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In vitro Bovine Oviduct Epithelial Cells response to presence of early embryo

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1. Summary

The positive effects of the oviduct on the embryo have been widely studied over the years. However, there is not as much literature about the effect of the embryo on the oviduct. The aim of this project was to evaluate the transcriptome response of a BOEC monolayer to the presence of early stage embryos. In a first experiment, embryos on Day 2 and Day 3 were co-cultured for 24 hours, and the expression of 4 genes (ROCK2, GPX4, SOCS3, PRELP) was analysed. No differences were found between the BOEC monolayer with or without embryos. In order to assess the possible local effect of the embryo on the cells, a second experiment was performed using a mesh to keep the embryos in the same place and in contact with the cells during 24 hours. Then, three different groups of samples were collected: cells in direct contact with the embryos (G1), cells in the same well that the embryos but not in direct contact (G2) and cells from another well without embryos (G3). The expression of 9 genes of interest (ROCK2, GPX4, SOCS3, ROCK1, SMAD6, NFE2L2, GPX4, EPSTI1, IGFBP3, SCN9A) was analysed. One gene (EPSTI1) was found up-regulated in both groups of cells with no direct contact with the embryos. Increased expression of EPSTI1 has been implicated in endometrial remodeling prior to embryo implantation in cattle. In conclusion, the embryo is probably exerting a local effect on the BOEC monolayer, however, based on the relatively small number of genes analyzed further studies are needed to standardize an adequate *in vitro* system to study embryo-maternal communication in the oviduct.

2. Resumen

Los efectos positivos del oviducto sobre el embrión han sido ampliamente estudiados a lo largo de los años. Sin embargo, no existe mucha bibliografía sobre el efecto que ejerce el embrión sobre el oviducto. El objetivo de este proyecto era evaluar la respuesta del transcriptoma de una monocapa de células epiteliales del oviducto bovino (CEOB) a la presencia de embriones tempranos. En un primer experimento, se co-cultivaron embriones de Día 2 y Día 3 durante 24 horas y se analizó la expresión de 4 genes (ROCK2, GPX4, SOCS3, PRELP). No se encontraron diferencias en su expresión entre la monocapa de CEOB con o sin embriones. Con tal de evaluar un posible efecto local de los embriones sobre las células, se realizó un segundo experimento en el que se usó una malla para mantener los embriones en el mismo lugar y en contacto con las células durante 24 horas. Se recogieron tres tipos de muestras: células en contacto directo con los embriones (G1), células en el mismo pocillo que los embriones pero no en contacto directo (G2) y células de otro pocillo sin embriones (G3). Se analizó la expresión de 9 genes de interés (ROCK2, GPX4, SOCS3, ROCK1, SMAD6, NFE2L2, GPX4, EPSTI1, IGFBP3, SCN9A). Uno de los genes (EPSTI1) estaba sobre-expresado en los dos grupos de células que no tenían contacto directo con los embriones. En bovino, un aumento de la expresión de EPSTI1 se ha relacionado con la remodelación del endometrio antes de la implantación del embrión. En conclusión, probablemente el embrión ejerce un efecto local sobre la monocapa de CEOB. No obstante, considerando el número relativamente bajo de genes analizados, más estudios son necesarios para estandarizar un sistema *in vitro* adecuado para el estudio de la comunicación embrio-maternal en el oviducto.

3. Introduction

3.1 Anatomy-histology and physiology of the Oviduct

The oviduct (uterine tube or Fallopian tube) is a large conduct that connects the ovary with the uterus. Thanks to the different anatomical regions and the dynamic environment of the oviduct that changes during the oestrus cycle, the oviduct is able to perform different functions (Yaniz *et al.* , 2000). The most important ones are: the final maturation of the gametes, the fertilization and the early embryo development (Avilés *et al.* 2015).

The oviduct is divided into 5 anatomic parts: the infundibulum, the ampulla, the ampullary-isthmic junction (AIJ), the isthmus and the utero-tubal junction (Figure 1). It has 3 different layers (mucous, muscular and serous) that varies in size and structure among the different regions. The serous layer is highly vascularized and innervated. The muscular layer is constituted by two layers: the inner circular and the longitudinal. The mucous layer also has two parts: the lamina propria and the epithelium. This layer forms folds whose number and size varies among the different segments of the oviduct, being more complex and numerous in the infundibulum and ampulla, and simpler and fewer in the isthmus (Yaniz *et al.* 2000). The epithelial layer is composed by different cell types. The most important ones are the secretory and the ciliated cells. The amount, proportion and size of these cell types vary along the oviduct and also during the oestrous cycle (Galina and Valencia, 2009).

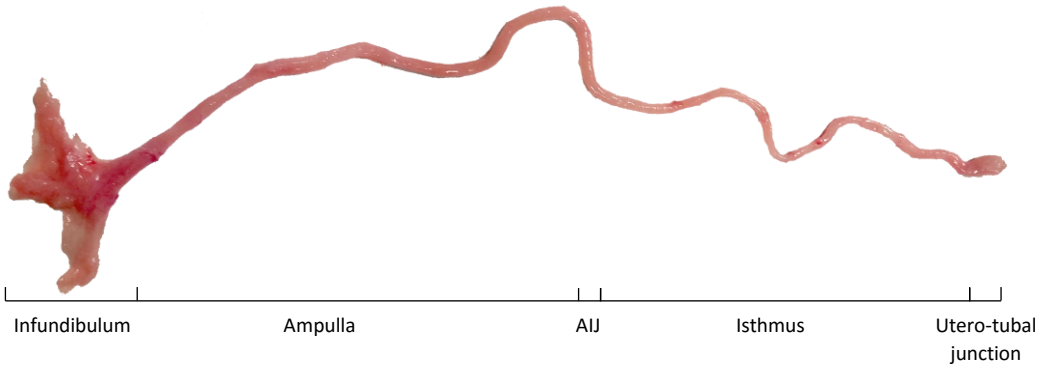

Figure 1: Anatomic parts of the oviduct. AIJ: Ampullary-isthmic junction.

In the cow, the ciliated cells are more abundant than secretory cells in the infundibulum and ampulla, but getting closer to the AIJ this proportion is reversed, being the isthmus rich in secretory cells (Leese 1988). The proportion and height of the ciliated cells falls dramatically during the luteal phase in the first segments, but it does not change in the isthmus and utero-tubal junction (Abe 1996).

The secretory cells secrete several macromolecules including oviduct-specific glycoprotein (OVGP1) and growth factors, usually by exocytosis, associated with the first days of the

oestrous cycle, which contribute to the development of the early embryo (for a review see Maillo *et al.* 2016). Apart from the own oviduct secretions, the oviductal fluid (OF) also has components from the plasma (Menezo and Guerin, 2016).

The final composition of the OF is very complex. Its components can be classified in different groups as: (i) growth factors, cytokines and receptors, (ii) hormones and receptors, (iii) proteases and inhibitors, (iv) antioxidant protective agents, (v) defence agents, (vi) glycosidases and glycosyl transferases, (vii) other enzymes, (viii) chaperones and heat shock proteins, (ix) other proteins, (x) glycosaminoglycans and proteoglycans and (xi) other components (Avilés *et al.* 2010). This composition varies among the different phases of the oestrous cycle (Bauersachs *et al.* 2004) and with the presence of gametes and early embryo (Kodithuwakku *et al.* 2007; Georgiou *et al.* 2007).

3.2The role of the oviduct

3.2.1 Final maturation and transportation of the gametes

First of all, the oocyte must suffer a maturation process in the ovarian follicle to be able to be fertilized later. After the LH surge, the ovulation takes place and the oocyte is released along with the cells that surround it (*cumulus oophorous*). The oocyte is ovulated in metaphase II in most mammals, and meiosis does not finish until it is fertilized. The cumulus-oocyte complex (COC) is collected by the infundibulum, and it is transported close to the AIJ where the fertilization occurs. The transport of the oocyte or zygote down the oviduct towards the uterus is due to a combination of cilia action and muscular contractions which are hormone regulated: a high concentration of estrogens (follicular phase) accelerate its transport (Orihuela *et al.* 2003), and a high concentration of progesterone (luteal phase) slow it down (Wessel *et al.* 2004).

