (Fig 1A).

In vitro production of embryos and their conditioned media

Immature COCs recovered from the ovaries of heifers slaughtered at a local abattoir were submitted to IVM *and* IVF as previously described (Garcia *et al*, 2017). Approximately 18-22 h

post-insemination, presumptive zygotes were denuded of cumulus cells by vortexing and groups of 50 were initially cultured in 500 μL of SOF supplemented with 5% of FCS in a four-well dish, under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5 °C. Embryos reached 2- and 8- cell stage were respectively selected at 31 and 52 hours post-insemination (hpi), and then randomly cultured in groups of 50 to produce embryo conditioned media (embryo-CM) or to be used in co-culture with BOEC monolayer. In order to produce fresh embryo-CM and early embryos for the co-culture with BOEC, embryo and embryo-CM replicates were synchronized with BOEC replicates (See experimental design Fig 2)

Mesh preparation and co-culture condition

In order to limit and establish a local area of contact between the embryos and the BOEC monolayer, a nontoxic woven polyester mesh (Sefar Petex; Sefar, Bury, Lancashire, UK) was used as previously described (Garcia *et al.* 2017). Grids with size of 41x41 openings covering an area of 121 mm^2 were prepared. First grid containing delimited marked areas of 7x7 openings was placed outside at the bottom of the wells, where BOEC monolayer are cultured and it was used as a guide to localize the co-culture area. Then, another mesh square of the same size, was washed once with 70% ethanol, three times with PBS and then with SOF media, it was introduced into the well, so that the external and internal mesh were overlapped (Fig 1B). During the co-culture 50 embryos (at 2- or 8- cell stage) were placed inside the BOEC well, over the 7x7 marked area. Preferably, the central marked area was used. After 48 h of co-culture, BOEC from experimental groups were collected by mechanical scraping with a micropipette tip, snap frozen in liquid nitrogen and then kept at-80C° until gene expression analysis.

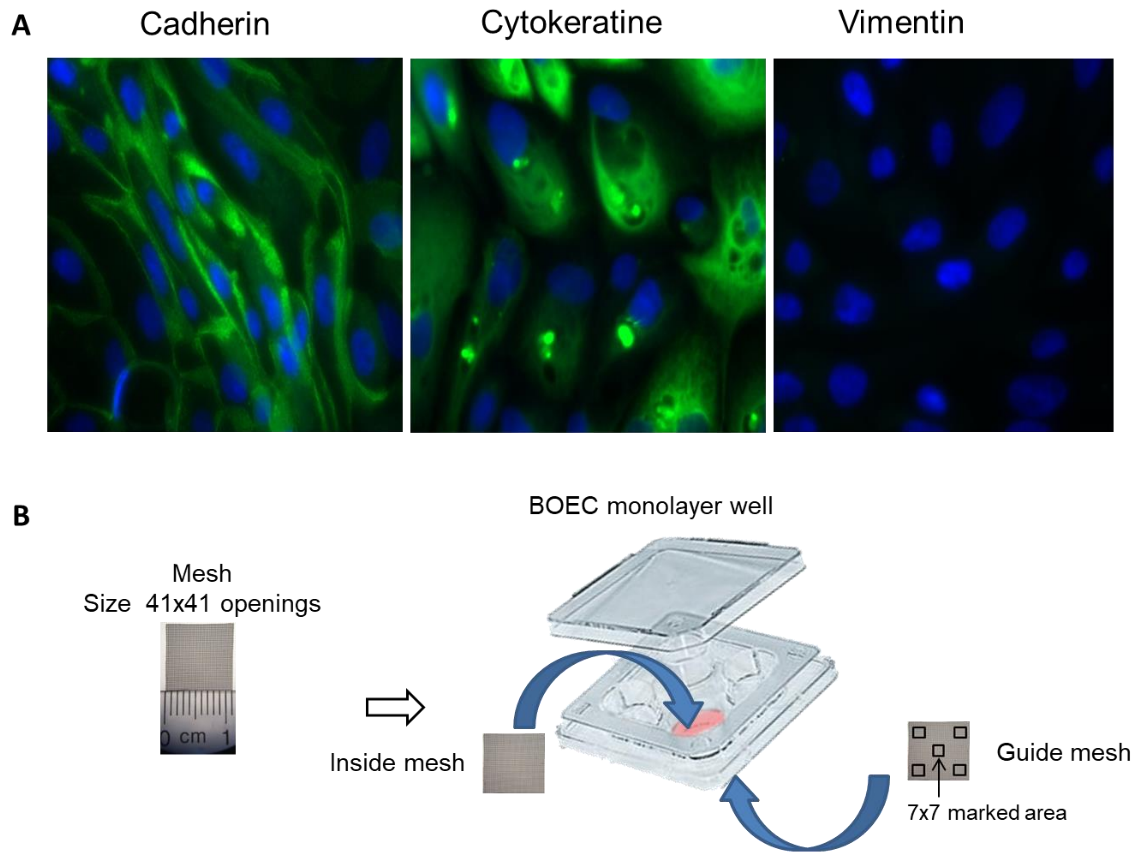

Figure 1. (**A**) Immunofluorescence analysis of BOEC monolayers showing positive staining for the epithelial markers, cadherin and cytokeratin, and negative staining for the fibroblast marker, vimentin. Cell nuclei were counterstained with Hoechst stain (blue). (**B**) Representative image of the polyester meshes employed to establish a local area of contact between the embryos and the BOEC monolayer. A guide mesh containing delimited marked areas of 7x7 openings was placed outside, at the bottom of the wells, where BOEC monolayer were cultured and was used as a guide to localize the co-culture area. Then, another mesh was introduced into the well, so that the external and internal meshes were overlapped during the co-culture.

RNA isolation and reverse transcription

mRNA from each experimental group of BOEC was extracted from 4 biological replicates using Dynabeads mRNA Direct Extraction Kit (Dynal Biotech, Oslo, Norway), following the manufacturer's instructions with minor modifications as it was described previously by (Bermejo-Alvarez *et al* 2008). After 10 min of incubation in lysis buffer with Dynabeads, poly(A) RNA attached to the Dynabeads was extracted with a magnet and washed twice in washing buffer A and washing buffer B. RNA was eluted with Tris-HCl. Immediately after extraction, the reverse transcription reaction was performed as recommended by the manufacturer (Epicentre Technologies Corp, Madison, WI, USA). Briefly, oligo-dT (0.2 µM) and random primers (0.5 µM) were added to the RNA and were then heated for 5 min at 70ºC to denature the secondary RNA structure. Next, the tubes were incubated at 25°C for 10 min to promote the annealing of random primers. Then, the RNA was reverse-transcribed for 60 min at 37ºC in a final volume of 40 µl containing 0.375mM dNTPs (Biotools, Madrid, Spain), 6.25U RNasin RNAse inhibitor (Promega, Madison,WI, USA), 10X MMLV-RT buffer with 8mM dithiothreitol, and 5U MMLV high performance reverse transcriptase (Epicentre Technologies Corp, Madison, WI, USA), followed by incubation at 85 °C for 5 min to inactivate the RT enzyme.

Gene expression analysis by quantitative real-time polymerase chain reaction

The mRNA expression levels of the selected genes were determined by quantitative real-time PCR (qRT-PCR) using specific primers (Table 1) designed with Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to span exon-exon boundaries when possible. All PCR reactions were performed in a final volume of 20 µL, containing 0.25 mM of forward and reverse primers, 10 µL of GoTaq qPCR Master Mix (Promega) and 2 µL of each cDNA sample derived from BOEC (≈60ng/µL) using a Rotorgene 6000 Real Time Cycler (Corbett Research, Sydney, Australia) and SYBR Green as double stranded DNA-specific fluorescent dye. The PCR program consisted of an initial denaturalization step at 94°C for 2 min, followed by 35 cycles of denaturalization at 94°C for 10 s, annealing at 56°C for 30 s, extension at 72°C for 15 s and 10 s of fluorescence acquisition defined for each primer. At the end of each PCR run, melt curve analyses were performed for all genes to ensure single product amplification and exclude the possible interference of dimers.

Relative expression levels were quantified by the comparative cycle threshold (ΔΔCT) method (Schmittgen and Livak 2008). Values were normalized using two housekeeping genes (*H2AFZ* and *ACTG1*). Fluorescence was acquired in each cycle to determine the threshold cycle during the log-linear phase of the reaction at which fluorescence increased above background for each

sample. According to the comparative CT method, the ΔCT value was determined by subtracting the mean CT value of the two housekeeping genes for each sample from each gene CT value of the sample. Calculation of ΔΔCT involved using the highest sample ΔCT value (i.e. the sample with the lowest target expression) as an arbitrary constant to subtract from all other ΔCT sample values. Fold changes in the relative gene expression of the target were determined using the formula 2–ΔΔCT (Livak and Schmittgen 2001)

Table 1 Set of primers used for qRT-PCR assays

Gene		Primer sequence (5´-3´)*	Fragment Size, bp	Gene Bank Accession
H2AFZ	Forward	AGGACGACTAGCCATGGACGTGTG	209	NM_174809
	Reverse	CCACCACCAGCAATTGTAGCCTTG		
ACTG1	Forward	GAGAAGCTCTGCTACGTCG	255	XM_003357928.3
	Reverse	CCAGACAGCACCGTGTTGG		
GPX4	Forward	TGTGGTGAAGCGGTATGGTC	266	NM_174770.3
	Reverse	TATTCCCACAAGGCAGCCAG		
NFE2L2	Forward	GCTCAGCATGATGGACTTGGAG	390	NM_001011678.2
	Reverse	GGGAATGTCTCTGCCAAAAGC		
ROCK1	Forward	ACGTGACCTAGTGCCTTGTG	164	XM_002697789.5
	Reverse	CCTCAGTGTGCTTTTGTGCC		
ROCK2	Forward	CTTGGCTGCTCAACTGGAGA	276	NM_174452.2
	Reverse	TGCTCTTGGGCTTCCTTCAG		
AGR3	Forward	TGTCACACTCAGTTCTGGTCC	119	NM_001191502.1
	Reverse	GTCATCTCCCCACCCTCTTGA		
SMAD6	Forward	GGAGAAATTCGCTCCAAGTGC	242	NM_001206145.1
	Reverse	CCCTGCCTTTAAAACCCAAGC		
TDGF1	Forward	ATGGTGAGAGACGGGCTGCTAG	201	NM_001080358.1
	Reverse	GCCCTTGTCTCATACAGCTTCC		
SCN9A	Forward	GTTGATAACCCTGTGCCTGGA	250	XM_005202453.3
	Reverse	CTTCAAAAGCCAGAGCACCAC		
SOCS3	Forward	GCGAGAAGATCCCTCTGGTG	167	NM_174466.2
	Reverse	CTAAAGCGGGGCATCGTACT		
IGFBP3	Forward	GAGTCCAAGCGTGAGACAGAA	150	NM_174556.1
	Reverse	GCGGCACTGCTTTTTCTTGTA		
PRELP	Forward	CAGCATCGAGAAAATCAATGGGA	158	NM_174434.3
	Reverse	AGCACATCATGAGGTCCAGC		
EPSTI1	Forward	AAACGACAGCAACAGGAGGAA	89	XM_002700904.4
	Reverse	CCTTGGAGTCGGTCCAGAAAA		

*All the primers were designed with NCBI Primer-BLAST online tool of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/tools/primer-blast/)

Metabolic Analysis

Glucose Lactate and Pyruvate consumption*:*

These energy substrates were measured using fluorometric assays described by Gardner and Leese (1990). The assays were based on the enzymic phosphorylation of substrate and the subsequent consumption or generation of NADH or NADPH in coupled reactions which causes an increase in fluorescence which was measured using FluoStar Omega Microplate Reader (BMG LabTech) (excitation 355 nm, fluorescence 460 nm and above). All values were expressed as mM and were relativized to blank control to calculate consumption rate.

Amino Acid Profiling*:*

The analysis of 18 amino acids was performed using high-performance liquid chromatography (HPLC) as previously described (Houghton *et al.* 2002). An aliquot of 2.4 µl of the media was diluted 1:12.5 in HPLC-grade water. Amino acid degradation/appearance was calculated with respect to control. All values were expressed in µM and were relativized to blank control to calculate consumption rate.

Experimental design

Experiment 1: *Effect of early embryo on the BOEC transcriptomic response: is it due to a contact-dependent signaling effect or the result of interaction with embryo secretions?*

An *in vitro* co-culture system was established to provide a local and temporal interaction between BOEC and early bovine embryos. Isthmus epithelial cells obtained from a pool of three post-ovulatory stage oviducts (4 replicates) were cultured in a monolayer. In parallel, four replicates of 50 embryos at 2- or 8- cell stage selected at 31 or 52 hpi respectively were established to produce fresh embryo CM. In addition, another four embryo replicates were performed to produce early embryos at 2- or 8-cell stage selected at 31hpi or 52 hpi respectively (Fig 2A). BOEC were co-cultured during 48h as following: (1) directly beneath to 50 embryos at 2- or 8- cell stage (BOEC EMB+); (2) in the same well as 2- or 8-cell embryos but not in direct contact with embryos (BOEC EMB-); (3) cultured with CM previously produced from 50 embryos at 2- or 8-cell stage (BOEC CM); (4) without embryos (BOEC CTR) (Fig 2B). BOEC from different experimental groups were recovered to assess gene expression analysis performed by RT-qPCR. The mRNA expression levels of genes that previously shown to be affected by the presence of embryo from *in vivo* (Maillo *et al*. 2015): *SMAD6, TDGF1*, *ROCK1*, *ROCK2, SOCS3*, *PRELP*, *AGR3,* and *in vitro* (Schmaltz-Panneau *et al.* 2014; Schmaltz-Panneau

et al. 2015): *GPX4*, *NFE2L2*, *SCN9A*, *EPSTI1, IGFBP3*; studies were analyzed according to the procedure described above.

Experiment 2: *Effect of early embryo on BOEC metabolism of energy and amino acid.*

In order to assess the metabolic changes that may result from BOEC/embryo or embryo-CM interaction, the media obtained after 48h of co-culture from the experiment 1 was collected to perform metabolic analysis. The experimental groups were: (1) BOEC co-cultured with 50 embryos at 2-or 8-cell stage (BOEC EMB), (2) BOEC cultured with CM produced from 2- or 8-cell stage (BOEC CM), (3) BOEC alone, (4) embryos at 2- or 8-cell stage (EMB) (Fig 2C). As blank control, embryo/BOEC-free media was treated in the same manner as the experimental groups.