In cows, the fertile life of the oocyte in the oviduct is 20-24 hours, but after 9-14 hours it loses the cumulus cells which can affect fertilization (Galina & Valencia 2009). Due to the disintegration and expansion of the cumulus cells, the oocyte is more exposed to the oviductal fluid (OF) which triggers a series of changes, like favour hardening of the zona pellucida (ZP), the extracellular glycoprotein coat surrounding the egg. The main molecules responsible for this changes are OVGP1 and the heparin-like glycosaminolgycans (Coy *et al.* 2008). The culture of matured pig and cows oocytes during 30 minutes in OF increases its resistance to proteolytic digestion from seconds to hours, decreases its affinity for sperm binding and makes

it less penetrable, resulting altogether in reduced levels of polyspermy (Mondéjar *et al.* 2013; Coy *et al.* 2008).

Meanwhile, the spermatozoa have to overcome a series of morphological barriers such as the cervix and the utero-tubal junction. The small proportion of spermatozoa that reaches the isthmus is bound to the ciliated epithelial cells, creating a sperm reservoir (Talevi & Gualtieri 2010). It is gradually released but the reasons of its release are still unknown. It is hypothesized that it could be due to a loss of binding sites on the oviductal epithelium, to the peri-ovulatory changes in the OF or to a change in the spermatozoa, for example, those associated to capacitation like membrane modifications and the hyperactive motility (Suarez 2008b; P. Coy *et al.* 2012).

Once released, the sperm has to travel from the isthmus to the AIJ. There are two mechanism described that guide the spermatozoa towards the oocyte: chemotaxis, which is the movement of cells up a concentration gradient of chemoattractant (i.e. the progesterone released during the ovulation and also produced by the cumulus cells), and thermotaxis, which is the directed movement of cells along a temperature gradient (Eisenbach & Giojalas 2006). The mucus present in the oviduct also helps the sperm transport towards the ampulla due to a reduction in its viscosity after the ovulation which allows an accentuated flagellar beat (Hunter *et al.* 2011).

3.2.2 Fertilization

The hyperactive motility acquired by the spermatozoa after being released from the isthmus epithelium is essential for the fertilization (Quill *et al.* 2003). This new pattern is the consequence of the intracellular increase of Ca^{2+} (Suarez *et al.* 1993). The calcium is mobilized into the sperm cytoplasm through a sperm-specific ion channel called CatSper. The activation of this channel depend on many physiological changes occurring in the oviduct, like the progesterone increase after the ovulation (Publicover *et al.* 2007) and the pH changes in the oviductal fluid during oestrus (Suarez 2008a).

When the hyperactive spermatozoon reaches the oocyte, it has to get through the cumulus cells with the aid of the hyaluronidase produced by itself, bind and penetrate the ZP and finally fusion with the oocyte. The traditional believe was that the union of the spermatozoon with the ZP induces the acrosome reaction which consist in the merge of the outer acrosomal membranes at the anterior region of the spermatozoon head with the plasma membrane (Senger 2005). However, recent studies showed that many spermatozoa have undergone the

acrosome reaction before having any contact with the ZP, in the upper isthmus (Muro *et al.* 2016; La Spina *et al.* 2016).

Some molecules presents in the OF have been describe to have an influence on the spermatozoon-oocyte binding, like the OVGP1 and the heparin-like glycosaminolgycans (Coy *et al.* 2008), as mentioned above. A group of five glycosidases, which activity has been proved to vary along the cycle, have been also suggested to have a role in this process by hydrolysing a ZP structural component (Carrasco *et al.* 2008). Finally, the plasminogen, a serum zymogen mainly produced by the liver, has also been quantified in the OF and demonstrated to bind oocytes at ZP and oolema level (Mondéjar *et al.* 2012). When converted to plasmin, it seems to remove spermatozoa attached to the ZP, thus contributing to the regulation of sperm penetration in the oocyte (Coy *et al.* 2012).

In general, the spermatozoon motility and the enzymes released in the exocytosis of the acrosome components have been postulated to explain spermatozoon penetration through the ZP (Kim *et al.* 2008). After crossing the ZP and the perivitelline space, the plasmatic membranes of both oocyte and spermatozoon fuse, and the spermatozoon head is incorporated to the oocyte cytoplasm. This fusion causes many changes in the oocyte, for example the finalization of the second meiotic division (with the expulsion of the second polar body) and the cortical reaction (exocytosis of the cortical granules). Then the female and male pronuclei migrate to the centre and merge (syngamy), what marks the end of the fertilization and the start of the embryo development (Senger 2005).

3.2.3 Early embryo development

The *in vivo* embryo production involves a series of complex events that take place in different environments (oviduct and uterus). *In vivo* approximately, 24 hours post fertilization the first cleavage occurs; this process comprises a series of mitotic cell divisions that lead the embryo from the 2-cell stage (24 hours post fertilization), 4-cells (36 hours), 8-cells (72 hours), to the morula stage (day 4 to 7) and blastocyst (day 7 to 12) (Senger 2005). In the blastocyst stage, the cells differentiate into two types: trophoectoderm cells, which will form the extraembryonic tissues, and the inner cell mass, which will be the foetus. The embryo increases in size (expanded blastocyst), which causes the ZP rupture and the released of the embryo (hatched blastocyst) from day 9 to 11 (Senger 2005). From that moment, the embryo acquires a tubular or ovoid form and is then termed a conceptus (Hue *et al.* 2012). Around day 15, the elongation begins and will finish with the formation of a filamentous structure which will occupy the entire uterine horn (Geisert & Bazer 2015).

The establishment of pregnancy in domestic ruminants (cow, goat, sheep) starts at the blastocyst stage and entails the recognition of pregnancy, implantation and placentation (Guillomot 1995; Spencer *et al.* 2007).

The most important events of early embryo development are the first mitotic division, the embryonic genome activation (8-16 cell stage), which is crucial for the zygote differentiation and its implantation (Schultz *et al.* 1999), the morula compaction and the cell differentiation at the blastocyst stage. The first two occur in the oviduct as the cow embryo reaches the uterus about day 4 after fertilization at the morula stage (Geisert & Bazer 2015).

The oviduct is the first environment to which the embryo is exposed, and although they can be produced *in vitro* and transferred successfully to a recipient, the oviduct has an important role regarding the quality of those embryos. The *in vitro* produced embryos are of inferior quality than the *in vivo* counterparts (Rizos *et al.* 2002), in terms of cryotolerance, gene expression patterns (altered in the *in vitro* production) (Rizos *et al.* 2008), inner cell mass/trophoectoderm cells ratios (Plourde *et al.* 2012) and pregnancy rates after transfer to recipients (Pontes *et al.* 2009).

The positive effects of the oviduct have been attributed to its capacity to regulate metabolites in the OF according to the needs of the embryo in the different phases, also being able to remove toxic compounds from the medium to protect the embryo against oxidative stress, and to the secretion of growth factors that contribute to cell proliferation (Bongso & Fong 1993).

Approximately 80% of embryonic losses occur between days 8 and 16 post insemination (Diskin *et al.* 2006). This interval coincides with the maternal recognition of pregnancy. The high percentage of losses highlights the complexity and importance of the events occurring during this phase.