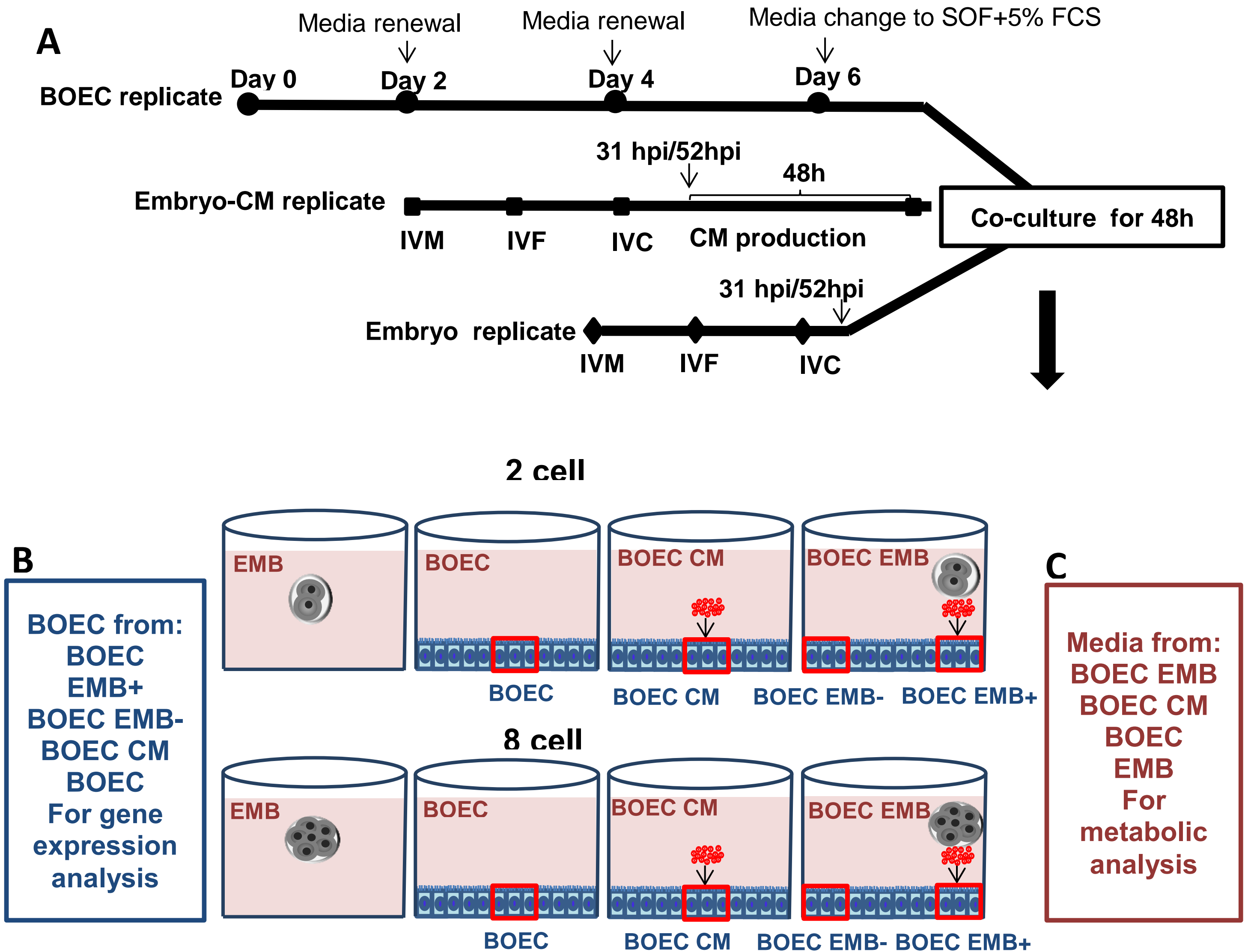

Figure 2: Experimental design. BOEC replicates (n=4 replicates) were synchronized with embryos (n=4 replicates) and embryo-CM (n=4 replicates). Early embryos selected at 2 cell- stage (at 31hpi) or at 8-cell stage (at 52hpi) either for fresh embryo CM production or for co-culture with BOEC monolayer during 48h (Fig 2A).The following experimental groups of BOEC were established: BOEC directly beneath to 50 embryos at 2- or 8- cell stage (BOEC EMB+). BOEC in the same well as 2- or 8-cell embryos but located away (BOEC EMB-). BOEC cultured with CM produced from 50 embryos at 2- or 8-cell stage (BOEC CM). BOEC cultured without embryos (BOEC CTR). After 48h of co-culture BOEC were recovered to perform gene expression analysis (Fig 2B). In order to perform metabolic analysis of energy substrates and amino acid, the culture media obtained after 48h from the following groups was used: Co-culture of BOEC with 50 embryos at 2-or 8-cell stage (BOEC EMB). Culture of BOEC with CM produced from 50 embryos at 2- or 8-cell stage (BOEC CM). BOEC cultured without embryos (BOEC). Embryos at 2- or 8-cell stage cultured

Statistical analysis

The statistical analysis was performed with the SigmaStat software package (Jandel Scientific, San Rafael, CA). Differences in the relative mRNA abundance levels and metabolic results were analyzed using one-way analysis of variance (ANOVA), followed by Holm–Sidak post hoc test, when applicable, to determine statistical differences between the experimental groups. When normality and/or homogeneity tests failed Kruskal-Wallis test was conducted. Values were considered significantly different when p value was lower than 0.05.

Results

Experiment 1: *Effect of early embryo on the BOEC transcriptomic response: is it due to a contact-dependent signaling effect or the result of interaction with embryo secretions?*

Gene expression analysis in BOEC revealed that direct interaction or not with 2-cell embryos (BOEC EMB+, BOEC EMB-) or with their CM (BOEC CM) significantly decreases the expression of *GPX4*, *ROCK2* and *SCN9A* in comparison with control group (BOEC CTR). However, the mRNA expression level of *SMAD6* was only decreased in BOEC EMB+ and BOEC EMB- in comparison with BOEC CM and BOEC CTR. No differences were observed between BOEC EMB+ and BOEC EMB- except for *NFE2L2* which decreased in BOEC EMB+ and BOEC CM compared to BOEC EMB- and BOEC CTR. The expression of *IGFBP3* was significantly increased in BOEC CM compared with BOEC CTR, but no significantly difference was found with the presence of the 2-cell embryos ($P < 0.05$) (Fig 3A). The remaining genes did not display an alteration of their expression in any condition ($P > 0.05$) (Fig 3A).

On the other hand, when BOEC were co-cultured in presence of 8-cell embryos or their CM, the expression pattern of many of the genes studied was different to that observed above. *PRELP* mRNA expression level was significantly decreased in presence of 8-cell embryo (BOEC EMB+, BOEC EMB-) and its CM (BOEC CM) in comparison with the control group. Also, BOEC directly exposed to 8-cell embryos (BOEC EMB+) or their CM (BOEC CM) decreased the expression of *SOCS3* and increased the expression of *SCN9A* in comparison with BOEC cultured away from embryos (BOEC EMB-) and with control BOEC (BOEC CTR) ($P<0.05$). *GPX4* transcript was only decreased in BOEC cultured directly to embryos (BOEC EMB+) in comparison with the other groups (BOEC EMB-, BOEC CM and BOEC CTR). Furthermore, the expression level of *NFE2L2* was increased in BOEC CM compared to BOEC CTR and BOEC EMB- but similar to BOEC EMB+. Also, *ROCK1* and *ROCK2* transcripts were increased in BOEC CM in comparison to BOEC EMB+ and BOEC CTR but similar to BOEC EMB. In the opposite, *SMAD6* conserves the same expression pattern in BOEC co-

cultured with 8- cell embryo stage similarly to the co-culture with 2 cell embryos. Indeed, *SMAD6* decreased in BOEC EMB+ and BOEC EMB- in comparison with BOEC CM and BOEC CTR (P < 0.05) (Fig 3B). The rest of genes did not display an alteration of their expression in any condition (P > 0.05) (Fig 3B)

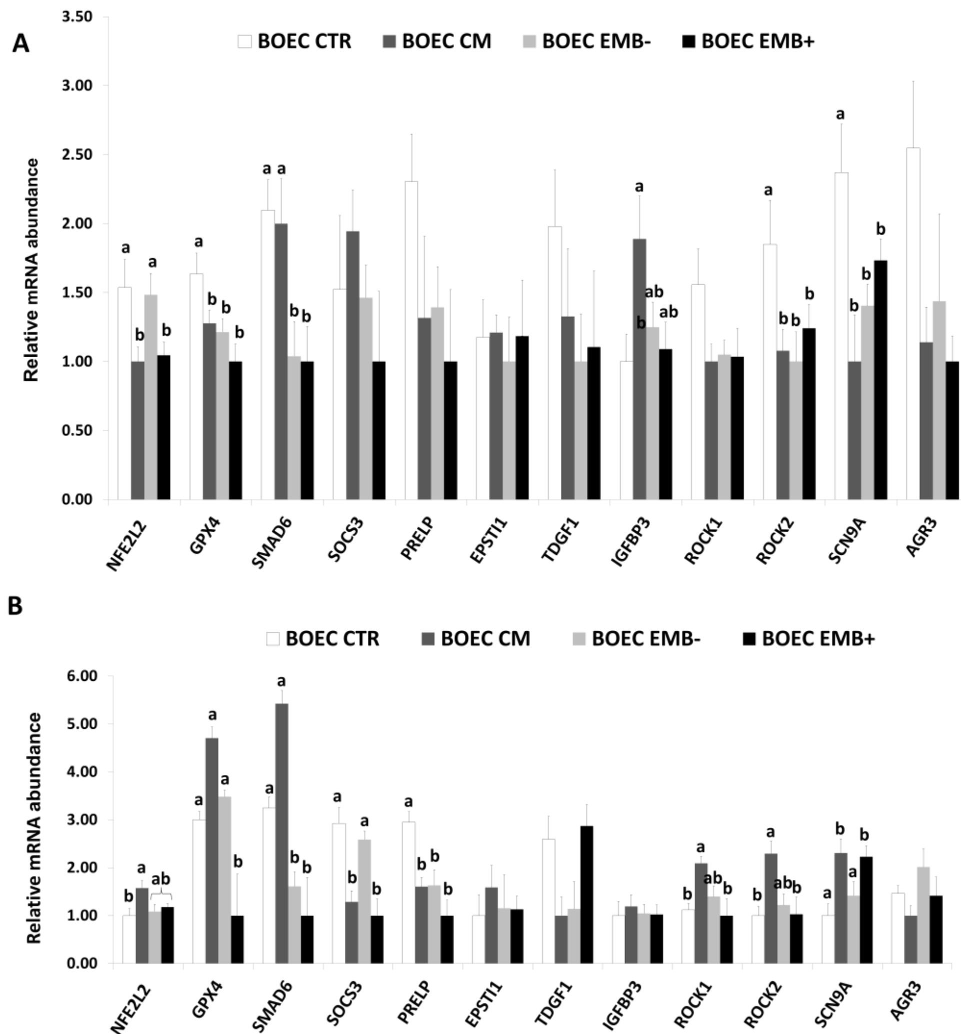

Figure 3: Relative mRNA abundance of genes in BOEC cultured *in vitro* in: (**A**) direct contact or not with 2-cell embryos or with their CM. (**B**) direct contact or not with 8-cell embryos or with their CM. Experimental groups correspond to: BOEC directly beneath to 50 embryos at 2- cell stage: BOEC EMB+; BOEC in the same well as 2- cell embryos but located away: BOEC EMB-; BOEC cultured with CM produced from 50 embryos at 2- cell stage: BOEC CM; BOEC control without embryos: BOEC CTR. Bars represent the relative abundance of the transcripts analyzed and normalized to *H2AFZ* and *ACTG1* as housekeeping genes. Results are expressed as means ± S.E.M. Different superscripts indicate significant differences (P < 0.05) between treatments. Data were obtained from four replicates of BOEC samples per experimental group.

Experiment 2: *Effect of early embryo on BOEC consumption of energy and amino acid*

Glucose Lactate and Pyruvate consumption:

The results of glucose lactate and pyruvate consumption by BOEC had a similar pattern in both 2- cell and 8-cell groups (Fig 4 A and B). Indeed, a similar consumption of BOEC to glucose and pyruvate was observed whether they were cultured with embryo (BOEC EMB), with embryo-CM (BOEC CM) or without embryos (BOEC). Interestingly, BOEC consumption was significantly higher than embryo consumption ($P<0.001$ for 2-cell group; $P<0.005$ for 8-cell group). For lactate consumption, it was significantly higher in BOEC cultured with 2- cell embryos (BOEC EMB) or without embryos (BOEC) in comparison with BOEC cultured with CM of 2- cell embryos (BOEC CM) or 2-cell embryo groups ($P<0.001$) (Fig 4A). However, lactate consumption was significantly higher in BOEC co-cultured with 8-cell embryos (BOEC EMB), without embryos (BOEC) and in 8-cell embryos in comparison with BOEC cultured with CM of 8-cell embryos (BOEC CM) ($P<0.005$) (Fig 4B). Regarding embryo metabolism, 2-cell embryo stage did not reveal a consumption of energy substrates when they were compared to the blank control.

Amino Acid Profiling

The results of HPLC revealed that among the 18 amino acids analyzed, 11 had the same pattern of consumption/ production in 2 and 8-cell groups (Fig 5 A and B). Indeed, these amino acids were significantly highly consumed in all BOEC groups (BOEC EMB, BOEC CM, BOEC) in comparison with embryos group (EMB) ($P<0.005$). For alanine, that was highly produced in BOEC EMB, BOEC CM and BOEC in comparison with embryos group ($P<0.01$). However, regarding serine and lysine significant differences were observed in 8-cell embryo group, being significantly highly consumed in BOEC compared to embryo group ($p<0.005$) (Fig 5B). No differences of consumption in histidine, threonine, tyrosine, tryptophan, methionine and phenylalanine were observed between the experimental groups neither in 2- or 8-cell embryos. Regarding embryo metabolism, 2-cell or 8-cell embryo did not reveal a consumption of amino acids substrates when they were compared to the blank control.

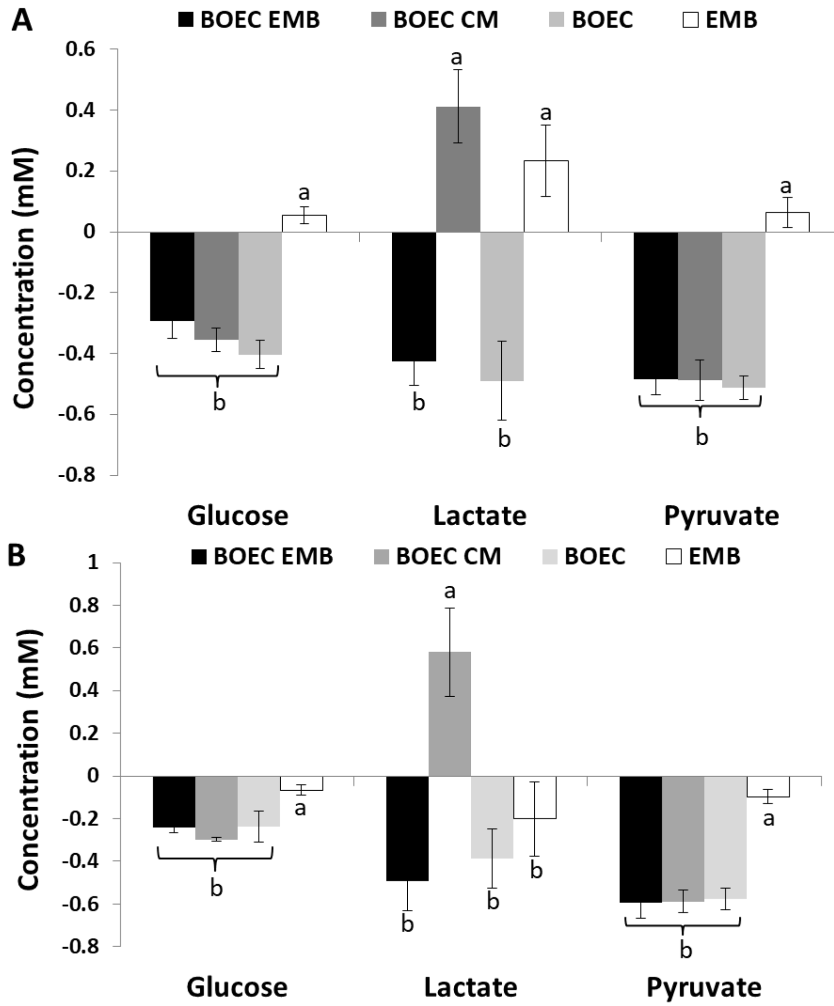

Figure 4: Energy substrates metabolism of BOEC co-cultured with 2-cell (Fig 4A) or 8-cell embryos (Fig 4B). Data of experimental groups were relativized with data of blank media. Values are expressed as means+SEM. Different superscripts indicate significant differences between treatments (Fig 4A, P<0.001); (Fig 4B, P<0.005). BOEC EMB: BOEC co-cultured with 2-cell or 8-cell embryos during 48h; BOEC CM: BOEC cultured with CM obtained from 50 embryos at 2-cell or 8-cell stage; BOEC: BOEC cultured neither with embryos nor with their CM; EMB: embryos at 2-cell or 8 cell stage cultured without BOEC