3.3 Early embryo-maternal communication

In ruminants, the principal pregnancy-recognition signal sent by the embryo is interferon tau, secreted by the trophoblast from day 10 up to Day 21-25 (Spencer 2004). However, some studies postulate that the *in vitro* embryos begin to express interferon tau as soon as the blastocyst forms (Lonergan, Rizos, Gutierrez-Adán, *et al.* 2003) or even sooner stages, from day 3 (Yao *et al.* 2009). Furthermore, another early pregnancy signal has been recently reported by Muñoz *et al.* (2012), which is a downregulation of a nuclear factor that plays a key role in immune responses in the cattle uterus on Day 8 of pregnancy. As mentioned above, this period between days 8 - 16 of pregnancy is the most critical, when most of the embryo losses occur, and it is when the embryo is already in the uterus. However, to have a successful pregnancy

establishment the embryo must be of an excellent quality and that depends also on the oviduct environment and on a good gametes/embryo-maternal communication. Evidence showed that the embryo when in the oviduct suffers epigenetic changes that will have an effect on its development, implantation and postnatal phenotype (Wrenzycki *et al.* 2005).

Some substances present in the OF may play an important role in the early embryo-maternal communication, like growth factors (Growth Hormone (GH) – Insulin-like Growth Factor (IGF)) and the hyaluronic acid (HA) (Wolf *et al.* 2003). The blastocysts quality was improved after culture with GH by inhibiting apoptosis (Kölle *et al.* 2002). Similarly, the quality and also the blastocyst rate was enhanced after culture with HA (Stojkovic *et al.* 2002; Palasz *et al.* 2006). The main functions of HA are cell adhesion and migration, dynamic processes that are mediated through interaction with extracellular matrix components, regulation of protein secretion, gene expression, and cell proliferation and differentiation (Stojkovic *et al.* 2002). Variable effects have been observed when the embryos have been cultured with IGF, depending on the dose (Wolf *et al.* 2003). The IGF may have a positive effect on the embryo development by modulating the oviduct secretions, or directly on the embryo, through the insulin-like growth factor binding proteins (IGFBPs)(Pushpakumara *et al.* 2002).

In the last years, there is a growing interest in the extracellular vesicles, like microvesicles and exosomes, after the discovery of its immune effects (Raposo *et al.* 1996; Lopera-Vasquez, Meriem Hamdi, *et al.* 2016), and the possibility of its implication in the intercellular communication (Théry 2011). Nowadays it is known that those type of membrane vesicles have an endosomal and plasma membrane origin, which are released by several types of cells from both hematopoietic and non-hematopoietic origin (Raposo & Stoorvogel 2013). Its role in the intercellular communication has been describe as transporters of proteins, lipids, nucleic acids, ligands and receptors from their cell of origin to the recipient cell (Cocucci *et al.* 2009). The extracellular vesicles are able to deliver its content also *in vitro* (Burns *et al.* 2014). There is evidence that microvesicles and exosomes are present in the follicular fluid (Sohel *et al.* 2013), oviductal fluid (Lopera-Vasquez, M. Hamdi, *et al.* 2016) and also in the uterine fluid of pregnant and cyclic ewes (Burns *et al.* 2014). Its specific function in the field of reproduction has been linked with an increase in the expression of PMCA4a in the oviduct, related to the increase in calcium concentration and probably, the hyperactivated motility of the sperm (Al-Dossary *et al.* 2013). Burns *et al.* (2014) observed differences in the exosomes content from pregnant and non-pregnant ewes, supporting the hypothesis of their role in the embryo-maternal communication and the establishment of pregnancy.

In the oviduct, the proportion between ciliated and secretory cells change between the follicular and the luteal phase (Yaniz *et al.* 2000) as well as the final composition of the OF (Seytanoglu *et al.* 2008). The gene expression of BOEC in the follicular and luteal phase of a cow was analysed by Cerny *et al.* (2015) and it revealed differential expression patterns between the two phases with several differentially expressed genes (DEG) exclusive to the ampulla and others exclusive to the isthmus. The protein expression pattern of the epithelial cells is also altered by the presence of the gametes in the oviduct, as it was shown in *in vitro* (Kodithuwakku *et al.* 2007) and *in vivo* studies (Georgiou *et al.* 2007). This last study in human and mouse oviducts detected a different oviductal response among the presence of oocytes or sperm. Furthermore, the response is different whether the spermatozoa carries an X- or Y-chromosome; 60-70% of the genes upregulated in the presence of Y-spermatozoa were related to signal transduction and immune system, compared with X-spermatozoa (Almiñana *et al.* 2014).

The presence of an early embryo in the oviduct also changes the expression patterns. Several DEG have been identified in litter-bearing species like mice (Lee *et al.* 2002) and pigs. In pigs, Chang *et al.* (2000) found that most of the newly expressed genes were detected at 4-cell stage and beyond and that the down-regulated genes were related with the immune system (Almiñana *et al.* 2012). However, in mono-ovulatory species, it is more difficult to find differences. In a recent study from Maillo *et al.* (2015), no differences were found at the transcriptome of the bovine oviduct with a presence of one embryo; nevertheless, after transferring of 50 embryos into the oviduct, they were able to detect 278 DEG (123 upregulated and 155 down-regulated). These findings suggested that failure to detect changes in the oviduct transcriptome in the presence of a single embryo may be due to the fact that any effect is very local and would not be easy detected by the use of the complete oviduct epithelium. However, the presence of multiple embryos in the oviduct resulted in the detection of DEGs in the oviductal isthmus, suggesting a reciprocal crosstalk between the early embryo and the oviduct (Maillo et al., 2015).

The metabolic status of a dairy cow (heifers *vs*. lactating cows and dry *vs*. lactating cows) can influence early embryo development as well. Maillo *et al.* (2012) transferred *in vitro* – produced zygotes to the oviducts of dry and lactating cows around Day 60 postpartum and the embryo development was affected dramatically, with significantly lower blastocyst yield in lactating compared with dry cows.

It is obvious then that the oviduct interacts with the gametes and/or embryo since the very beginning and that its environment adapts accordingly to the circumstances. More studies are needed to elucidate the mechanisms involved in this maternal-embryo communication, largely unknown till the date (Fazeli & Pewsey 2008).

3.4 *In vitro* embryo production (IVP)

In 1951, (Chang 1951; Austin 1951) realized that the sperm needed to undergo some form of capacitation before it was able to penetrate the zona, and that this process occurred in the oviduct. Thanks to this observation, the first successful *in vitro* fertilization (IVF) was performed by Chang (1959) in rabbits with semen recovered from the uterine horn 12 hours after mating. It was not until 1977 when the first IVF was achieved in cattle by Iritani & Niwa (1977), and until 1982 when the first calf was born after IVF in the USA (Brackett *et al.* 1982). By this time, the oocytes were recovered after ovulation through laparoscopy and the culture after fertilization was performed after transfer in the rabbit oviduct.

In 1988 Lu *et al. (*1988) developed a method to produce embryos entirely *in vitro* with the advances in the knowledge of oocyte maturation, sperm capacitation and co-culture of the embryos with bovine oviductal cells (Gandolfi & Moor 1987). The fact of being able to mature oocyte *in vitro* also opened the possibility of recovery oocytes from ovaries collected at the slaughterhouse, therefore significantly decreasing the cost of the IVP. As a consequence, the IVP became quickly a commercial reality and a tool for breeding-improvement purposes.

Almost 30 years after, the production process of *in vitro* embryos is far from optimal. From the oocytes recovered from slaughterhouse ovaries, 90% undergo matured (nuclear and cytoplasmic maturation). Of those, 80 % are successfully fertilized and divide into 2 cells stage (Lonergan, Rizos, Gutiérrez-Adán, *et al.* 2003). However, the problem is that only 30% of the matured oocytes manage to reach the blastocyst stage (Rizos *et al.* 2008). In addition, after transfer of these embryos into recipient cows, pregnancy rate is between 40-60% compared to about 70% when *in vivo* embryos are transferred (Hasler *et al.* 1995).

3.4.1 *In vitro* maturation (IVM)

Although the rate of oocytes maturation is high (90%)(Lonergan, *et al.* 2003), there are many factors to consider achieving it. Oocyte origin is what determines the final rate of blastocysts; those which have matured *in vivo* and then have been fertilized and cultured *in vitro* are more

likely to reach the blastocyst stage than those which have undergone the whole process *in vitro* (Rizos *et al.* 2002).

Another factor that influences the quality and therefore the blastocyst rate, is the size of the follicle aspirated. Oocytes recovered from follicles of more than 6 mm yield a higher proportion of blastocyst compared with follicles between 2-6 mm (65.3% and 34.3% respectively) (Lonergan *et al.* 1994).

After obtaining the COCs, a good selection is essential to obtain a good maturation rate. The two parameters used for this purpose are the presence or absence of cumulus cells and the appearance of the cytoplasm. According to these features the COCs are classified into 4 groups:

- Grade 1: oocytes with more than 4 layers of cumulus cells, complete and compact, and a homogeneous cytoplasm.
- Grade 2: oocytes with fewer layers of cumulus cells, between 1 and 3, with homogeneous cytoplasm or a darker area on the perimeter of the oocyte.
- Grade 3: oocytes without cumulus cells or not completely surrounded by them, with darker and/or irregular cytoplasm.
- Grade 4: expanded oocytes, and picnotic or very dark and/or irregular oocyte cytoplasm (de Loos *et al.* 1989).

Once selected, another important factor is the maturation medium, which can be defined, when all of the chemical components are known, or semi-defined, when adding some biological component for example, which composition varies between batches. The most widely used for maturation is the TCM-199, which is a complex medium, usually supplemented with foetal calf serum (FCS) or bovine serum albumin (BSA), whose composition varies between different batches and is not fully known (Gordon 2003). The maturation media can also be supplemented with gonadotropins, steroids and growth factors, and a small increase is obtained in the rate of blastocysts (Lonergan & Fair 2008).

Finally, oocytes must be matured between 22 and 24 hours in a suitable environment with 38.5°C, 5% CO_2 and saturated humidity (Gordon 2003).

3.4.2 *In vitro* fertilization (IVF)

In this step, the most important factor is to make a good selection of sperm to remove non-motile sperm cells and unwanted components of semen. There are several products and protocols to do so: the swim-up (Parrish *et al.* 1986), and gradients such as Percoll (Saeki 1991)

and BoviPure ™. The Percoll and BoviPure ™ have similar results regarding the final rate of blastocysts, but the advantage of BoviPure ™ is that it is less toxic (Samardzija *et al.* 2006) .

After selection, the sperm concentration is calculated and the proper quantity of sperm is added to the fertilization medium to have a final concentration of $1x10^6$ sperm/mL.

The IVF medium most commonly used is the TALP (Bavister & Yanagimachi 1977), a modification of the Tyrode that contains sodium bicarbonate, albumin as a source of protein, lactate and pyruvate as an energy source (Gordon 2003). Heparin can also be added to the medium to trigger sperm capacitation (Parrish *et al.* 1986), and prepare it for the acrosome reaction and fertilization (Parrish *et al.* 1985).

The co-culture of sperm with matured oocytes is done for 18-22 hours under the same conditions as maturation: 38.5 °C, 5% CO_2 and saturated humidity (Gordon 2003).

3.4.3 *In vitro* culture (IVC)

While oocyte origin is the main factor affecting the rate of blastocysts, the factors that most influence the quality of the embryos are the conditions after fertilization (Rizos *et al.* 2002). Some of the main factors affecting the development and the embryo quality are: the culture medium, the number of embryos per micro-droplet, the ratio embryo/culture medium, the temperature and the gas balance in the incubator (Lonergan *et al.* 2006).

The most used media for the culture of the zygotes is synthetic oviductal fluid (SOF) which is usually supplemented with 5% of FCS and/or BSA (Holm *et al.* 1999; Tervit *et al.* 1972). Both have pros and cons, its use increases the final percentage of blastocysts compared to serum free media. Serum causes an increase in the speed of development, many embryos reach the blastocyst stage on day 6 (Gutierrez-Adan *et al.* 2001); however, affect negatively the embryo quality compared to the once produced with albumin in a concentration of 3mg/mL (Rizos *et al.* 2002). The addition of FCS to the culture media has also been linked to the large offspring syndrome, which is characterized by large calves that can cause dystocia and also have a high postnatal mortality due to defects in many organs (Young *et al.* 1998); which represents an important economic loss to the farmer.

Beside the defined or semi-defined media, the embryos can also be cultured with oviductal, granulosa or Vero cells; or with conditioned media (Mermillod *et al.* 2010; Maeda *et al.* 1996). The conditioned media is the result of the co-culture of the media with somatic cells. This co-culture systems are supposed to help overcoming the embryonic blockage that happens in the stage of 8-16 cells (Vansteenbrugge *et al.* 1994; Minami *et al.* 1992).

The *in vitro* culture of bovine embryos can be performed in an isolated oviduct. Rizos *et al.* (2007) demonstrated that the mouse oviduct can support the development of a bovine embryo and improve its quality.

3.5 Embryo co-culture with bovine oviductal epithelial cells (BOEC)

The introduction of co-culture of cattle and sheep embryos with somatic cells a quarter-century ago was regarded at the time as being an important milestone in overcoming the arrest of development at the 8 to 16-cell stage (Gandolfi & Moor 1987).

Nowadays, the improved cultured media allow avoiding the co-culture to produce embryos for commercial or research purposes. However, this method has been described as one of the most appropriate *in vitro* models, that "mimic" the physiological conditions, to study the gametes and embryo interactions with the oviduct (Van Soom *et al.* 2010).

The positive effects of the co-culture with BOEC on the embryo quality are well known, the number of cells in the blastocyst stage is slightly increased and the survival rate post-thawing is significantly better (Mermillod *et al.* 2010). Those effects are attributed to embryotrophic substances, such as growth factors secreted by the cells (Tse *et al.* 2008). A recent study by Cordova *et al.* (2014) showed that embryo co-culture with BOEC during the four first days of embryo development accelerated the kinetics of blastocyst development and improved the expression of genes related with apoptosis and oxidative stress in the developed embryos. Furthermore, Lopera-Vasquez *et al.* (2016) reported that an established BOEC line can be used successfully after freezing and thawing, avoiding the lack of reproducibility between replicates, as an *in vitro* embryo co-culture system. However, the effect of the embryo on the epithelial cells is not fully known. The only evidence in the literature comes from a study by Schmaltz-Panneau *et al.* (2014) where the transcriptome profile of a BOEC monolayer cultured for 8 days with or without blastocysts was compared. The analysis revealed 34 DEG from which at least 25 were already described as induced by interferons. Based on the difficulty to study the oviduct-embryo interaction *in vivo*, the challenge today is to develop an *in vitro* model "mimicking" the *in vivo* conditions.

4. Motivation and objective of the project

The motivation of this work is to establish an *in vitro* model for embryo maternal communication in bovine based on a previous *in vivo* study (Maillo *et al.* 2015). In this study, two experiments were performed. First, the effect of a single embryo in the oviduct was assessed, but no signal was perceived. Therefore, a second experiment was performed transferring 50 embryos into the oviduct. The microarray analysis revealed 278 differentially expressed genes after the multiple transfer.

The specific objective of this project was to evaluate the transcriptome response of BOEC monolayer to the presence of early stage embryos.

5. Experimental design

Experiment 1

Objective: determine the BOECs response to the presence of early bovine embryos cultured *in vitro* under a monolayer system.

BOECs monolayer were co-incubated for 24 h with Day 2 (monolayer Day 2 embryos group) or Day 3 (monolayer Day 3 embryos group) bovine embryos produced *in vitro* to determine the embryonic effect on BOECs. A control group without embryos was included in each replicate (monolayer no embryos group). Cells were snap frozen in liquid nitrogen and stored at -80ºC. mRNA extraction was carried out and the relative abundance of specific genes was measured by quantitative real time PCR (qPCR).