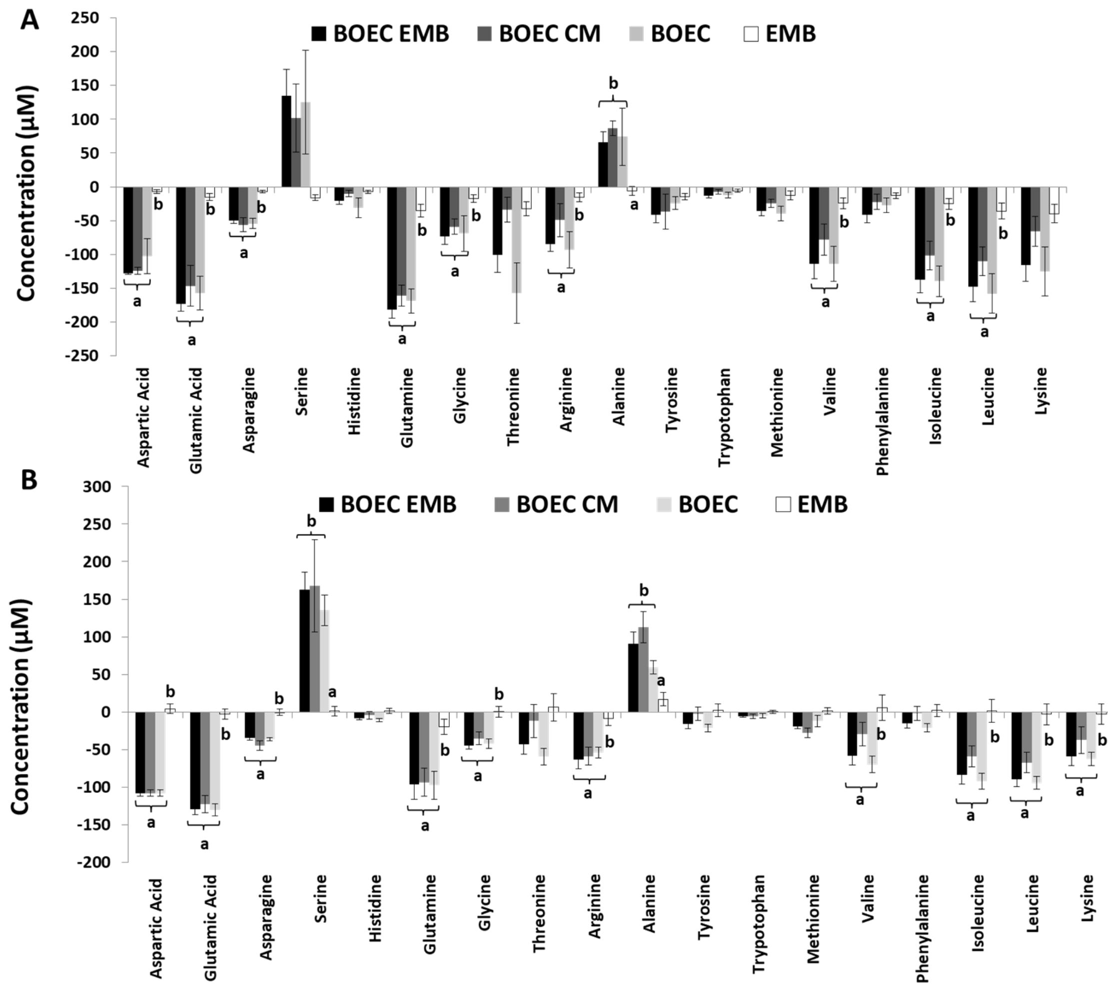

Figure 5: Amino acid metabolism of BOEC co-cultured with 2-cell (Fig 5A) or 8-cell embryos (Fig 5B). Data of experimental groups were relativized with data of blank media. Values are expressed as means+SEM. Different superscripts indicate significant differences between treatments (P<0.005). BOEC EMB: BOEC co-cultured with 2-cell or 8-cell embryos during 48h; BOEC CM: BOEC cultured with CM obtained from 50 embryos at 2-cell or 8-cell stage; BOEC: BOEC cultured neither with embryos nor with their CM; EMB: embryos at 2-cell or 8 cell stage cultured without BOEC

Discussion

The main objective of the present study was to examine whether the preimplantational embryo is able to modulate transcriptome and metabolic changes in BOEC and to elucidate if this effect is due to a contact-depending signaling with the embryo or the result of the BOEC interaction with its secretions, using an *in vitro* co-culture system.

It was suggested that single embryo may have a very local effect on the oviduct (Maillo *et al.* 2015; Smits *et al.* 2016) which make it difficult to detect. The challenge now is to identify appropriate *in vitro* models to facilitate the study of early embryo-maternal communication. Recent advances in different species, including bovine, porcine and human have enabled the development of new *in vitro* culture techniques that better mimic the morphological and physiological characteristics of the oviduct (Chen *et al.* 2017; Jordaens *et al.* 2017; Ferraz *et al.* 2017b). However, considering the complexity of studies that aim to understand the regulatory mechanisms controlling embryo-maternal communication, we judged more suitable to initiate our investigation with a less complex system using *in vitro* BOEC monolayer, allowing a more controlled interaction between embryo and oviduct cells in terms of area and contact time, by the use of polyester mesh. According to our previous study (Garcia *et al.* 2017) and others (Matoba *et al.* 2010) no negative effect was observed on BOEC or embryo development related to the use of this device.

We evidenced that the overall transcriptional profile between BOEC co-cultured with 2-cell embryos or with their CM was different from the one observed after co-culture with 8-cell embryos or with their CM. It is important to emphasis that the difference between both embryo stages might reside in the EGA. In bovine, the activation of transcription occurs during the 8- to 16-cell stages and it is associated with a phase called the maternal-to-embryonic transition where maternal mRNA are replaced by embryonic ones (Graf *et al.* 2014). A very high proportion of genes expressed in the 8-cell stage embryo have a demonstrated involvement in gene transcription or RNA processing (Vigneault *et al.* 2009). These events may be accompanied with a specific embryo signaling that could be implicated in embryo-maternal-communication.

Our results of gene expression analysis revealed that early embryo (especially 8-cell embryo) or their CM affect *SMAD6, ROCK1, ROCK2, SOCS3* and *PRELP* transcripts in the same manner as 8-cell embryo affected the *in vivo* expression in the oviduct (Maillo *et al.* 2015). In our results, *SMAD6* decreased in presence of both 2- and 8-cell embryo. This is also consistent with previous data obtained from our group evidencing the relevance of *SMAD6* as a component of BMP signaling pathway (Garcia *et al.* 2017). Beyond that, our current result

suggests that this effect seems to be due to a direct contact between the embryo and the BOEC, as far as *SMAD6* did not decrease in BOEC cultured with embryo CM. This could be also the case of *GPX4* that decreased in BOEC co-cultured in direct contact with 8-cell embryos (BOEC EMB+). *GPX4* is known to be implicated in oxidative stress response. Previous studies reported an increased expression of *GPX4* in BOEC cultured *in vitro* due to the increasing level of ROS (Schmaltz-Panneau *et al.* 2015). However, decreased BOEC expression of *GPX4* may be explained by the fact that BOEC had already performed their role of embryo protection against oxidative stress by producing antioxidant enzymes such as copper, zinc superoxide dismutase or *GPX4* (Schmaltz-Panneau *et al*., 2015). Thus, the decreased *GPX4* abundance of BOEC revealed an unbalanced ROS level and maintain it in the physiological amount as it is very important to produce mitotic signal for cells growth (Tsunoda *et al.* 2014). Nevertheless, further studies are required to confirm that possibility.

Moreover, *ROCK1* and *ROCK2* are members of rho-family, known to be implicated in multiple aspects of establishment and maintenance of epithelial cell polarity and cell morphology. Rho proteins are considered as key players in mediating the effects of extracellular signals (including secreted factors, cell surface-associated ligands, and adhesion molecules) to the downstream cellular machinery that controls the organization of the actin cytoskeleton, determining cell shape, adhesion, contractility, and movement (Van Aelst and Symons 2002). Previous studies using mouse embryo suggested the importance of Rho-ROCK signaling at the early stages of preimplantation development for polarization and compaction at the eight-cell stage (Clayton *et al.* 1999). In our study, BOEC decreased the expression of *ROCK2* after co-culture with 2-cell embryos and their CM. Conversely an increase of *ROCK1* and *ROCK2* expression was observed in BOEC cultured with CM of 8-cell embryos. This could be the result of specific factors secreted from 8-cell embryos in the media, which target *ROCK1* and *ROCK2* activity in BOEC. Further studies, such immune-cytological analysis would be relevant to detect those proteins and their distribution in BOEC and whether they are implicated in embryo-maternal communication.

Our result revealed a gene expression alteration of *SOCS3, PRELP* and *SCN9A* in BOEC cultured in direct contact with 8-cell embryos (BOEC EMB+) and their conditioned media (BOEC CM). The no effect observed in the BOEC EMB-, could be due to a capture of the signal from the BOEC surrounding the embryos (BOEC EMB+) avoiding the signal to arrive to the rest of the cells including the BOEC EMB-. The fact that 8-cell embryo decreased the expression of *SOCS3* and *PRELP;* genes implicated in immune defense supports the idea that embryo could be able to avoid the maternal immune response by decreasing inflammation (Maillo *et al.* 2015). This is in accordance with data obtained from the comparison of pregnant

and cyclic ipsilateral oviducts in the mare, showing that embryo affects the expression of immune response-related genes with marked upregulation of interferon-associated genes (Smits *et al.* 2016). This is also consistent with data from pigs showing that most of transcripts differentially expressed in pregnant sows were down-regulated in the uterine horn in response to blastocysts when compared to oocytes, many of which were related to the immune system (Almiñana *et al.* 2012).

Bovine oviduct epithelial cells co-cultured with 8-cell embryos and their CM has their expression of *SCN9A* up-regulated. This is in the same line as the study of Schmaltz-Panneau et al. (2014), where they hypothesized that there is a neurotrophin signaling by the embryo at the time of blastocyst formation (Schmaltz-Panneau *et al.* 2014). Furthermore, *SCN9A* mediates the voltage-dependent sodium ion permeability of excitable membrane. Previous study showed the relevance of sodium ion transport during embryo development and blastocoel formation in mouse (Jones *et al.* 1997). Taken together these finding, *SCN9A* may be implicated in early embryo-maternal communication.

Regarding *IGFBP3* transcript, it increased in BOEC cultured with embryos CM. Schmaltz-Panneau et al. (2014) observed an increase of *IGFBP3* in BOEC monolayer co-cultured during 8 days with 20 early embryos (Schmaltz-Panneau *et al.* 2014). IGFBP3 belongs to IGFBP, a family of six binding proteins that bind insulin-like growth factor 1 and 2 (IGF-1 and –2) and which regulate the bioavailability of the IGFs to bind to the receptors (Rechler and Clemmons 1998). It was demonstrated that bovine oviduct markedly expresses IGFBP-3 in the epithelial lining of the isthmus during estrus. In the same study, it was also suggested the existence of a region-specific expression of IGFBP-3 and that this binding protein may help to regulate the concentration of IGF-1 in the OF. This pattern of distribution might help to create an IGF-1 gradient along the oviduct as the embryo travels through it (Pushpakumara *et al.* 2002).

Regarding energy substrates and amino acids analysis our results indicated that BOEC metabolism was not affected by presence of early embryos (2-cell and 8-cell stage) or by their CM. Furthermore, the results indicated that BOEC metabolism is different to that of embryos. BOEC clearly showed a consumption of glucose, lactate and pyruvate as well as amino acids, excepting of serine and alanine that were produced by cells. Previously, it was proposed that BOEC altered the composition of culture medium in a manner consistent with embryo development. Actually in co-culture media, BOEC decreased glucose concentration and provide lactate and pyruvate, two key metabolites required for successful development of the early embryo (Edwards *et al.* 1997). This was in disagreement with our results, where BOEC

metabolism was not altered by early embryo suggesting that both BOEC and embryos have metabolic requirements that may be independently satisfied by the culture media

Interestingly embryo before EGA seems to be independent as no consumption of energy substrate or amino acids by 2- to 8-cell embryo was evidenced. However, this was not the case for embryos at 8- to 16-cell stage. The fact that 2- to 8-cell embryo did not exhibit consumption of energy substrates or amino acids may be supported by the possibility that embryo may have sufficient endogenous energy sources; most likely fat but possibly protein to support its early development (Sturmey *et al.* 2009; Leese 2015). Data reinforcing this view obtained for cattle was demonstrated that supplementing early bovine embryo culture media with L-carnitine, a cofactor of β-oxidation, improved embryo development in the absence of carbohydrates, this improvement was in part due to increased utilization of endogenous lipid stored (Sutton-McDowall *et al.* 2012). Furthermore, when bovine embryos were cultured in the absence of exogenous energy sources, oxygen consumption by 5- to 8-cell embryos was unchanged indicating that endogenous energy reserves are able to sustain development for considerable periods of time (Ferguson and Leese 2006). Thus, our result suggests that this period of time might coincide with EGA, since that 8-cell to 16-cell stage displayed an active metabolism by consumption of energy substrates and amino acids. During EGA embryo starts to produce its own transcripts, which could be more dependent of exogenous source of energy.

The result of amino acid metabolism revealed a production of serine and alanine by BOEC independently to their co-culture with embryos or not. Alanine is involved in the regulation of intracellular pH and may have a role in protecting the embryo from osmotic stress (Van Winkle 2001) and it has shown to promote bovine pre-implantation development *in vitro* (Moore and Bondioli 1993). We suggest that BOEC could be predisposed to increase alanine concentration in the media for sustain embryo needs.

In conclusion, we confirm that early embryo affects the transcriptome of BOEC *in vitro* but this effect seems to be embryo stage depending. Moreover, embryo effect could result from a direct contact with BOEC and/or from their secretions released into the media. Under our experimental conditions early embryo did not affect BOEC metabolism but interestingly embryo metabolism before EGA seems to be independent of exogenous source of energy.

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Chapter 3

Bovine embryo-oviduct interaction *in vitro* reveals an early cross talk mediated by BMP signaling

Elina V García, Meriem Hamdi, Antonio D Barrera, María J Sánchez-Calabuig, Alfonso Gutiérrez-Adán and Dimitrios Rizos. Reproduction (2017) 153 631–643

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Bovine embryo-oviduct interaction *in vitro* reveals an early cross talk mediated by BMP signaling

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Abstract

Signaling components of bone morphogenetic proteins (BMPs) are expressed in an anatomically and temporally regulated fashion in bovine oviduct. However, a local response of this signaling to the presence of the embryo has yet to be elucidated. The aim of the present study was to evaluate if early embryo-oviduct interaction induces changes in the gene expression of BMP signaling components. For this purpose, we used an *in vitro* co-culture system to investigate the local interaction between bovine oviductal epithelial cells (BOEC) from the isthmus region with early embryos during two developmental periods: before (from the 2-cell to 8-cell stage) or during (from the 8-cell to 16-cell stage) the main phase of embryonic genome activation (EGA). Exposure to embryos, irrespective of the period, significantly reduced the relative abundance of *BMPR1B*, *BMPR2*, *SMAD1*, *SMAD6* and *ID2* mRNAs in BOEC. In contrast, embryos that interacted with BOEC before EGA showed a significant increase in the relative abundance of *SMAD1* mRNA at the 8-cell stage compared to embryos cultured without BOEC. Moreover, embryos at the 16-cell stage that interacted with BOEC during EGA showed a significant increase in *BMPR1B*, *BMPR2* and *ID2* mRNA. These results demonstrate that embryo-oviduct interaction *in vitro* induces specific changes in the transcriptional levels of BMP signaling, causing a bidirectional response that reduces the expression levels of this signaling in the oviductal cells while increases them in the early embryo. This suggests that BMP signaling pathway could be involved in an early cross talk between the bovine embryo and the oviduct during the first stages of development.

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Introduction

Maternal-embryo communication during the preimplantation period plays a critical role for the establishment and maintenance of pregnancy. Any disturbance in this reciprocal cross talk can lead to pregnancy failure and can also have long-term consequences in the developmental potential and health of the offspring (Wolf *et al.* 2003, Fazeli 2008, Rizos *et al.* 2016).

In mammals, the first maternal site that makes contact with the embryo is the oviduct. In the particular case of cattle, this contact is established during the first four days after fertilization (Maillo *et al.* 2016). Within this window of time, important developmental events occur in the embryo, including the first cleavage divisions (Lonergan *et al.* 1999) and the epigenetic reprogramming and activation of the embryonic genome (Memili & First 2000, Graf *et al.* 2014). To provide an optimal environment and to ensure the correct synchronization of these events, the oviduct needs to initiate a molecular dialog with the developing embryo. Studies in mice, pigs and horses have provided evidence for this initial cross talk, demonstrating that the presence of the embryo(s) produces transcriptional changes in the oviductal cells (Lee *et al.* 2002, Almiñana *et al.* 2012, Smits *et al.* 2016). In cattle, although the presence of a single embryo *in vivo* has no detectable effect on the oviductal transcriptome, the transfer of 50 embryos into the oviduct of heifers produces changes in the gene expression pattern of the oviductal cells, suggesting that the effect of the embryo, particularly in mono-ovulatory species, seems to be more local than was expected (Maillo *et al.* 2015).