Experiment 2

Objective: evaluate the expression of several genes of interest from the BOEC monolayer in response to direct or indirect contact with an early embryo.

In order to limit the area of contact between the embryos and the BOEC, embryos were cultured in a nontoxic woven polyester mesh (Sefar Petex; Sefar, Bury, Lancashire, UK) in a 7x7 grid (i.e., 49 embryos/well). After 24h co-culture, the BOECs directly beneath the embryos (Group 1) were recovered as well as cells in the same well but outside this area (i.e., not in direct contact with the embryos (Group 2, control+)) and cells from a different well without embryos (Group 3, control-). Cells were snap frozen in liquid nitrogen and stored at -80ºC. mRNA extraction was carried out and the relative abundance of specific genes was measured by qPCR.

6. Material and Methods

Unless otherwise stated, all chemicals were purchased from Sigma Aldrich Química S.A Company (Madrid, Spain).

6.1 *In vitro* Embryo Production (IVP)

6.1.1 *In vitro* Maturation (IVM)

Ovaries from heifers and cows were collected in the slaughterhouse and transported in saline solution (9g/L NaCl) supplemented with gentamicin (0.1%) at 38 ºC. The immature COC's (n=500) were obtained through aspiration of follicles between 2 and 8 mm of diameter. Only grade 1 and 2 COCs were selected and matured for 22-24 h in 500 µL of maturation media (Annex – Table 1) in a four well NUNC® dish, in groups of 50 COCs per well at 38.5 ºC under an atmosphere of 5% CO_2 in air, with maximum humidity.

6.1.2 *In vitro* Fertilization (IVF)

After 24 hours, the COCs were washed twice in fertilization medium (Annex – Table 2) and transferred into a four well dish with 250 µL of this medium in groups of 50 COC's per well.

Meanwhile, frozen-thawed sperm from a proven fertile bull (Asturgen S.L, Gijón, Asturias, Spain) was selected with BoviPure® gradient. The content was deposited on top of the gradient and centrifuged for 10 minutes at 300 x g. Then the supernatant was carefully removed and the pellet was resuspended and transferred into another tube with 1 mL of BoviWash®. This tube was spin at 300 x g for 5 minutes. The supernatant was removed, and the pellet resuspended in 300 µL of FERT. The sperm concentration was calculated with a Thoma chamber and the pellet resuspended in the appropriate volume of FERT to achieve a concentration of 2 $x10^6$ sperm/mL. A 250 µL of this last solution were added to each well containing the COC's (final sperm concentration of 1 $x10^6$ sperm/mL). The gametes were co-incubated for 18 to 22 hours under an atmosphere of 5% CO_2 in air, with maximum humidity at 38.5 ºC.

6.1.3 *In vitro* Culture (IVC)

After 18, the presumptive zygotes were deposited into a tube with 3 mL of PBS and vortexed for 3 min to remove the cumulus cells and also to eliminate the remaining sperm. The denuded presumptive zygotes were selected based on their morphological characteristics like

homogeneity of the ZP, perivitelline space and cytoplasm. They were washed 3 times in PBS and one in culture medium (Annex – Table 3). The culture was performed in a 35 mm dish in micro-droplets of 25 µL of SOF covered with mineral oil, at 38.5 ºC under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 and in groups of 25 zygotes/drop.

After 48 (Day 2) and 54 (day 3) hours post-insemination 2-4 cell and 6-8 cell embryos were isolated respectively and used for co-culture with BOEC monolayer (Experiment 1 and 2) .

6.2 BOEC's culture

Samples of oviducts corresponding to early luteal phase based on corpus luteum morphology (2 to 4 days after ovulation) (Ireland *et al.* 1980) were collected from the local slaughterhouse and transported on ice. Once in the laboratory, the oviducts were trimmed free of surrounding tissues and washed 3 times in PBS. The isthmus was isolated and squeezed, and the epithelial cells of 3 individuals were pooled. These cells were centrifuged twice with 10 mL of PBS (300 x g; 7 min), removing the supernatant after the first centrifugation and adding fresh media. After the second centrifugation the pellet was resuspended in 1 mL of culture medium (TCM 199 - M4530 supplemented with 10% (v⁄v) foetal calf serum (FCS) and 2.5% (v⁄v) gentamycin) and the cells were mechanically individualized with a syringe of 1 mL and a 25G needle. The concentration of cells was calculated with the same procedure as for the sperm and the proper dilutions were made to have a final concentration of 2 x 10^6 cells/mL. The cells were incubated in 500 mL of culture medium in a four-well NUNC® dish at 38.5 ºC under an atmosphere of 5% CO_2 in air, with maximum humidity.

After 24 hours, the supernatant was centrifuged (300 x g; 7 min), the pellet was resuspended in fresh culture medium and incubated under the same conditions. Half volume of the medium was changed every 48 hours.

Six days later, the, medium was replaced with synthetic oviductal fluid (SOF) supplemented with 10% FCS.

6.3 Mesh preparation

In order to limit the area of contact between the embryos and the BOEC in the second experiment, a nontoxic woven polyester mesh (Sefar Petex; Sefar, Bury, Lancashire, UK) was used with a size of 41x41 grid corresponding to the size of a well of a 4-well dish NUNC.

Once the monolayer were confluent, one of the squares was stuck outside the well with the BOEC monolayer, in order to use it as a guide, it had a delimited area of 7x7 squares

highlighted in blue. Another square of mesh, after being washed once with 70% ethanol and twice with the medium SOF, was introduced into the well so that the two meshes were overlapped (Figure 2).

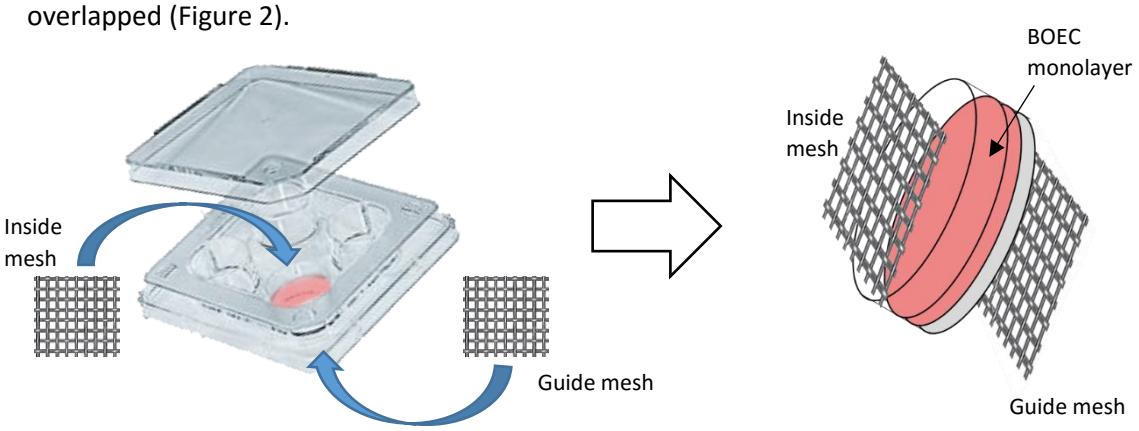

Figure 2. Mesh preparation in a NUNC 4-well dish.

6.4 Co-culture

In Experiment 1, the IVP and the BOEC monolayer culture were coordinated to have Day 2 (2-4-cells) and Day 3 (8-cells) embryos on day 7 of the BOEC, when the confluency was 75 to 80%. At that time, 50 embryos were placed over the monolayer. Then they were co-cultured at 38.5ºC for 24h under an atmosphere of 5% CO_2 and saturated humidity.

In Experiment 2, we used 6-8 cell embryos (reached approximately 52 hours post-fertilization) also on Day 7 of the BOEC. Then, 49 embryos were placed in the mesh within the well in an area of 7x7 squares to keep the embryos fixed in the same area and in direct contact with the cells (Figure 3). They were co-cultured for 24h under the same conditions as experiment 1.