It is known that epithelial cells lining the lumen of the mammalian oviduct can synthesize and secrete a wide range of proteins, including diverse growth factors and cytokines that can mediate a local interaction with the

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embryo through different signaling pathways (Buhi *et al.* 2000, Lee & Yeung 2006, Aviles *et al.* 2010). Among the candidate signaling pathways that could actively participate in this local embryo-maternal cross talk in the oviduct, we focused our attention on the signaling mediated by bone morphogenetic proteins (BMPs). BMPs are a subfamily of growth factors that belongs to the TGF-β superfamily (Bragdon *et al.* 2011). These factors exert their effects as ligand dimers by binding to tetrameric complexes of type I (BMPR1A, BMPR1B or ACVR1A, also known as ALK3, ALK6 and ALK2 respectively) and type II (BMPR2, ACVR2A or ACVR2B) serine/threonine kinase receptors leading to signal transduction through SMAD-dependent pathways which regulates transcriptional responses (Beyer *et al.* 2013). Several BMPs are expressed in the maternal tract (ovary, uterus and placenta) and play important roles as autocrine and paracrine regulators of ovarian follicular development, blastocyst implantation in the uterus, and morphogenesis and organogenesis during embryo development (Shimasaki *et al.* 2004, Kishigami & Mishina 2005, Jones *et al.* 2006, Knight & Glister 2006). In addition, different components of the BMP signaling pathway are expressed by the embryo in a developmentally regulated fashion during the preimplantation development (Roelen *et al.* 1997, Zhang *et al.* 2007, Lee *et al.* 2014). Particularly in mice, knockout experiments and inhibition assays demonstrated that during the preimplantation period BMP signaling is critical for cell cleavage and the normal development of extra-embryonic lineages (Graham *et al.* 2014, de Mochel *et al.* 2015).

Previous studies have revealed that different members of the BMP family are expressed in the epithelial cells of the bovine oviduct in an anatomically and temporally regulated fashion during the estrous cycle (Garcia *et al.* 2014). In particular, BMP5 exhibits differential expression in the isthmus region and high transcriptional levels during the periovulatory phase. The presence of this factor in the oviductal fluid suggests that BMP ligands produced by the oviduct could have a paracrine action on the bovine embryo (Garcia *et al.* 2014). In fact, the addition of BMP5 to the culture medium during the first two days after fertilization produces an increase in the blastocyst rate and the relative mRNA abundance of pluripotency-related genes (Garcia *et al.* 2015). Interestingly, mRNA expression of BMP receptors is greater in early stage embryos (from 2-cell to 8-cell stage), suggesting that the embryo could be target of BMPs during its transit through the oviduct (Garcia *et al.* 2015). Moreover, in a recent study by RNA sequencing, Maillo and coworkers (Maillo *et al.* 2015) observed that the presence of multiple embryos induces changes in the expression levels of several genes in the bovine oviduct *in vivo*, included among them are various genes associated with the BMP signaling pathway. However, until now the participation of this signaling pathway in an initial cross talk between the embryo and the oviduct during the preimplantaion period has not been explored in depth.

Considering this evidence, BMP signaling could be a candidate pathway for embryo-maternal communication during the preimplantation period. Within this context, the objective of this study was to evaluate whether early embryo-oviduct interaction induces changes in the gene expression levels of BMP signaling components both in the oviductal cells and in the preimplantation embryos. For this purpose, an *in vitro* co-culture system allowing a local and temporal interaction between early bovine embryos and isthmus epithelial cells was established.

Materials and methods

Chemicals

All reagents were purchased from Sigma Chemical Química S.A Company unless otherwise stated.

Bovine oviduct epithelial cell isolation and in vitro culture

Bovine oviducts at the early luteal phase (corresponding to Days 3–5 of the estrous cycle) were obtained from slaughtered heifers and selected based on ovarian morphology according to Ireland and coworkers (Ireland *et al.* 1980). The oviducts were sealed in a plastic bag and transported to the laboratory on ice within 2 h of slaughter. The surrounding fat and connective tissues were carefully removed and each oviduct was washed three times in sterile PBS (pH 7.4). The isthmus region was separated and the oviductal mucosa was collected by squeezing and was washed 2 times with PBS by centrifugation at 300 ***g*** for 10 min. The cell pellet was then resuspended in 1 mL of synthetic oviductal fluid (SOF) supplemented with 10% (v/v) of fetal calf serum (FCS) and was passed 10 times through a 25 G syringe needle to obtain a single cell suspension. Bovine oviduct epithelial cells (BOEC) were counted in a hemocytometer, diluted to a final concentration of 2×10^6 cells/mL and then cultured in 500 µL of SOF + 10% FCS in four-well plates at 38.5°C, 5% CO_2, 5% O_2, 90% N_2 and saturated humidity until confluence. Half of the media (SOF + 10% FCS) was replaced every 48 h. On Day 5 of culture, the medium was changed to SOF with 5% FCS; and 24 h later, half of the media was renewed and the cells were used for co-culture with embryos.

Immunocytochemical analysis with antibodies anti-bovine-pancadherin (C1821), anti-bovine-pancytokeratin (C2931) and anti-bovine-vimentin (V2258), following the protocol described by Lopera-Vásquez and coworkers (Lopera-Vásquez *et al.* 2016), confirmed the epithelial nature of the cultured cells showing positive staining for the epithelial markers cadherin and cytokeratin, and negative staining for the fibroblast marker vimentin (Fig. 1A).

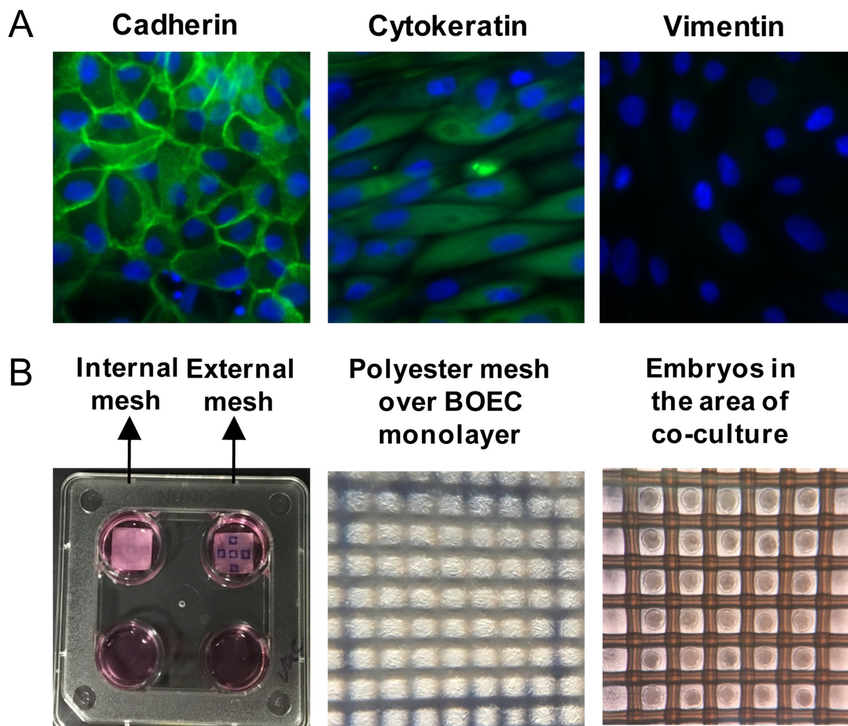

Figure 1 *In vitro* co-culture system. (A) Immunofluorescence analysis of BOEC monolayers showing positive staining for the epithelial markers, cadherin and cytokeratin, and negative staining for the fibroblast marker, vimentin. Cell nuclei were counterstained with Hoechst stain (blue). (B) Representative image of the polyester meshes employed to establish a local area of contact between the embryos and the BOEC monolayer. Thirty embryos/well were transferred to the mesh and placed in a 6 × 5 openings grid covering an area of 2.1 mm^2 over the monolayer. Mesh dimensions: 170 µm opening and 96 µm filament diameter.

Oocyte collection and in vitro maturation

Immature cumulus–oocyte complexes (COCs) were recovered by aspirating follicles (2–8 mm in diameter) from ovaries of heifers slaughtered at local abattoir and *in vitro* maturation was performed as described by Lopera-Vásquez and coworkers (Lopera-Vásquez *et al*. 2015). Only oocytes with a compact, non-atretic cumulus of at least three layers and a homogeneous ooplasm (Class 1 and 2 COCs) were selected and matured for 24 h in 500 µL of maturation medium (TCM 199 (M4530) supplemented with 10% (v/v) FCS and 10 ng/mL epidermal growth factor (E4127)) in four-well dishes (50 COCs per well) at 38.5°C under an atmosphere of 5% CO_2, with maximum humidity.

In vitro fertilization

Frozen semen from a single Asturian Valley bull (ASEAVA, Asturias, Spain), previously tested for IVF, was thawed at 37°C in a water bath for 1 min and selected on a gradient of Bovipure (Nidacon Laboratories AB, Göthenborg, Sweden) following the protocol described by Lopera-Vázquez and coworkers (Lopera-Vázquez *et al*. 2016). Sperm concentration was determined and adjusted to a final concentration of 1×10^6 sperm cells/mL. Gametes were coincubated for 18–22 h in 500 µL of fertilization medium (Tyrode's medium with 25 mM bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate and 6 mg/mL fatty acid-free bovine serum albumin (BSA) supplemented with 10 µg/mL heparin sodium salt (Calbiochem)) in a four-well dish, in groups of 50 COCs per well under an atmosphere of 5% CO_2, with maximum humidity at 38.5°C.

In vitro embryo culture and co-culture conditions

After the fertilization period, presumptive zygotes were completely denuded of cumulus cells by vortexing and groups of 50 were initially cultured in 500 µL of SOF supplemented with 4.2 mM sodium lactate (L4263), 0.73 mM sodium pyruvate (P4562), 30 µL/mL basal medium eagle (BME) amino acids (B6766), 10 µL/mL minimum essential medium (MEM) amino acids (M7145), 1 µg/mL phenol red (P0290) and 3 mg/mL bovine serum albumin (BSA; A9647) in a four-well dish, under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C. At 33 and 54 h post-insemination (hpi); those embryos that reach the 2-cell stage and the 8-cell stage respectively were selected and randomly cultured in groups of 30 embryos in SOF supplemented with 3 mg/mL of BSA or in SOF supplemented with 5% (v/v) of FCS in the presence or absence of a BOEC monolayer, depending on the experimental group to which they were assigned (see 'Experimental design' described below for more details). In order to limit and establish a local area of contact between the embryos and the BOEC monolayer, a nontoxic woven polyester mesh (Sefar Petex; Sefar, Bury, Lancashire, UK) was used with a grid size of 41 × 41 openings, covering an area of 121 mm^2 that fitted perfectly inside the well of a NuncTM 4-well dish. First of all, one mesh square containing delimited marked areas of 6 × 5 openings was placed outside at the bottom of the wells and was used as a guide to localize the co-culture area (Fig. 1B). Secondly, another mesh square of the same size was washed once with 70% ethanol, thrice with PBS and twice with SOF media, and then was introduced into the well so that the external and internal mesh were overlapped. Then, 30 embryos/well were

transferred to the mesh and placed in a 6 × 5 grid, covering an area of 2.1 mm^2 over the monolayer (Fig. 1B). Preferably, the central grid was selected. Following culture, embryos were transferred and cultured in 25 µL droplets of SOF with BSA until Day 9 pi.

RNA isolation and reverse transcription

Five independent samples of BOEC cultured with and without embryo under each experimental condition, and also three independent groups of 10 embryos per stage (8-cell, 16-cell and blastocyst stage) and per experimental group were collected and processed for poly(A) RNA extraction. Poly(A) RNA was extracted using the Dynabeads mRNA Direct Extraction Kit (Dynal Biotech, Oslo, Norway) following the manufacturer's instructions and with minor modifications as it was described previously by Bermejo-Alvarez and coworkers (Bermejo-Alvarez *et al.* 2008). After 10 min of incubation in lysis buffer with Dynabeads, poly(A) RNA attached to the Dynabeads was extracted with a magnet and washed twice in washing buffer A and washing buffer B. RNA was then eluted with Tris–HCl. Immediately after extraction, the reverse transcription (RT) reaction was performed as recommended by the manufacturer (Epicentre Technologies Corp, Madison, WI, USA). Briefly, oligo-dT (0.2 µM) and random primers (0.5 µM) were added to each RNA sample and were heated for 5 min at 70°C to denature the secondary RNA structure. Next, the tubes were incubated at 25°C for 10 min to promote the annealing of the primers. Then, the RNA was reverse-transcribed for 60 min at 37°C in a final volume of 50 µL containing 0.375 mM dNTPs (Biotools, Madrid, Spain), 6.25 U RNasin RNAse inhibitor (Promega), 10× MMLV-RT buffer with 8 mM dithiothreitol and 5 U MMLV high performance reverse transcriptase (Epicentre Technologies Corp, Madison, WI, USA), followed by incubation at 85°C for 5 min to inactivate the RT enzyme.

Gene expression analysis

The mRNA expression levels of the selected genes were determined by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) using specific primers designed with Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to span exon-exon boundaries when possible. Primers (Table 1) were previously validated for adequate primer efficiency; and specificity of their PCR products was confirmed by electrophoresis on a 2% agarose gel. All target genes showed efficiencies between 97 and 100% and correlation coefficients close to 1.0.

All PCR reactions were performed in a final volume of 20 µL, containing 0.25 mM of forward and reverse primers, 10 µL of GoTaq qPCR Master Mix (Promega) and 2 µL of each cDNA sample derived from BOEC (≈60 ng/µL), 8-cell embryos (≈4 ng/µL), 16-cell embryos (≈20 ng/µL) or blastocysts (≈60 ng/µL), using a Rotorgene 6000 Real Time Cycler (Corbett Research, Sydney, Australia) and SYBR Green as double-stranded DNA-specific fluorescent dye. For each experimental group, five different cDNA samples from BOEC and three different cDNA samples from each embryonic stage were used in two repetitions for all genes of interest. The PCR program consisted of an initial denaturalization step at 94°C for 2 min, followed by 35 cycles of denaturalization at 94°C for 10 s, annealing at 56°C for 30 s, extension at 72°C for 15 s and 10 s of fluorescence acquisition defined for each primer. At the end of each PCR run, melt curve analyses were performed for all

Table 1 Set of primers used for qRT-PCR assays.

Gene		Primer sequences (5′–3′)[a]	Amplicon length (bp)	GenBank accession number
BMP5	Forward	GATGTGGGTTGGCTTGTCTT	271	NM_001305016.1
	Reverse	CCTGATGAGAGCCGGATTTA		
BMP7	Forward	AACCATGCCATCGTGCAGACGC	250	NM_001206015.1
	Reverse	AAGCCCGGACAACCATGTTTGC		
BMPR1A	Forward	TTGGGAAATGGCTCGTCGTT	142	NM_001076800.1
	Reverse	AGACACAATTGGCCGCAAAC		
BMPR1B	Forward	TTTGGGAGGTCGCTAGGAGA	132	NM_001105328.1
	Reverse	GCCGCAGCTTCTTGATACAC		
BMPR2	Forward	TCTTTCAGCCACCAATGTCCT	158	NM_001304285.1
	Reverse	GTTCAGTGGAAATGACCCAGG		
SMAD1	Forward	TGTGAACCACGGCTTCGAGACG	99	NM_001076223.2
	Reverse	TCCTGGCGGTGGTATTCTGCTC		
SMAD5	Forward	TTGCTCAGCTTCTGGCTCAGTC	119	NM_001077107.3
	Reverse	TTGCCGGTGATACTCTGCTCC		
SMAD6	Forward	GGAGAAATTCGCTCCAAGTGC	242	NM_001206145.1
	Reverse	CCCTGCCTTTAAAACCCAAGC		
ID2	Forward	CGACATCAGCATCCTGTCCTT	145	NM_001034231.2
	Reverse	AGAGCCTGTGGATTTGTTGT		
H2AFZ	Forward	AGGACGACTAGCCATGGACGTGTG	209	NM_174809.2
	Reverse	CCACCACCAGCAATTGTAGCCTTG		
ACTB	Forward	GAGAAGCTCTGCTACGTCG	263	NM_173979.3
	Reverse	CCAGACAGCACCGTGTTGG		

[a]All the primers were designed with NCBI Primer-BLAST online tool of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

genes to ensure single product amplification and exclude the possible interference of dimers.

Relative expression levels were quantified by the comparative cycle threshold (ΔΔCT) method (Schmittgen & Livak 2008). Values were normalized using two housekeeping genes (*H2AFZ* and *ACTB*) that were tested in previous studies (Maillo *et al.* 2015). Fluorescence was acquired in each cycle to determine the threshold cycle during the log-linear phase of the reaction at which fluorescence increased above background for each sample. According to the comparative CT method, the ΔCT value was determined by subtracting the mean CT value of the two housekeeping genes (*H2AFZ* and *ACTB*) for each sample from each gene CT value of the sample. Calculation of ΔΔCT involved using the highest sample ΔCT value (i.e. the sample with the lowest target expression) as an arbitrary constant to subtract from all other ΔCT sample values. Fold changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta CT}$ (Livak & Schmittgen 2001). All the study were carried out following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments recommendations (Bustin *et al.* 2009).