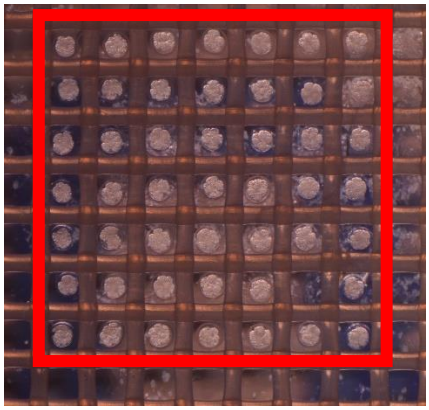

Figure 3. 6 to 8-cell stage embryos placed in the mesh in co-culture with a 7d BOEC monolayer.

6.5 Sample collection

In the first experiment, the embryos were removed and the whole monolayer was lifted, after being washed twice with PBS, using a 100 µL/well of lysis buffer from the Dynabeads mRNA Direct Kit (Ambion; Thermo Fisher Scientific Inc., Oslo, Norway) during 3 minutes. Three groups were differentiate: monolayer Day 2 embryos group, monolayer Day 3 embryos group and monolayer no embryos group. The samples were snap frozen and stored at -80ºC.

In the second experiment, the embryos and the mesh were removed and three different samples of BOEC were collected: G1, the BOEC in direct contact with the embryos; G2, the proximal control, BOEC which were not in direct contact with the embryos but belonged to the same well; G3, the distal control, BOEC from another well without embryos.

To collect the samples, the wells were washed twice with PBS and a minimum volume to cover the well was left. Then, with a small pipette tip (5-10 µL), the area corresponding to a 7x7 squares was lifted. The cells were collected with a micropipette in a volume of 2 to 4 µL which was placed in a 1.5 mL Eppendorf tube, snap frozen and stored at -80 °C for further analysis. Embryos were also snap frozen and stored at -80 to later analyse.

6.6 Gene expression Analysis

6.6.1 RNA extraction, reverse transcription and quantification of mRNA transcript abundance

Five replicates of BOECs from Experiment 1 and 2 were analysed separately. The extraction of the BOECs mRNA was made using the Dynabeads mRNA Direct Kit following the manufacturer instructions (Ambion; Thermo Fisher Scientific Inc., Oslo, Norway). After 10 min incubation in lysis buffer with Dynabeads, poly(A) RNA attached to the Dynabeads was extracted with a magnet and washed twice with washing buffer. Next, RNA was eluted with Tris-HCl. Immediately after extraction; the reverse transcription (RT) reaction was carried out following the manufacturer's instructions (Epicentre Technologies Corp., Madison, WI, USA). The primer mix was prepared adding Oligo(dT) primers, random primers, dNTPs (deoxyribonucleotide triphosphates), DTT (Dithiothreitol, used to stabilize enzymes and other proteins) to the buffer, in a total volume of 40 mL to prime the RT reaction and to produce cDNA. First, the tubes were heated to 70ºC for 5 min to denature the secondary RNA structure and then the RT mix was completed with the addition of 50 units of a high performance reverse transcriptase enzyme, the Moloney Murine Leukemia Virus (MMLV). The tubes were then incubated at 25ºC for 10

min to favour the annealing of the primers, followed by 60 min at 37ºC to allow RT of RNA, and finally 85ºC for 5 min to denature the enzyme.

6.6.2 Quantification

Quantitative polymerase chain reactions (qPCR) were carried out in duplicate on the Rotorgene 6000 Real-Time Cycler TM (Corbett Research, Sydney, Australia) by adding a 2µL aliquot of each sample to the PCR mix (GoTaq qPCR Master Mix; Promega Corporation, Madison, WI, USA) containing the specific primers previously designed using Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to amplify 10 genes of interest: Rho associated coiled-coil containing protein kinase 1 and 2 (ROCK1 and ROCK2), proline/arginine-rich end leucine-rich repeat protein (PRELP), suppressor of cytokine signalling 3 (SOCS3), SMAD family member 6 (SMAD6), Glutathione peroxidase 4 (GPX4), Nuclear factor, erythroid 2 like 2 (NFE2L2), Sodium channel, voltage gated, type IX alpha subunit (SCN9A), Epithelial stromal interaction 1 (EPSTI1) and Insulin-like growth factor binding protein 3 (IGFBP3). Primer sequences and the approximate sizes of the amplified fragments of all transcripts are shown in Table 4 (Annex).

Cycling conditions were 94ºC for 3 min followed by 35 cycles of 94ºC for 15s, 56ºC for 30s, 72ºC for 10s and 10s of fluorescence acquisition. Each pair of primers was tested to achieve efficiencies close to 1 and then the comparative cycle threshold (ΔΔCt) method was used to quantify expression levels as described by (Schmittgen & Livak 2008). To avoid primer dimer artefacts, fluorescence was acquired in each cycle at a temperature higher than the melting temperature of primer dimers (specific for each product, 80–86ºC). Then the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background was determined for each sample. The ΔCt value was determined by subtracting the endogenous control Ct value (an average of H2A Histone Family and Actin Gamma 1 (ACTG)) 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 a constant to subtract from all other ΔCt sample values. Fold-changes in the relative gene expression of the target were determined using the equation $2^{-\Delta\Delta Ct}$.

6.6.3 Immunocytochemical staining

The epithelial origin of the isolated BOEC was confirmed by subjecting the cells to immunocytochemical staining after culturing them on glass coverslips previously coated with

5% gelatin. Those were fixed on day 7 of culture with 4% paraformaldehyde (Panreac®) for 30 minutes. Then the cells were permeabilized with Triton x-100 0.5% for 5 minutes. Three coverslip were incubated separately with primary and secondary antibodies for epithelial cells (anti-bovine-pancadherin, anti-bovine-pancytokeratin) and for stromal cells (anti-bovine-vimentin). Then samples were mounted with Hoechst 33342-containg Prolong (Invitrogen) and visualized under an epifluorescence microscope.

6.6.4 Statistical analysis

Relative abundance of the target transcripts was analysed with the statistical program SigmaStat (Jandel Scientific, San Rafael, CA) and a one-way analysis of variance (ANOVA) was made. Differences were considered significant at $P < 0.05$.

7. Results

7.1 Immunostaining results:

Cells positive for cytokeratin, cadherin and vimentin were green stained and nuclei were blue counterstained with Hoechst 33342 (Figure 4). Overall analysis of the immunocytochemical staining showed approximatively >95% of cells were positive for anti-bovine-pancadherin and anti-bovine-pancytokeratin, confirming the epithelial origin, while less than 5% were positive for anti-bovine-vimentin (stromal cells) confirming the purity of the cells isolated from the isthmus part of the oviduct.