Experimental design

Experiment 1: Effect of BOEC co-culture on early embryo development in vitro

In the first experiment, an *in vitro* co-culture system was established to provide a local and temporal interaction between a BOEC monolayer and early bovine embryos during two developmental periods: (a) Experimental Group 1 (G1): from 2-cell to 8-cell stage, before the main phase of embryonic genome activation (EGA); and (b) Experimental Group 2 (G2): from 8-cell to 16-cell stage, during the main phase of EGA (Fig. 2). To determine if this *in vitro* co-culture system had an effect on early embryonic development, cleavage rate and blastocyst yield were evaluated.

For this purpose, isthmus epithelial cells obtained from a pool of three post-ovulatory stage oviducts (*n* = 21, 3 oviducts per experimental replicate) were cultured in a monolayer as described previously. On Day 6 of culture, the BOEC monolayer was cultured in the absence or presence of *in vitro*-produced embryos from 2- to 8-cell stage (G1 BOEC; 33–54 hpi) or from 8- to 16-cell stage (G2 BOEC; 54–98 hpi) in SOF medium supplemented with 5% FCS, under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C. As mentioned above, 30 embryos/well were placed in a 6 × 5 grid over the monolayer. In addition, as control without BOEC, groups of 30 embryos in both developmental stages were cultured either in SOF + 5% FCS (G1 FCS and G2 FCS) or in SOF + 3 mg/mL BSA (G1 BSA and G2 BSA). At 54 hpi (G1 BOEC/FCS/BSA) or 98 hpi (G2 BOEC/FCS/BSA), embryos that reached the 8- or 16-cell stage respectively were transferred to SOF + BSA and cultured until Day 9, maintaining the different experimental groups separately (Fig. 2).

Considering that during the experiment it was necessary to preselect the embryos at different stages of development, the developmental parameters were calculated as follows: (I) developmental rate at 33 hpi: percentage of presumptive zygotes that developed to the 2-cell stage at 33 hpi; (II) developmental rate at 54 hpi: percentage of selected 2-cell embryos that developed to the 8-cell stage at 54 hpi; (III) developmental rate at 98 hpi: percentage of selected 8-cell embryos that developed to the 16-cell stage at 98 hpi; and

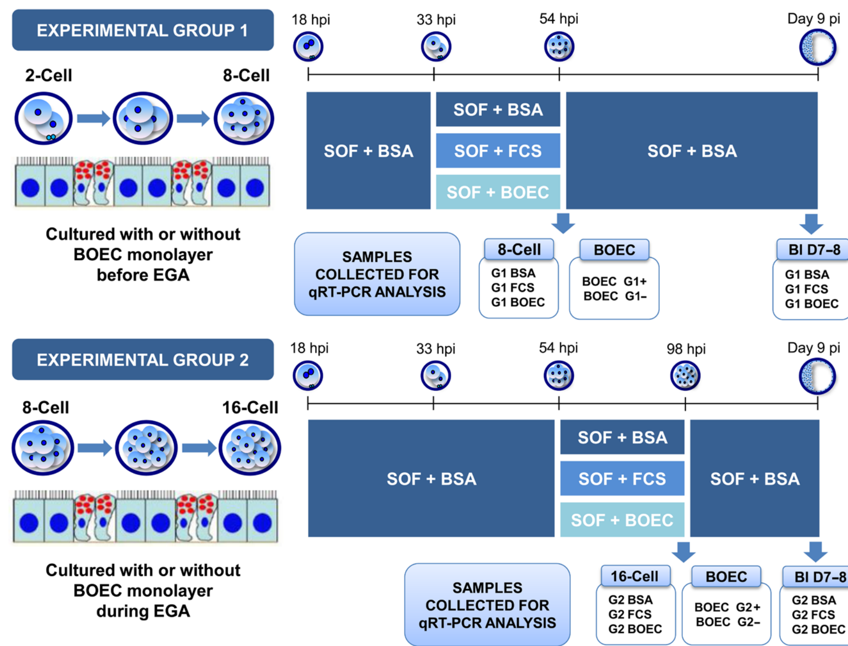

Figure 2 Experimental design. Experimental group 1: Two-cell IVF bovine embryos were selected at 33 hpi and cultured before the main phase of EGA (from 2-cell to 8-cell stage: 33–54 hpi) in medium SOF + BSA (G1 BSA), SOF + FCS (G1 FCS) or co-cultured with BOEC monolayer (G1 BOEC). At 54 hpi, embryos that reached the 8-cell stage were transferred to SOF + BSA and cultured until Day 9. Experimental group 2: Eight-cell IVF bovine embryos were selected at 54 hpi and cultured during the main phase of EGA (from 8-cell to 16-cell stage: 54–98 hpi) in medium SOF + BSA (G2 BSA), SOF + FCS (G2 FCS) or co-cultured with BOEC monolayer (G2 BOEC). At 98 hpi, embryos that reached the 16-cell stage were transferred to SOF + BSA and cultured until Day 9. Samples of BOEC cultured with (BOEC G1+/G2+) or without embryos (BOEC G1−/G2−) before or during EGA, and embryos at 8-cell (54 hpi), 16-cell (98 hpi) and blastocyst stage (Day 7–8) were collected from the different experimental groups to evaluate the mRNA expression levels of BMP signaling components by qRT-PCR. EGA, embryonic genome activation; hpi, hours post insemination.

(IV) blastocyst yield: percentage of selected 8-cell embryos or 16-cell embryos that continued in culture and developed to the blastocyst stage at Days 7, 8 and 9 after insemination.

Seven experimental replicates were performed under the same assay conditions and a total of 2550 presumptive zygotes were *in vitro* cultured: 1192 were used for experimental groups 1 (G1 BOEC/FCS/BSA) and 1358 for groups 2 (G2 BOEC/FCS/BSA).

Experiment 2: Effect of early embryo-oviduct interaction in vitro on mRNA expression of BMP signaling components in oviductal epithelial cells

To evaluate whether *in vitro* interaction with early embryos induces changes in the gene expression levels of BMP signaling components in the oviductal cells, an expression analysis was performed by qRT-PCR in 5 samples of BOEC cultured with or without embryos before (G1 BOEC) or during EGA (G2 BOEC), obtained from 5 different experimental replicates of Experiment 1.

After the co-culture period, the polyester mesh and the embryos were removed and the BOEC monolayer was quickly washed twice with PBS. The cell area directly beneath the embryos was recovered from G1 BOEC at 54 hpi (BOEC G1+, $n=5$) and from G2 BOEC at 98 hpi (BOEC G2+, $n=5$) by mechanical scraping with a micropipette tip. At the same time, an area of the same size but from a different well of BOEC, cultured without embryos, was collected as control (BOEC G1− and BOEC G2−; $n=5$). The BOEC samples collected were immediately snap-frozen in liquid nitrogen and kept at −80°C until RNA extraction.

The mRNA expression levels of two BMP ligands (*BMP5/7*), three BMP receptors (*BMPR1A/1B/2*), two signaling proteins (*SMAD1/5*), one inhibitor (*SMAD6*) and one target gene (*ID2*) were analyzed by qRT-PCR according to the procedures described above.

Experiment 3: Effect of early embryo-oviduct interaction in vitro on mRNA expression of BMP signaling components in preimplantation embryos

The objective of the third experiment was to evaluate if the *in vitro* interaction with the oviductal cells induces changes in the expression levels of BMP signaling components in the embryos at specific developmental stages. For this purpose, gene expression analysis by qRT-PCR was performed in three independent groups of 10 embryos per stage (8-cell, 16-cell and blastocyst stage) obtained from each experimental group that was cultured with or without BOEC, before or during EGA (corresponding to groups 1 (G1 BOEC/BSA/FCS) and groups 2 (G2 BOEC/BSA/FCS) respectively) in Experiment 1.

After the co-culture period, at 54 hpi (G1 BOEC/FCS/BSA) or 98 hpi (G2 BOEC/FCS/BSA), embryos that reached the 8- or 16-cell stage respectively were removed, quickly washed in PBS; and 10 embryos per stage and experimental group were immediately snap-frozen in liquid nitrogen and kept at −80°C until RNA extraction. The remaining embryos were transferred to 25 µL droplets of SOF + BSA under mineral oil and cultured until Day 9 pi. At Day 7–8 pi, pools of 10 expanding blastocysts per treatment were collected, snap-frozen and stored at −80°C until use for gene expression analysis.

The mRNA expression levels of two BMP ligands (*BMP5/7*), three BMP receptors (*BMPR1A/1B/2*), two signaling proteins (*SMAD1/5*), one inhibitor (*SMAD6*) and one target gene (*ID2*) were analyzed by qRT-PCR according to the procedures described above.

Statistical analysis

Data were analyzed using SigmaStat 3.5 and SigmaPlot 10.0 statistical software (Systat Software, Richmond, CA, USA). Cleavage rate, blastocyst yield and relative mRNA abundance levels were analyzed using one-way analysis of variance (ANOVA), followed by Holm–Sidak *post hoc* test, when applicable, to determine statistical differences between the experimental groups. Probability values less than 0.05 ($P \leq 0.05$) were considered statistically significant.

Results

Experiment 1: Effect of BOEC co-culture on early embryo development in vitro

For all the experimental groups, only embryos that reached the 2-cell stage at 33 hpi were selected for the study. As shown in Fig. 3A cleavage rates at 33 hpi were similar for G1 and G2 (G1: 66.8 ± 2.0% vs G2: 67.6 ± 1.2%; $P > 0.05$). In general, most of the embryos reached the 2-cell stage at 33 hpi (G1: 53.9 ± 3.8% and G2: 58.8 ± 3.5%) and a low percentage of embryos were at a more advanced stage (G1: 12.8 ± 3.5% and G2: 8.8 ± 2.7%).

At 54 hpi in the G1 BOEC group, a lower proportion of embryos reached the 8-cell stage than control groups (G1 BOEC: 57.1 ± 4.9% vs G1 BSA: 78.6 ± 4.0% and G1 FCS: 79.0 ± 7.4% $P < 0.05$) (Fig. 3B). As a consequence, a significantly higher proportion of embryos with a delayed development (<8 cells) was observed in G1 BOEC than control groups (G1 BOEC: 42.9 ± 4.9% vs G1 BSA: 21.4 ± 4.0% and G1 FCS: 21.0 ± 7.4%, $P < 0.05$) (Fig. 3B).

Similarly, at 98 hpi, the G2 BOEC group showed a lower but not significant proportion of embryos reaching the 16-cell stage than control groups (G2 BOEC: 52.4 ± 8.4%, G2 BSA: 66.7 ± 5.9% and G2 FCS: 65.2 ± 6.8%) (Fig. 3C). In this case, a significantly higher proportion of embryos with a delayed development (<16 cells) was observed in the G2 BOEC group in comparison with the control groups (G2 BOEC: 47.6 ± 8.4% vs G2 BSA: 33.3 ± 5.9% and G2 FCS: 34.8 ± 6.8%, $P < 0.05$) (Fig. 3C).

Despite this initial delay observed in the developmental kinetics of the embryos, no significant differences were observed in the blastocyst rate between the control groups (G1 BSA/FCS and G2 BSA/FCS) and the groups that were co-cultured with BOEC before or during EGA (G1 BOEC or G2 BOEC) (Fig. 3D and E).

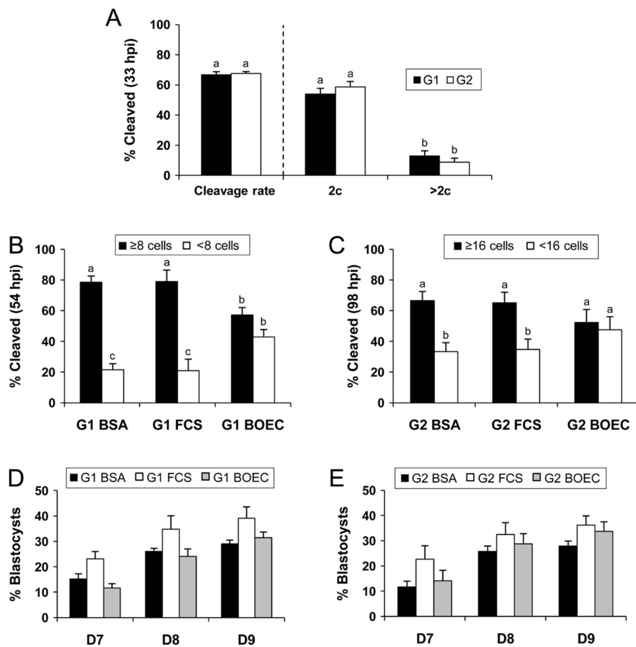

Figure 3 Developmental rates of *in vitro*-produced bovine embryos cultured before or during embryonic genome activation with or without BOEC. (A) Cleavage rate at 33 h post-insemination (hpi) and percentages of IVF embryos that reached the 2-cell stage (2c) or more (>2c), from G1 and G2 experimental groups. (B) Developmental rate at 54 hpi for experimental groups 1 (G1 BSA/FCS/BOEC). (C) Developmental rate at 98 hpi for experimental groups 2 (G2 BSA/FCS/BOEC). (D) Blastocyst rate at Days 7–9 pi (*in vitro* fertilization = Day 0) derived from experimental groups 1 (G1 BSA/FCS/BOEC). (E) Blastocyst rate at Days 7–9 pi derived from experimental groups 2 (G2 BSA/FCS/BOEC). Results are expressed as mean ± s.e.m. Significant differences ($P < 0.05$) are indicated with different letters. G1: embryos cultured from 2-cell stage to 8-cell stage in medium SOF + BSA (G1 BSA), SOF + FCS (G1 FCS) or co-cultured with a BOEC monolayer (G1 BOEC). G2: embryos cultured from 8-cell stage to 16-cell stage in medium SOF + BSA (G2 BSA), SOF + FCS (G2 FCS) or co-cultured with a BOEC monolayer (G2 BOEC).

Experiment 2: Effect of early embryo-oviduct interaction in vitro on mRNA expression of BMP signaling components in oviductal epithelial cells

Gene expression analysis in BOEC showed that the interaction with the embryos, before and during the main phase of EGA, produced a decrease in the mRNA expression levels of *BMP7*, *BMPR1B*, *BMPR2*, *SMAD1*, *SMAD6* and *ID2* compared with the significant higher levels detected in those transcripts when BOEC were cultured without embryos (Fig. 4). The embryonic presence produced a similar transcriptional response in the oviductal cells, irrespective of the embryonic stage.

On the other hand, expression levels of *BMPR1A* and *SMAD5* mRNA tended to be similar to those levels observed for *BMP7* mRNA. However, the relative abundance of *BMPR1A* and *SMAD5* transcripts did not show significant differences between BOEC cultured with or without embryos (Fig. 4). In the particular case of *BMP5*, the expression of its mRNA was not detected in the oviductal cells cultured *in vitro*. In this case, to confirm that *BMP5* primers work efficiently, samples of bovine oviduct epithelial cells freshly obtained from the isthmus region (BOEC *in vivo*) were used as positive control (data not shown).

Experiment 3: Effect of early embryo-oviduct interaction in vitro on mRNA expression of BMP signaling components in preimplantation embryos

Gene expression analysis in early stage embryos revealed a different transcriptional response to the interaction with BOEC, depending on the embryonic stage in which they interacted. At the 8-cell stage, embryos from G1 BOEC group showed a significant increase in the relative abundance of *SMAD1* mRNA, compared to the levels detected in embryos derived from control groups (G1 BSA and G1 FCS) (Fig. 5A). Moreover, at the 16-cell stage, embryos from G2 BOEC group showed a significant increase in the relative abundance of *BMPR1B*, *BMPR2* and *ID2* mRNA compared to controls (G2 BSA and G2 FCS) (Fig. 5B). Despite these transcriptional changes detected in early stage embryos, in general, no differences were observed in the mRNA expression levels of BMP signaling components between the blastocysts obtained from the different experimental groups cultured in the presence or absence of BOEC (Fig. 6).

Discussion

The *in vitro* culture of oviductal cells has provided a suitable model for the study of the mechanisms involved in the interaction of gametes and embryos with the mammalian oviduct. Recent advances in *in vitro* modeling of the oviductal epithelium of different species, including bovine, porcine and human, have enabled the development of new culture techniques that better mimic the morphological and physiological characteristics of the oviduct (Gualtieri *et al.* 2012, Chen *et al.* 2013, Lawrenson *et al.* 2013, Simintiras *et al.* 2017). However, considering the increasing complexity of studies aimed at understanding the regulatory mechanisms that control embryo-maternal communication at a molecular level, it is necessary to establish novel strategies for *in vitro* culture that allow a more controlled interaction, in terms of area and contact time, between the embryo and the oviductal cells.

The main objective of the present study was to evaluate if the interaction of the embryo with the oviduct can induce changes in the gene expression of BMP signaling components. Considering the limitation of carrying out this study *in vivo*, an *in vitro* co-culture system was established to study the interaction between isthmus epithelial cells and *in vitro*-produced bovine embryos at developmental stages during which, under

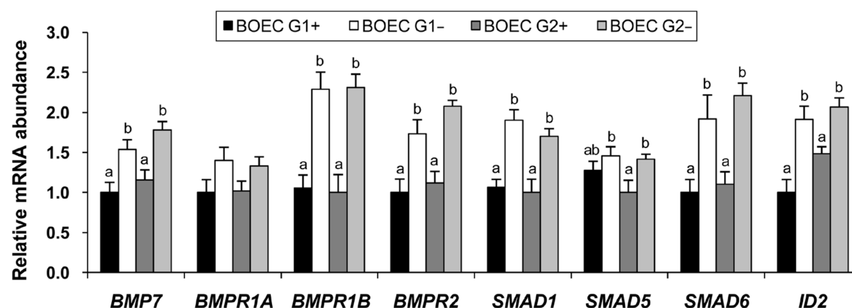

Figure 4 Relative mRNA abundance of BMP signaling-related genes in BOEC cultured in the presence or absence of early embryos, before or during embryonic genome activation. BOEC G1+: bovine oviductal epithelial cells cultured with embryos from 2-cell to 8-cell stage (33–54 hpi). BOEC G1−: bovine oviductal epithelial cells cultured in the absence of embryos from 33 hpi to 54 hpi. BOEC G2+: bovine oviductal epithelial cells cultured with embryos from 8-cell to 16-cell stages (54–98 hpi). BOEC G2−: bovine oviductal epithelial cells cultured in the absence of embryos from 54 hpi to 98 hpi. Bars represent the relative abundance of the transcripts analyzed and normalized to *H2AFZ* and *ACTB* as housekeeping genes. The experimental groups are represented by columns. Results are expressed as means ± s.e.m. Different superscripts letters indicate significant differences ($P < 0.05$) between treatments. Data were obtained from five replicates of independent BOEC samples per experimental group.

physiological conditions, the embryo makes contact with the oviduct. To provide a local interaction, the area of contact between the embryo and the monolayer of oviductal cells was controlled and delimited with a polyester mesh. Moreover, a temporal interaction was established by analyzing two periods of embryonic development: before (from the 2-cell to 8-cell stage) and during (from the 8-cell to 16-cell stage) the main phase of embryonic genome activation (EGA). These two time windows were established taking into account that bovine embryonic genome is gradually activated, starting with minor activation, presumably initiated at the 2-cell stage and followed by a major activation at the 8- to 16-cell stage (Gad *et al.* 2012, Graf *et al.* 2014).

Embryos derived from the different experimental groups that were co-cultured with BOEC showed an early delay in developmental kinetics compared to embryos cultured without oviductal cells. However, this initial delay had no impact on the final production rate of blastocysts. A negative effect of the polyester mesh

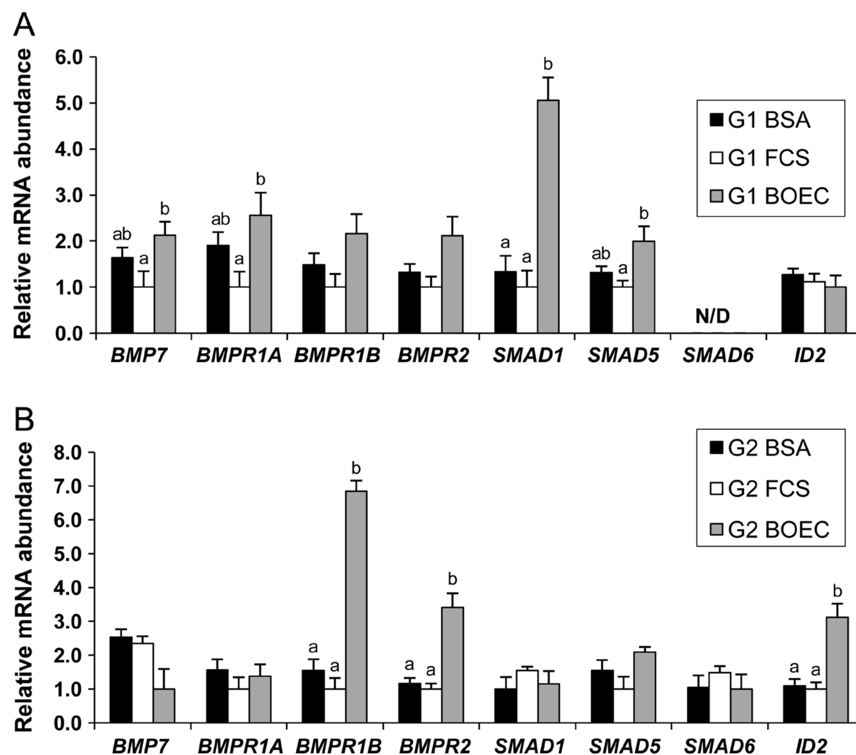

Figure 5 Relative mRNA abundance of BMP signaling-related genes in *in vitro*-produced bovine embryos cultured in the presence or absence of BOEC, before or during embryonic genome activation. (A) Bars represent the relative abundance of BMP signaling transcripts measured in 8-cell stage embryos obtained from experimental groups that were cultured from 2-cell stage to 8-cell stage in medium SOF + BSA (G1 BSA), SOF + FCS (G1 FCS) or co-cultured with a BOEC monolayer (G1 BOEC). (B) Bars represent the relative abundance of BMP signaling transcripts measured in 16-cell stage embryos obtained from experimental groups that were cultured from 8-cell stage to 16-cell stage in medium SOF + BSA (G2 BSA), SOF + FCS (G2 FCS) or co-cultured with a BOEC monolayer (G2 BOEC). The relative abundance of the transcripts was normalized to *H2AFZ* and *ACTB* as housekeeping genes. The control groups (G1/G2 BSA and G1/G2 FCS) are represented by black and white columns, while embryos cultured with oviductal cells are represented by grey columns. Results are expressed as mean ± s.e.m. Different superscripts letters indicate significant differences ($P < 0.05$) between experimental groups. Data were obtained from three replicates of independent groups of 10 embryos per stage and experimental group.

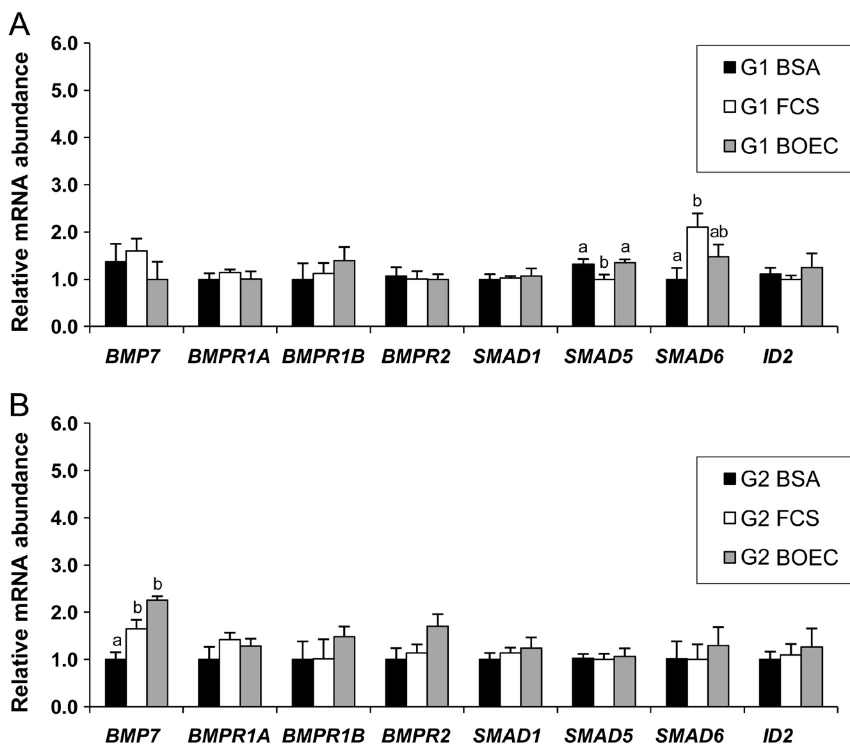

Figure 6 Relative mRNA abundance of BMP signaling-related genes in bovine blastocysts obtained from embryos co-cultured *in vitro* before or during embryonic genome activation in the presence or absence of BOEC. (A) Bars represent the relative abundance of BMP signaling transcripts measured in blastocysts derived from embryos cultured from the 2-cell stage to the 8-cell stage in the absence (G1 BSA and G1 FCS) or presence of a BOEC monolayer (G1 BOEC). (B) Bars represent the relative abundance of BMP signaling transcripts measured in blastocysts derived from embryos cultured from the 8-cell stage to the 16-cell stage in the absence (G2 BSA and G2 FCS) or presence of a BOEC monolayer (G2 BOEC). The relative abundance of the transcripts was normalized to *H2AFZ* and *ACTB* as housekeeping genes. The control groups (G1/G2 BSA and G1/G2 FCS) are represented by black and white columns, while groups cultured with oviductal cells are represented by grey columns. Results are expressed as mean ± s.e.m. Different superscripts letters indicate significant differences ($P < 0.05$) between groups. Data were obtained from three replicates of independent groups of 10 blastocysts per experimental group.

was ruled out as control embryos cultured without BOEC showed normal development in the presence of the mesh. In concordance with this fact, Matoba and coworkers (Matoba *et al*. 2010) showed that the same type of mesh has no effect on embryonic development *in vitro*.

Other studies reported that the *in vitro* co-culture of BOEC with bovine embryos during the first 4 days (Rief *et al*. 2002, Cordova *et al*. 2014) or the entire 8 days of embryo development (Schmaltz-Panneau *et al*. 2014, 2015) can improve the early cleavage rate and/or the development to the blastocyst stage. Undoubtedly, the time of co-culture and the culture conditions are variables that have to be considered before evaluating and comparing these results. In particular, in the present study, the time of co-culture evaluated was shorter than that used in those studies. This allows to infer that an extended co-culture time between the embryo and the oviductal cells seems to be necessary to improve embryo development rate. In fact, other authors have shown that the embryotrophic effect of human oviductal cells cultured with mouse embryos is enhanced when the time of co-culture is longer and it lasts for at least four days (Xu *et al*. 2001).

On the other hand, gene expression analysis showed that the interaction with the embryos, before and during EGA, induced a significant decrease in the transcriptional levels of BMP signaling components in oviductal cells cultured *in vitro*. In particular, this reduction was observed in the mRNA levels of one ligand (*BMP7*), two receptors (*BMPR1B* and *BMPR2*), one signaling mediator (*SMAD1*) and two BMP target genes (*SMAD6* and *ID2*). As can be seen, the effect is observed in all the genes encoding factors required for the signal transduction, suggesting a possible decrease in the activity of BMP pathway in the oviduct when the embryo is present.

It has been reported that BMP7 has a critical role in the oviduct, by acting as an inductor of apoptosis during tissue remodeling (Monroe *et al*. 2000). In fact, estrogen exerts a protective role by reducing the expression of this factor in the oviductal epithelium (Monroe *et al*. 2002, Kusumegi *et al*. 2004). Considering this, it would be interesting to explore if the downregulation of *BMP7* expression, induced by the presence of the embryo, could be associated with a protective effect in order to maintain the integrity of the oviduct as the embryo passes.

In reference to BMP receptors, previous studies demonstrated the expression of *BMPR1B* and *BMPR2* in the bovine embryo, showing high abundance of their transcripts at the early embryonic stages (from 2-cell stage to 8-cell stage) (Garcia *et al*. 2015). This temporal gene expression pattern of the receptors suggests that the embryo could respond to BMP ligands during the first developmental stages during transit through the oviduct. Based on the present study, the mRNA expression of the same receptors was detected in the oviductal cells and the presence of the embryo downregulated their expression. In this sense, the embryo could be sending

specific signals to reduce the expression levels of these receptors in the oviduct to avoid competence. If so, BMP ligands present in the oviductal microenvironment could act preferably on the embryo. However, further studies are required to confirm this possibility.

Downregulation of mRNA expression of *SMAD1* and the target genes *ID2* and *SMAD6* is also indicative of a suppressed BMP pathway as a response of the oviduct to the embryo. This coincides with a previous study performed *in vivo* in which the transfer of 50 embryos to the bovine oviduct produced changes in the gene expression pattern of the oviductal cells; *SMAD6* was among the genes downregulated by the embryo presence (Maillo *et al.* 2015). This gene is induced via SMAD1/5 pathway, and at protein level, it acts as an inhibitor self-regulated by the BMP signaling pathway (Ishida *et al.* 2000, Li 2015). A recent study in mice demonstrated that SMAD signaling is required for structural integrity of the female reproductive tract and uterine function during early pregnancy (Rodriguez *et al.* 2016). However, until now, little is known about its role in the oviduct, in particular during the early stages of embryo development.

Similarly to other studies (Cordova *et al.* 2014, Schmaltz-Panneau *et al.* 2014, Maillo *et al.* 2015) these results confirm that bovine embryos can have a direct effect on the transcriptome of the oviductal cells. However, it is important to note that this study, in contrast to others, demonstrates that a short period of interaction with the early embryo is sufficient to induce specific transcriptional changes in the oviductal cells. Furthermore, a similar transcriptional response was observed irrespective of the embryonic stage, suggesting that oviductal cells do not show a temporal sensitivity to the different stages of embryo development, at least in the conditions assayed and for the genes analyzed.

On the other hand, gene expression analysis of the embryos revealed significant changes in the expression profile of BMP genes depending on whether they interact with oviductal cells before or during EGA. Embryos that interacted with BOEC before the main phase of EGA (G1 BOEC) showed higher levels of transcripts associated with BMP signaling. Particularly, a significant increase in the relative abundance of *SMAD1* mRNA was observed at the 8-cell stage, compared to control groups cultured without BOEC. SMAD1 is a key component of the BMP signaling pathway that allows the transduction of the signal from membrane receptors to the nucleus (Bragdon *et al.* 2011). Depending on cell response, SMAD1 or SMAD5 can be activated (Li 2015). Thus, the significant increase in the mRNA levels for *SMAD1* in the embryos suggests that the interaction with oviductal cells may induce the activation of BMP signaling in the embryos through the SMAD1 pathway. In mouse embryos, *Smad1* coordinates the growth of extra-embryonic structures necessary to support embryo development within the uterine environment. As a consequence, the failure of *Smad1* signal induces defects in extra-embryonic tissues and knockout embryos die due to problems in placentation (Tremblay *et al.* 2001, Arnold *et al.* 2006, Li 2015). Transcriptional analysis of mouse and bovine embryos reveals high abundance of *SMAD1* mRNA at early stages before the main phase of EGA and a significant reduction after EGA up to the blastocyst stage (Wang *et al.* 2004, Lee *et al.* 2014) suggesting that this factor is necessary during the early stages in which the embryo transits through the oviduct. In this sense, our results suggest that early embryo-oviduct interaction could have a critical role in maintaining high transcriptional levels of this factor before EGA, during the early stages in which the embryo interacts with the oviduct.

In the case of the embryos that interacted with BOEC during EGA (G2 BOEC), a significant increase in the relative abundance of *BMPR1B*, *BMPR2* and *ID2* mRNA was detected at the 16-cell stage. This suggests that contact with the oviductal cells might induce the activation of the BMP signaling pathway in the embryo during the early stages of development. However, the precise mechanisms that control this upregulation are still unknown. It is well known that both BMP receptors, BMPR1B and BMPR2, play a critical role in preimplantation development and female fertility (Soyun *et al.* 2001, Nagashima *et al.* 2013, Graham *et al.* 2014, de Mochel *et al.* 2015). In addition, their transcript levels in the embryo are specifically elevated at early stages before EGA (Lee *et al.* 2014, Garcia *et al.* 2015). In the case of *ID2*, it is a target gene of BMP signaling and it acts as a transcription factor required for maintenance and differentiation of trophoblast cells (Janatpour *et al.* 2000, Roberts *et al.* 2004, Schiffmacher & Keefer 2013). In agreement with our results, transcriptome analysis by microarray in *in vivo*-produced bovine embryos reported that *ID2* increases its transcription during the activation of the bovine embryonic genome (Kues *et al.* 2008). The fact that the transcriptional levels of BMP receptors and the *ID2* gene were higher during the early stages and stimulated by the interaction with the oviduct allows us to infer that BMP signaling pathway could be mediating an active cross talk between the embryo and the mother during the early stages of development.