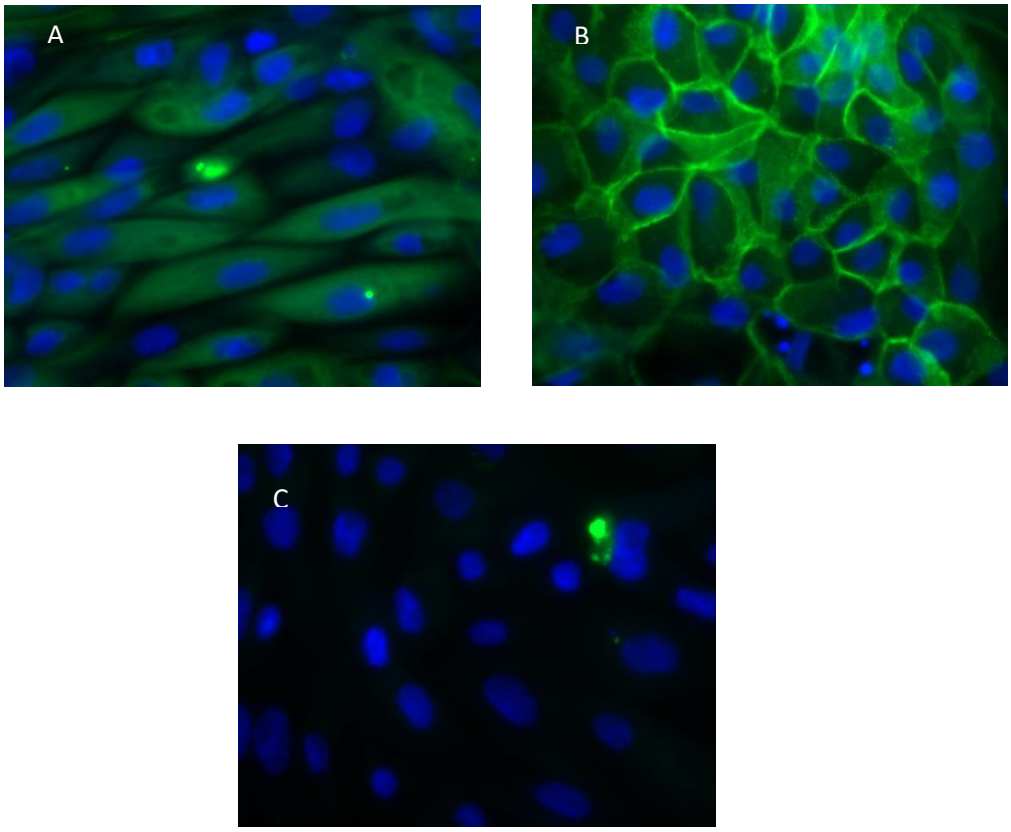

Figure 4. BOEC monolayers immunostaining. The green staining correspond to (A) epithelial cells positive to anti-bovine-pancytokeratin; (B) epithelial cells positive to anti-bovine-pancadherin; (C) stromal cells positive to anti-bovine-vimentin. The blue counterstaining correspond to the cells nuclei.

7.2 Gene expression analysis

Experiment 1

We compared gene expression profiles of BOEC at day 7 of culture after 24 hours in the three groups. The genes analysed could be classified in 3 groups according to their molecular function: antioxidant activity (GPX4), catalytic activity (GPX4, ROCK2, SOCS3) and binding (PRELP) (Protein Analysis Through Evolutionary Relationships, http://pantherdb.org). No effect of the embryo in the BOECs related with the expression of these genes was detected among groups ($p>0.05$) (Figure 5).

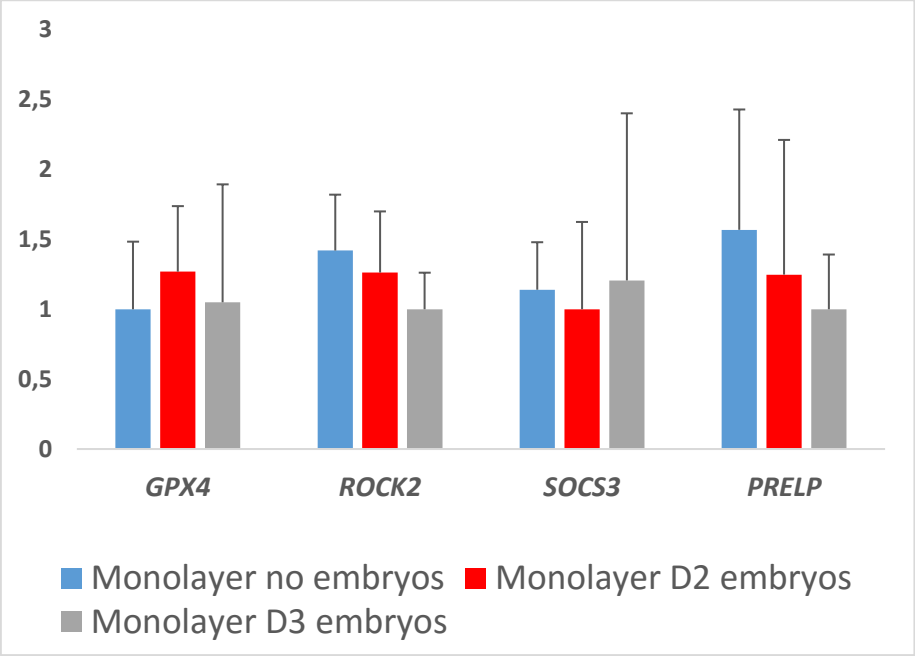

Figure 5. Relative abundance of the target transcripts in the BOEC monolayer cultured with or without early embryos

Experiment 2

We compared gene expression profiles of BOEC at day 7 of culture after 24 hours in the three groups. The genes analysed could be classified in 5 groups according to their molecular function: antioxidant activity (GPX4), binding (SMAD6, IGFBP3, NFE2L2), catalytic activity (GPX4, ROCK1, ROCK2, SOCS3), transporter activity (SCN9A) and one with unknown function (EPSTI1) (Protein Analysis Through Evolutionary Relationships, http://pantherdb.org). The

statistical analysis revealed only one DEG (Figure 6). The EPSTI1 expression was increased ($p<0.05$) from almost 2-fold in the groups of cells that were not in direct contact with the embryos (G2 and G3). The presence of early embryos did not seem to have an effect in the rest of genes expression. However, a similar pattern can be appreciate in the expression of ROCK1, ROCK2 and GPX4, all of which have a catalytic activity, and also the NFE2L2, involved in binding processes. In all of them the G2 group is slightly overexpressed and the distal control (G3), has the lowest expression.

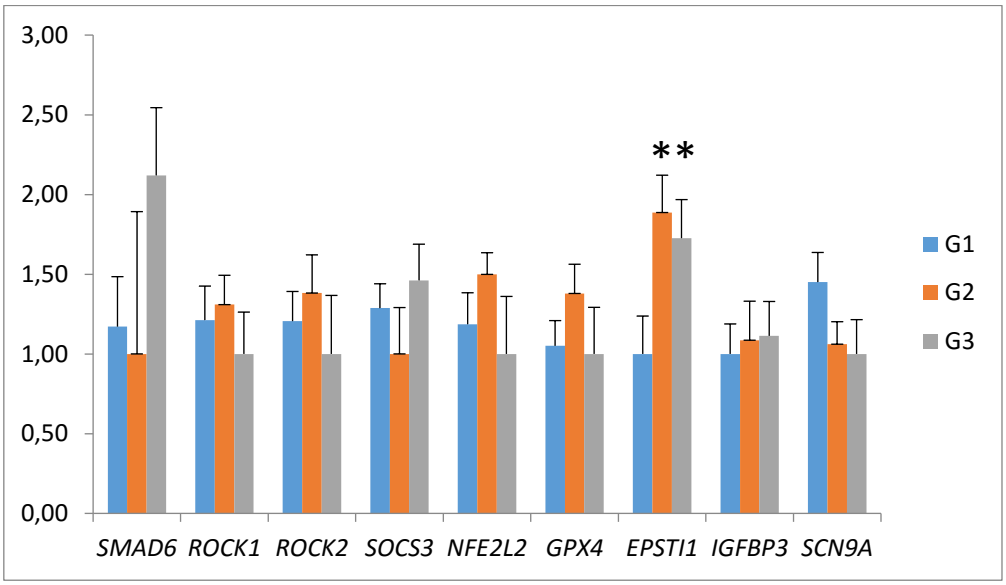

Figure 6: Relative abundance of the target transcripts in the BOEC monolayer in response to direct contact with early embryos (G1), the proximal control (G2) and the distal control (G3).