Coincident with other studies (Wang *et al.* 2004, Zhang *et al.* 2007, Kues *et al.* 2008, Lee *et al.* 2014), our results show that during early embryogenesis, the transcript abundance of BMP signaling components is differentially regulated and that early maternal-embryo interaction can have a critical impact on the transcriptional regulation of this signaling. Moreover, the low levels detected at the blastocyst stage and the fact that at this stage there were no differences in the mRNA expression levels of BMP signaling components supports the hypothesis that BMP signaling is more important during the early stages of development. In fact, the inhibition of this signaling at the beginning

of embryo culture produces a significant reduction in the cleavage and developmental rates of mouse (de Mochel *et al.* 2015) and bovine embryos (La Rosa *et al.* 2011), suggesting that a correct balance of BMP signaling, during early stages, is needed for a proper preimplantation development. Thus, considering our results, the interaction with the oviduct could play a critical role to regulate this balance.

In conclusion, this study provides a new strategy for the study of the maternal-embryonic dialog and gives evidence about possible signaling pathways involved in the communication between the embryo and the bovine oviduct during the preimplantation period. The data presented demonstrate that local embryo-oviduct interaction *in vitro* induces changes in the transcriptional levels of BMP signaling, causing a bidirectional response that reduces the expression levels of this signaling in the oviductal cells while increasing them in the embryo at the early stages. These findings suggest that BMP signaling pathway could be involved in an early cross talk between the bovine embryo and the oviduct during first stages of development. Increased understanding of this intrinsic signaling for maternal-embryo communication is important for future improvements in ART and further understanding of the signaling pathways that potentially contribute to infertility in cattle and humans.

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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GENERAL DISCUSSION

The physiological environment in which early embryo development occurs has a critical influence on its subsequent growth in short and long term (Rizos *et al.* 2017). Moreover, this development is related to embryo-maternal communication in which most of the mechanisms involved remain unknown. *In vivo* studies are difficult to perform, especially in cattle, since experiments with live animals are expensive and most importantly it is not possible to differentiate between the outcome of fertilization and early embryonic death (Maillo *et al.* 2016). Thus, *in vitro* studies mimicking the physiological mechanisms and interactions in the maternal reproductive tract are essential to this understanding. Moreover, such studies will help to support the development of assisted reproductive techniques including *in vitro* embryo production that seek to mimic physiological conditions to optimize *in vitro* culture and generate good quality embryos (Maillo *et al.* 2016).

Mimicking bovine preimplantational embryo development by using OF and UF in IVC

Early embryonic development is one of the most critical and complex periods of mammalian development. Despite the successful improvement of IVP in cattle, the development of bovine oocytes to blastocyst following IVM, IVF and IVC is limited to about 30-40 % (Rizos *et al.* 2002b). Several strategies have been used to improve IVC of bovine embryo by mimicking the physiological conditions. Bovine embryo spends the first 4 days in the oviduct, where important morphological and metabolic changes occur, including the first mitotic division and embryonic genome activation (Duranthon *et al.* 2008). Then, it enters the uterus and remains free floating until the initiation of implantation at approximately days 19–22 (Bazer *et al.* 2009). During this journey the growing conceptus is solely dependent on OF and UF nutrients (Hugentobler *et al.* 2007). The result of the chapter 1 clearly showed that alternating OF and UF in low concentration in serum-free culture support embryo development. Previous studies have also reported a positive effect of OF when it was used during the entire period of IVC (Lopera-Vasquez *et al.* 2017b). Furthermore, in other species, such as porcine it was related with increasing of ZP hardening, reducing the incidence of polyspermy without decreasing the penetration rate and improving embryo quality (Cebrian-Serrano *et al.* 2013; Ballester *et al.* 2014; Batista *et al.* 2016). Studies concerning the effects of UF during IVC are limited. However, it has been observed that IVC supplementation with uterine proteins such as HDGF (Gómez *et al.* 2014) and vascular endothelial growth factor (Binder *et al.* 2014) have a positive effect on embryo development, blastocyst cell numbers, embryo implantation rates and limb development. Furthermore, our results also suggest that sequential culture of OF and UF during IVC may mimic more adequately the physiological conditions of the reproductive tract, where the early embryo normally develops to the blastocyst stage, thus, being a good alternative to serum supplementation.

In vitro blastocyst quality improved by OF and UF supplementation at low concentration in IVC

It is well known that culture environment during embryo development has an impact on the quality of the produced embryos in terms of cryotolerance (Rizos *et al.* 2008), ultrastructure morphology (Fair *et al.* 2001); embryo cell number (Trigal *et al.* 2011) and gene expression (Rizos *et al.* 2002a; Lonergan *et al.* 2003a). The use of FCS as supplement to *in vitro* culture was related with reduced quality of blastocysts (Rizos *et al.* 2003), while embryos produced under serum-free conditions show aberrations in genes involved in lipid metabolism, indicating that the serum-free conditions require further optimization (Heras *et al.* 2016). The result of chapter 1 showed that the sequential culture with OF and UF improved blastocyst quality, by increasing both the survival rate after cryotolerance (61–65%) and increasing the expression of epigenetic marker genes (*DNMT3A* and *IGF2R*) indicating a better control of embryo methylation, predominantly due to OF effect. In addition, the decreased expression of oxidative stress genes (*CLIC1* and *GPX1*) was supported by a low accumulation of intracellular ROS level in the produced blastocysts. This reveals a possible antioxidant activity derived primarily from UF. This result confirms the role of physiological fluids in determining embryo quality highlighting the importance of embryo exposure to the maternal environment (Tesfaye *et al.* 2007; Lazzari *et al.* 2010; Rizos *et al.* 2010b)

In vitro embryo-maternal communication: using co-culture with BOEC monolayer as starting point

To understand more precisely how the embryo develops physiologically, it is necessary to ascertain the crucial role of the maternal environment on development. Focusing our interest on the positive influence of the oviduct on the early embryo, it is necessary to examine whether the embryo itself is able to modulate changes in the oviduct and consequently induce such influence. Based on the current knowledge gained from *in vivo* studies, interactions of the embryo with the maternal epithelium may be very local. Furthermore, the small size of the embryo and the difficulty of identifying its exact position in the oviduct make difficult to detect this interaction (Maillo *et al.* 2015). Therefore, an *in vitro* model to facilitate the study of early embryo-maternal communication was necessary. The *in vitro* culture of BOEC has provided a suitable model for the study of the mechanisms involved in the interaction of gametes and embryos with the mammalian oviduct. Recent advances in *in vitro* modeling of the oviductal epithelium have enabled the development of new culture techniques that better mimic the morphological and physiological characteristics of the oviduct (Gualtieri *et al.* 2012; Chen *et al.* 2013; Lawrenson *et al.* 2013; Simintiras *et al.* 2017). However, considering the increasing complexity of studies, it was necessary to establish novel strategies for *in vitro* culture that

allow a more controlled interaction, in terms of area and contact time, between the embryo and the oviductal cells. Thus, the main objective of the chapter 2 was to examine whether the preimplantational embryo (2-cell or 8-cell stage) is able to modulate the transcriptome and metabolic changes in BOEC and to elucidate if this effect is due to a contact-depending signaling with embryo or the result of the BOEC interaction with embryo secretions (embryo CM). Accordingly, an *in vitro* co-culture system allowing a local interaction between BOEC and embryos using polyester mesh was established. The BOEC transcriptome response was assessed by analyzing the expression of 12 genes that have been already affected by embryo presence based on *in vitro* (Schmaltz-Panneau *et al.* 2014; Schmaltz-Panneau *et al.* 2015) and *in vivo* studies (Maillo *et al.* 2015). Similarly to the results observed in those studies, our results in chapter 2 confirmed that bovine embryos have a direct effect on the transcriptome of the oviductal cells. However, it is important to note that this study, in contrast to others, demonstrates that a short period (48h) of interaction with the early embryo is sufficient to induce specific transcriptional changes in the oviductal cells.

Gene expression and metabolic response of BOEC to early embryo

The overall transcriptional profile between BOEC co-cultured with 2 cell embryos or with their CM was different from the one observed after co-culture with 8-cell embryos or with their CM. It is important to emphasis that the difference between both embryo stages resides in the EGA. In bovine, the activation of transcription occurs during the 8- to 16-cell stages and it is associated with a phase called the maternal-to-embryonic transition (MET) where maternal mRNA are replaced by embryonic ones (Graf *et al.* 2014). These events may be accompanied with a specific embryo signaling that could be implicated in embryo-maternal-communication. Our results reported that early embryo induces changes in BOEC expression of genes related with binding, antioxidant and immune activities. This research with other *in vivo* (Maillo *et al*. 2015) provide further input on the targeted genes implicated in the early-maternal dialogue in bovine oviduct. Furthermore this study suggests that embryo affect BOEC transcriptome by a direct contact but also through its secretions released into the media.In terms of energy substrates and amino acid analysis, the results revealed that BOEC metabolism was not affected by the presence of early embryos (2-cell and 8-cell stage) or by their CM. Furthermore, embryo metabolism was different to cells, suggesting that both BOEC and embryos have metabolic requirements that may be independently satisfied by the culture media. Interestingly our results suggest that early embryo before EGA might be energy independent supporting previous studies providing that embryo may have sufficient endogenous energy to support its early development (Sturmey *et al.* 2009; Leese 2015)

Embryo-oviduct interaction *in vitro* induces specific changes in the transcriptional levels of BMP signaling

The epithelial cells lining the lumen of the mammalian oviduct synthesize and secrete a wide range of proteins, including diverse growth factors and cytokines able to mediate a local interaction with the embryo through different signaling pathways (Buhi 2002; Avilés *et al.* 2010). We focused on the signaling mediated by BMPs proteins, a subfamily of growth factors that belongs to the TGF-β superfamily (Bragdon *et al.* 2011). The main objective of chapter 3 was to evaluate if the interaction of the embryo, before and during EGA, with the *in vitro* BOEC can induce changes in the gene expression of BMP signaling components. Although the embryo co-culture with BOEC before or during EGA had no impact on the final production rate of blastocysts, gene expression result revealed a significant decrease in the transcriptional levels of BMP signaling components in BOEC, while in embryos it revealed significant changes in the expression profile of BMP genes depending on whether they interact with BOEC before or during EGA.

The decrease of BMP signaling components in BOEC particularly affects genes encoding factors required for the signal transduction: one ligand (*BMP7*), two receptors (*BMPR1B* and *BMPR2*), one signaling mediator (*SMAD1*) and two BMP target genes (*SMAD6* and *ID2*), suggesting a possible decrease in the activity of BMP pathway in the oviduct when the embryo is present. On the other hand, embryos that interacted with BOEC before the main phase of EGA (G1 BOEC) showed a significant increase in the relative abundance of *SMAD1*. While embryos that interacted with BOEC during EGA (G2 BOEC) displayed a significant increase in the relative abundance of *BMPR1B*, *BMPR2* and *ID2*.

Considering that BMP7 has a critical role in the oviduct epithelium, by acting as an inductor of apoptosis during tissue remodeling. Furthermore, its expression decreased under the effect of estrogen (Monroe *et al.* 2002). Thus, it would be interesting to explore if the downregulation of *BMP7* expression, could be associated with a protective effect in order to maintain the integrity of the oviduct as the embryo passes through.

It is well evidenced the importance of BMP receptors, BMPR1B and BMPR2 in embryo preimplantation development and female fertility (Leal *et al.* 1999; Nagashima *et al.* 2013; Graham *et al.* 2014). In addition, their transcript levels in the embryo are specifically increased at early stages before EGA (Lee *et al.* 2014; Garcia *et al.* 2015). Our results suggest that embryo could send specific signals to reduce the expression levels of these receptors in the oviduct to avoid competence. If so, BMP ligands present in the oviductal microenvironment could act preferably on the embryo. However, further studies are required to confirm this possibility.

According to *in vivo* studies the transfer of 50 embryos to the bovine oviduct produced changes in the gene expression pattern of the oviductal cells such as downregulation of *SMAD6* (Maillo *et al.* 2015). This gene is induced via SMAD1/5 pathway and at protein level acts as an inhibitor self-regulated by the BMP signaling pathway (Ishida *et al.* 2000). A recent study in mice demonstrated that SMAD signaling is required for structural integrity of the female reproductive tract and uterine function during early pregnancy (Rodriguez *et al.* 2016).

SMAD1 is a key component of the BMP signaling pathway that allows the transduction of the signal from membrane receptors to the nucleus (Bragdon *et al.* 2011). Thus, the significant increase in the mRNA levels for *SMAD1* in the embryo suggests that the interaction with oviductal cells may induce the activation of BMP signaling in the embryo through the SMAD1 pathway. Transcriptional analysis of mouse and bovine embryos reveals high abundance of *SMAD1* mRNA at early stages before the main phase of EGA and a significant reduction after EGA up to the blastocyst stage (Wang *et al.* 2004) suggesting that this factor is necessary during the early stages in which the embryo transits through the oviduct. Regarding the target gene of BMP signaling, transcriptome analysis by microarray in *in vivo*-produced bovine embryos reported that *ID2* increases its transcription during the activation of the bovine embryonic genome (Kues *et al.* 2008) that was in accordance with our results.

This research reveals the implication of BMP signaling pathway during early embryogenesis, coinciding with other studies (Zhang *et al.* 2007; Lee *et al.* 2014). Moreover the low levels detected at the blastocyst stage and the fact that at this stage there were no differences in the mRNA expression level of BMP signaling components supports the hypothesis that BMP signaling is more important during the early stage of development, representing an active cross talk, resulting in a bidirectional response in BOEC and embryo. Further understandings of that signaling pathway and others are important for the future improvement of ART and may contribute to decrease infertility in cattle and human

CONCLUSIONS

1. Mimicking the physiological conditions of early embryo development by alternating oviductal and uterine fluid in *in vitro* serum-free culture supports embryo development and improves blastocyst quality.
2. Oviductal fluid provides a better control of embryo methylation, whereas uterine fluid has an antioxidant activity.
3. Bovine oviduct epithelial cells primary culture represents an adequate starting point to study embryo-maternal interaction *in vitro*.
4. Early bovine embryo affects the transcriptome of the oviductal cells but does not affect their metabolism.
5. The embryo effect may be due to a direct contact with oviductal cells or its secretions released into the media.
6. Bovine embryo-oviduct interaction *in vitro* reveals an early cross talk mediated by bone morphogenetic proteins signaling pathway

CONCLUSIONES

1. La simulación de las condiciones de desarrollo embrionario temprano, alternando el uso del fluido oviductal y uterino en un medio de cultivo *in vitro* libre de suero, mantiene el desarrollo embrionario y mejora la calidad de los blastocitos.

2. El fluido oviductal proporciona un mejor control de la metilación embrionaria, mientras que el fluido uterino tiene una actividad antioxidante.

3. El cultivo de BOEC en monocapa representa un punto de partida adecuado para estudiar la interacción materno-embrionaria *in vitro*.

4. El embrión bovino temprano afecta el transcriptoma de las células oviductales pero no afecta a su metabolismo.

5. El efecto del embrión sobre las células oviductales puede ser debido a un contacto directo entre ambos o a las secreciones liberadas por el embrión en el medio.

6. La interacción oviducto-embrión *in vitro* en la especie bovina revela la implicación de un diálogo temprano mediado por la vía de señalización de BMP

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Van Wagtendonk-de Leeuw, A.M. (2006) Ovum pick up and in vitro production in the bovine after use in several generations: a 2005 status. *Theriogenology* **65**(5), 914-25

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Weber, J.A., Freeman, D.A., Vanderwall, D.K., and Woods, G.L. (1991) Prostaglandin E2 secretion by oviductal transport-stage equine embryos. *Biol Reprod* **45**(4), 540-3

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Wrenzycki, C., Herrmann, D., Lucas-Hahn, A., Korsawe, K., Lemme, E., and Niemann, H. (2005) Messenger RNA expression patterns in bovine embryos derived from in vitro procedures and their implications for development. *Reproduction, fertility, and development* **17**(1-2), 23-35

Wu, B., and Zan, L. (2012) Enhance beef cattle improvement by embryo biotechnologies. *Reprod Domest Anim* **47**(5), 865-71

Yániz, J.L., Lopez-Gatius, F., Santolaria, P., and Mullins, K.J. (2000) Study of the functional anatomy of bovine oviductal mucosa. *The Anatomical Record* **260**(3), 268-278

Curriculum Vitae

MERIEM HAMDI

EDUCATION

- **PhD** (Defending April 2018) in Veterinary Medicine. Dept. of Animal Reproduction, INIA, Madrid - University Complutense de Madrid, Spain**,** 2014-2018
- **Master** in "Animal Breeding and Reproduction Biotechnology". Autonomous University of Barcelona and Polytechnic University of Valencia, Spain, 2011-2013
- **DVM,** University of Mentouri. Constantine, Algeria. 2003-2005
- Languages knowledge: English (good), Spanish (very good), French (bilingual), Arabic (native speaker).

RESEARCH EXPERIENCE

- **PhD studies**: The main objective of my studies was to establish an in vitro model using bovine oviduct epithelial cells (BOEC) to study early maternal-communication in the bovine oviduct.
- **Master studies**: It was focused on the effect of bovine oviduct epithelial cells and their extracellular vesicles in *in vitro* embryo production
- Both PhD and MSc studies were developed in The Preimplantational Embryology Laboratory at the National Institute for Agricultural and Food Research and Technology (INIA) -Madrid-Spain, under the supervision of Dr. Dimitrios Rizos.
  During this time I acquired the following laboratory practices:

  - In vitro embryo Production including: Maturation, fertilization and embryo culture
  - Embryo vitrification using: Cryoloop, Cryotop…etc.
  - Embryo biopsy
  - Primary cell culture of bovine oviduct epithelial cells
  - RNA and DNA extraction, RTPCR, qPCR and Primer design
  - Extracellular vesicles isolation
  - Immunofluorescence
  - In vivo and in vitro BOEC sample preparation for Transmission and Scanning Electron Microscopy
  - Differential embryo cell count

TEACHING EXPERIENCE

- Training and supervising bachelor and master students on IVP, embryo vitrification etc. (During my PhD study). 2014-2018
- Training in in vitro embryo production for veterinary students University of Complutense, de Madrid, Spain. In October 2016 & October 2017
- Assisted reproduction seminar for veterinary students of University of Complutense, de Madrid, Spain. 2015

WORK EXPERIENCE

- Veterinary in hygienic qualification service. Annaba, Algeria. 2011
- Veterinary inspector in chicken slaughterhouse. Annaba, Algeria. 2010
- Veterinary Clinician. Annaba, Algeria. 2008

RESEARCH STAYS

- Avantea "laboratory of Advanced Technologies for Biotechnology Research and Animal Reproduction". Cremona Italy under the supervision of Dr. Giovanna Lazzari. From 1st of September to 27th of November 2015
- Lyons Research Farm, University College of Dublin, Agriculture & Food Science Centre, Ireland under the supervision of Prof. Patrick Lonergan. From 26th of November to 4th of December 2016 & 6th to 11th of November 2017

ATTENTED COURS

- Course of experimental design and analysis of molecular data. National Institute for Agricultural and Food Research and Technology (INIA), Spain. 2014
- Course of Hazard analysis and critical control points (HACCP), high institute of management (ISGA) ISGA. Annaba-Algeria. 2010

PUBLICATIONS IN INDEXED JOURNALS

1. **Hamdi M**, Sánchez Calabuig M, Rodriguez-Alonso B, Roussi K, Gutierrez-Adan A, Sturmey R, Lonergan P, Rizos, D (**2018**). Gene expression and metabolic response of bovine oviduct epithelial cells to early embryo in vitro. Reproduction (*under review)*
2. Torres V, **Hamdi M**, Millán de la Blanca MG, Urrego R, Echeverri J, López-Herrera A, Rizos D, Gutierrez-Adan A and Sánchez-Calabuig M (**2018**).Resveratrol-cyclodextrin complex affects the expression of genes associated with lipid metabolism in bovine in vitro produced embryos. Reproduction in Domestic Animals (*Under revision*)
3. Torres V, **Hamdi M**, Maillo V, Urrego R, Echeverria J, López–Herrera A, Gutierrez-Adan A, Rizos D and Sánchez-Calabuig M (**2018**). Ascorbic-acid–cyclodextrin complex improves embryo quality during in vitro maturation and in vitro culture. Theriogenology (*Under revision*)
4. **Hamdi M,** Lopera-Vasquez R, Maillo V, Sánchez Calabuig M, Núñez C, Gutierrez-Adan, A, Rizos D (**2017**). Bovine oviduct and uterine fluid support in vitro embryo development. Reproduction, Fertility and Development doi: 10.1071/RD17286.
5. García EV*, **Hamdi M***, Barrera AD, Sánchez Calabuig M, Gutierrez-Adan A, Rizos D (**2017**). Bovine embryo-oviduct interaction in vitro reveals an early crosstalk mediated by BMP signaling. Reproduction 153 (5) 631-643. (*both authors contributed equally).
6. Lopera-Vasquez R**, Hamdi M,** Maillo V, Gutierrez-Adan A, Bermejo-Alvarez P, Ramírez MÁ, Yáñez-Mó M, Rizos D (**2017a**). Effect of bovine oviductal extracellular vesicles on embryo development and quality in vitro. Reproduction 153(4):461-470.

7. Lopera-Vasquez R**, Hamdi M,** Maillo V, Lloreda V, Coy P, Gutierrez-Adan A, Bermejo-Alvarez P, Rizos D (**2017b**). Effect of bovine oviductal fluid on development and quality of bovine embryos produced in vitro. Reproduction, Fertility and Development, 29, 621–629.
8. Barrera AD, García EV**, Hamdi M,** Sánchez-Calabuig MJ, López-Cardona ÁP, Balvís NF, Rizos D, Gutiérrez-Adán A (**2017**). Embryo culture in presence of oviductal fluid induces DNA methylation changes in bovine blastocysts. Reproduction 154 (1) 1-12.
9. Fonseca Balvís N, Garcia-Martinez S, Pérez-Cerezales S, Ivanova E, Gomez-Redondo I**, Hamdi M,** Rizos D, Coy P, Kelsey G, Gutierrez-Adan A .(**2017**).Cultured bovine embryo biopsy conserves methylation marks from original embryo. Biol Reprod.1;97(2):189-196.
10. Lopera-Vásquez R**, Hamdi M**, Fernandez-Fuertes B, Maillo V, Beltrán-Breña P, Redruello A, Gutierrez-Adán A, Yañez-Mó M, Ramirez MA, Rizos D. (**2016**). Extracellular vesicles from BOEC in *in vitro* embryo development and quality. PLoS One 4;11(2):e0148083.
11. Maillo V, Sánchez-Calabuig M, Lopera-Vasquez **R, Hamdi M,** Gutierrez-Adan A, Lonergan P, Rizos D. (**2016**) Oviductal response to gametes and early embryos in mammals. Reproduction 152(4):R127-41.
12. Maillo V, Lopera-Vasquez R**, Hamdi M,** Gutierrez-Adan A, Lonergan P, Rizos D. (**2016**). Maternal-embryo interaction in the bovine oviduct: Evidence from in vivo and in vitro studies. Theriogenology 86(1):443-50.

ORAL COMMUNICATION AT NATIONAL AND INTERNATIONAL CONFERENCES

1. **Hamdi M,** Sánchez-Calabuig M, Gutiérrez-Adán A, Rizos D. Transcriptomic response of bovine oviduct epithelial cells to the early embryo. 33rd Annual meeting of the European Embryo Transfer Association (**AETE**). Bath, United Kingdom (2017). *Finalist for student competition*
2. **Hamdi M,** Lopera R, Maillo V, Sánchez-Calabuig M, Núñez C, Beltrán-Breña P, Gutiérrez-Adán A, Rizos D. Effect bovine oviduct and uterine fluid on in vitro embryo development and quality. 18th International Congress of Animal Reproduction (**ICAR**).Tours-France (2016)
3. **Hamdi M,** Lopera R, Maillo V, Nuñez C, Gutierrez-Adan A, Bermejo P, Rizos D. Bovine oviduct epithelial cells: an in vitro model to study early embryo-maternal communication.31st Annual meeting of the European Embryo Transfer Association (**AETE**). Ghent, Belgium (2015). *Finalist for student competition*
4. **Hamdi M,** Lopera R, Maillo V, Nuñez C, Gutierrez-Adan A, Bermejo P, Rizos D. "El tracto reproductor femenino bovino: Modelos in vitro para el estudio del desarrollo embrionario temprano y comunicación materno embrionaria". Primera Jornada de investigacion **VETINDOC UCM**. Madrid, Spain. (2015)
5. Lopera R, **Hamdi M,** Maillo V, Lloreda V, Nuñez C, Coy P, Rizos D. The effect of bovine oviductal fluid in in vitro embryo production. 30th Annual Meeting of the European Embryo Transfer Society (**AETE**). Dresden Alemania.(2014)

POSTERS

1. Lopes JS, **Hamdi M**, Rodríguez-Alonso B, Canha-Gouveia A, París-Oller E, Cánovas S, Rizos D, Coy P. First pregnancy outcomes of vitrified embryos developed in vitro with pure reproductive fluids as additives 34th Annual Meeting of European Society of Human Reproduction and Embryology (**ESHRE**). Barcelona, Spain. From 1 to 4 July 2018

2. Rodríguez-Alonso B, Sánchez JM, **Hamdi** M, Havlicek V, Besenfelder U, Lonergan P, Rizos D. Oviduct-embryo interaction in cattle: Effect of asynchrony between the embryo and the oviduct on subsequent embryo development. 34th Annual Meeting of European Society of Human Reproduction and Embryology (**ESHRE**). Barcelona, Spain. From 1 to 4 July 2018
3. **Hamdi M**, Rodríguez-Alonso B, Almansa-Ordonez A, Gutierrez-Adán A, Lonergan P, Rizos D. In vitro transcriptomic response of bovine oviduct epithelial cells to direct or indirect embryo contact. 44th Annual Conference of the International Embryo Transfer Society (**IETS**). Bangkok, Tailandia. 2018
4. Rodríguez-Alonso B, **Hamdi M**, Sánchez Gomez M, Gutierrez-Adán A, Lonergan P, Rizos D. In vivo transcriptomic response of bovine oviduct epithelial cells to the early embryo. 44th Annual Conference of the International Embryo Transfer Society (**IETS**). Bangkok, Tailandia. 2018
5. **Hamdi M**, Sanchez Calabuig M, Gutiérrez-Adán A, Lonergan P, Rizos D.Transcriptomic response of bovine oviduct epithelial cells to the early embryo. 33rd Annual meeting of the European Embryo Transfer Association (**AETE**). Bath. United Kingdom, 2017
6. Van Hoeck V, **Hamdi M**, Jordaens L, Beemster G, Rizos D, Sprangers K, Gutiérrez-Adán A, PEJ Bols, JLMR Leroy. Maternal metabolic disorders and early embryonic loss: pathways to bridge the gap between bovine embryo quality and endometrial receptivity. 33rd Annual meeting of the European Embryo Transfer Association (**AETE**). Bath. United Kingdom, 2017
7. Torres V, **Hamdi M**, Millán de la Blanca M.G, Urrego R, Echeverri J, López-Herrera A, Rizos D, Gutierrez-Adan A. and Sánchez-Calabuig M. Effect of resveratrol-cyclodextrin complexsupplementation during oocyte maturation or embryo culture in vitro in bovine. 33rd Annual meeting of the European Embryo Transfer Association (**AETE**). Bath. United Kingdom, 2017.
8. García EV, **Hamdi M**, Barrera AD, Sánchez Calabuig M, Gutierrez-Adan A, Rizos D. Bovine embryo-oviduct interaction in vitro reveals an early crosstalk mediated by BMP signaling. 43rd Annual Conference of the International Embryo Transfer Society (**IETS**). Austin, Texas. 2017
9. Barrera AD, García EV, **Hamdi M**, Sánchez-Calabuig M, Rizos D, Gutiérrez-Adán A. Effect of oviductal fluid on DNA methylation of bovine blastocysts produced in vitro. 43rd Annual Conference of the International Embryo Transfer Society (**IETS**). Austin, Texas. 2017
10. **Hamdi M**, Lopera R, Maillo V, Sánchez-Calabuig M, Núñez C, Beltrán-Breña P, Gutiérrez-Adán A, Rizos D. Bovine oviduct and uterin fluid support in vitro embryo development. 18th International Congress of Animal Reproduction (**ICAR**). Tours, France. 2016.
11. **Hamdi M**, Bages S, Gutiérrez-Adán A, Lonergan P, Rizos D. In vitro oviduct epithelial cells response to early embryo stage. 32nd Annual meeting of the European Embryo Transfer Association (**AETE**). Barcelona, Spain. 2016.
12. Lopera-Vasquez R, **Hamdi M**, Maillo V, Nunez C, Yanez-Mó M, Ramirez MA, Gutierrez-Adan A, Bermejo-Alvarez P, Rizos D. Extracellular vesicles of bovine oviductal fluid modify the gene expression on bovine in vitro derived embryos. 42nd annual conference of the International Embryo Transfer Society (**IETS**). Louisville, Kentucky,USA. 2016
13. Lopera-Vasquez R, **Hamdi M**, Maillo V, Lloreda V, Nuñez C, Coy P, Gutierrez-Adan A, Bermejo P, Rizos D. Effect of bovine oviductal fluid on development and quality of in

vitro-produced bovine embryos. 41st Annual Conference of the International Embryo Transfer Society (IETS). Versaille, France. 2015.

14. **Hamdi M**, Lopera R, Maillo V, Nuñez C, Gutierrez-Adan A, Bermejo P, Rizos D."Bovine oviduct epithelial cells: an in vitro model to study early embryo-maternal communication 31st Annual meeting of the European Embryo Transfer Association (**AETE**). Ghent, Belgium. 2015.
15. Lopera-Vasquez R, **Hamdi M**, Fuertes B, Maillo V, Beltran P, Redruello A, Yañez Mo M, Ramirez MA, Rizos D. Depletion of extracellular vesicles from fetal calf serum improves the quality of bovine embryos produced in vitro. 12º Congreso de la Asociación Española de Reproducción Animal (**AERA**). Alicante Spain. 2014.
16. Lopera-Vasquez R, **Hamdi M**, Maíllo V, Lloreda V, Núñez C, Gutiérrez-Adán A, Rizos D. Effect of extracellular vesicles of bovine oviductal fluid on in vitro embryo development and quality. Epigenetics and Periconception Enviroment **EPICONCEPT–COST**. Vilamora, Portugal. 2014.
17. Lopera-Vasquez R, **Hamdi M**, Maillo V, Lloreda V, Nuñez C, Coy P, Rizos D. The effect of bovine oviductal fluid in in vitro bovine embryo production. 30th Scientific Meeting of the European Embryo Transfer Society (**AETE**). Dresden, Germany. 2014
18. Lopera R, **Hamdi M**, Fuertes B, Maillo V, Beltran P, Redruello A, Gutierrez-Adan A, Yañez-Mo M, Ramirez MA, Rizos D. Effect of extracellular vesicles secreted by bovine oviductal epitelial cells in in vitro bovine embryo production. International Society of Extracellular Vesicles (**ISEV**). Rotterdam, Netherland. 2014.
19. Lopera-Vasquez R, **Hamdi M**, Fuertes B, Maillo V, Beltran P, Redruello A, Gutierrez-Adan A, Yanez-Mo M, Ramirez MA, Rizos D. Extracellular vesicles secreted by bovine oviductal epitelial cells increase the quality of in vitro produced bovine embryos. 29th Annual Meeting of the European Embryo Transfer Association (**AETE**), Istanbul, Turkey. 2013

AWARDS AND SCHOLARSHIPS

- Finalist for student competition in the 33rd Scientific Meeting of the European Embryo Transfer Society (AETE). Bath, United kingdom 2017
- Finalist for student competition in the 31st Scientific Meeting of the European Embryo Transfer Society (AETE). Ghent, Belgium 2015.
- Scholarship granted by Spanish Ministry of Economy and Competitiveness to do PhD studies, 2014-2018.
- Scholarship granted by the International Centre for Advanced Mediterranean Agronomic Studies-Zaragoza (CIHEAM-IAMZ) to do master degree, 2011-2013.