8. Discussion

The purpose of this study was to elucidate changes in the oviduct epithelial cells *in vitro* due to the presence of early embryos. The BOEC monolayers have been described as an adequate *in vitro* system to study embryo maternal interactions (Ulbrich *et al.* 2010). The positive effects of the co-culture of BOEC with embryos are largely known. Since it helped overcome the embryo blockage between the 8- to 16-cells stage (Gandolfi & Moor 1987), many researchers have focused their interest in the effects on the embryo (Eyestone & First 1989; Abe & Hoshi 1997; Mermillod *et al.* 2010; Cordova *et al.* 2014). However, there is not as much literature about the effect of the embryo on the oviduct epithelial cells *in vitro* (Lee *et al.* 2002; Tse *et al.* 2008; Schmaltz-Panneau *et al.* 2014).

The selection of the genes was made considering relevant genes for embryo development and maternal communication previously reported. Some of them were found differentially expressed (ROCK1 and ROCK2 were up-regulated, and PRELP and SOCS3 were down-regulated) between pregnant and cyclic heifers in the *in vivo* study by Maillo *et al.* (2015) after a multiple embryo transfer. Furthermore, ROCK1 and ROCK2 were described previously to be required for cohesion of inner cell mass cells and formation of segregated tissues in mouse blastocyst (Laeno *et al.* 2013; Kono *et al.* 2014). SOCS3 was found strongly upregulated during implantation, especially in embryos produced by somatic-cell nuclear transfer, suggesting a role in the immune response (Carvalho *et al.* 2014). High expression levels of NFE2L2 were previously linked to competent blastocysts cultured under oxidative stress (Amin *et al.* 2014). SMAD6 was reported to be involved in the negative regulation of the bone morphogenetic proteins signalling pathway, which play multiple roles in the regulation of growth, differentiation and apoptosis of numerous cell types and have been implicated in the regulation of ovarian follicular development (Fatehi *et al.* 2016; Hata *et al.* 1998). GPX4 has an important role in the detoxification of oxidative damage to membrane lipids (Ran *et al.* 2004), so it could exert a positive effect protecting the embryos against oxidative stress. Lapointe *et al.* (2005) observed that the highest expression of the GPX4 was in the isthmus proximal to the dominant follicle during the follicular stage and remained high during the postovulatory period. Finally, the SCN9A, EPSTI1 and IGFBP3 were found differentially expressed in the BOEC monolayer co-cultured during 8 days with 20 early embryos (Schmaltz-Panneau *et al.* 2014). However, there is no information regarding their exact function in the context of early embryonic development.

In the first experiment of the present study, even when 50 embryos were used to amplify the possible signal, no DEG were found. However, only 4 genes were assessed and the whole monolayer was recovered for analysis. We hypothesised that the embryo could exert a highly localised signal, only affecting the cells in direct contact with it. So, when analysing the whole monolayer the proportion of cells in direct contact with the embryos compared with the rest was very low, and the signal was probably silenced. With this hypothesis, the second experiment was designed using the mesh for the first time in a BOEC expression study. This allowed us to keep the embryos fixed in the same place and to be able to analyse only the cells in direct contact. One of the nine genes analysed was found differentially expressed, supporting our hypothesis.

In the study performed by Schmaltz-Panneau *et al.* (2014), 34 genes were differentially expressed. Nevertheless, at least 25 of those were induced by interferon, since they co-cultured the BOEC with the embryos until the blastocyst stage. From the other 9 genes, 8 were up-regulated (including the SCN9A, EPSTI1 and IGFBP3), and only one was down-regulated. The expression pattern of the SCN9A obtained in the present study, although there was no significance difference, seemed to be slightly overexpressed in the G1 group, which would be in concordance with the study by Schmaltz-Panneau *et al.* (2014). They hypothesised that there is a neurotrophin signalling by the embryo at the time of blastocyst formation, which will be consistent with a recent report in human were the expression of the SCN9A was up-regulated by neurotrophins (Diss *et al.* 2008). In contrast, the expression of the IGFBP3 and the EPSTI1 in the G1 group, was lower than in G2 and G3.

The EPSTI1 expression was reported for the first time in bovine endometrium in a transcriptome study by Klein *et al.* (2006) of endometrium samples from Day 18 pregnant vs. non-pregnant twin cows. In a similar study by Forde *et al.* (2011) were the aim was to determine the earliest response of the bovine uterine endometrium to the presence of the conceptus, they didn't find any difference between non-pregnant cow and pregnant cows until Day 16 post-estrus when they found 764 DEG. However, the EPSTI1 was not one of them. This gene has been linked to the endometrial remodelling before the attachment of the embryo (Klein *et al.* 2006). Therefore, the difference between the expression patterns of the two studies could be due to the time when the endometrium starts to be prepared for the implantation. In human, the most prominent expression of the EPSTI1 was found in the placenta, but it was also highly upregulated in invasive breast carcinomas compared with

normal breast tissue (Nielsen *et al.* 2002). In any case, high expression levels of EPSTI1 are shown in tissues characterized by extensive epithelial-stromal interaction which reflects an important role in tissue remodelling (Klein *et al.* 2006).

The difference between the results of the present study and the results from Schmaltz-Panneau *et al.* (2014) are possibly owing to the type of samples taken and the time of the co-culture. In the present study, the samples were divided in 3 groups as explained above, while in the second case were just divided between cells co-cultured with embryos or without embryos. Therefore, it could be possible that the presence of early embryos does not really upregulate the expression of EPSTI1 on the BOEC monolayer, as shown in the present results. The high expression of the gene in the G2 and G3 could be due to the fact that the BOEC, when cultured in a monolayer, are regularly dividing, which rarely happens *in vivo* (Gordon 2003) and probably exists an interaction with the stromal cells present in the culture. When the embryos are placed in direct contact with the monolayer, it seems to stop this tissue remodelling, which would be closer to their physiological behaviour. Another explanation could be the duration of the experiment, in the present study, the co-culture was only during 24 hours, while in the study by Schmaltz-Panneau *et al.* (2014) it lasted 8 days. However, their results contrast with the studies *in vivo* mentioned above, although those were focused on the endometrium, they were not able to detect a high expression of the EPSTI1 on Day 5, 7, 13 and 16 of pregnancy (Forde *et al.* 2011), and it was not until Day 18 (Klein *et al.* 2006) when it was upregulated.

In both cases, a primary culture of BOEC was used for the experiment. Every batch of BOEC differs from the other, as shown in the study by Diez *et al.* (1999) where they observed different levels of embryotrophic activity between batches. Therefore, there is a lack of repeatability, added to a complex methodology to obtain and isolate the cells, and a risk of contamination (Guerin *et al.* 1997). A possible solution could be the use of established cell lines, which could be cryopreserved, thus providing a continuous supply of cells (Eyestone *et al.* 1991; Ulbrich *et al.* 2010). The use of BOEC monolayer has been also questioned due to the dedifferentiation of the cells once they are attached to the well (Rottmayer *et al.* 2006), which implies the loss of important morphological features like the cell height, loss of cilia and loss of secretory granules and bulbous protrusions (Thibodeaux *et al.* 1992; Walter 1995). Many alternatives have been proposed to avoid the withdraws of the monolayer as culture the BOEC in suspension, in perfusion chambers, in polarized systems (for review see Maillo, *et al.* 2016) or performing a 3D culture (Ferraz *et al.* 2015). In general, with the use of this systems the cells conserve their physiologic morphology during more time, and in the case of the

suspension cells, the polarized systems and the 3D culture, some genes markers (such as OVGP1, estrogen and progesterone receptors) are still present (Rottmayer *et al.* 2006; Hamdi *et al.* 2015; Ferraz *et al.* 2015).

The use of the mesh implies more accurate results than analysing the whole monolayer. From 9 genes analysed, we found one DEG, related to tissue remodelling. It would be interesting in further studies, to add more genes or use an array to be able to detect more differences and identify genes involved in the embryo-maternal communication at early stages of pregnancy, when the embryo is still in the oviduct. Another possible improvement, would be to use a system that conserves the morphologic characteristics more faithfully, like the ones already mentioned. However, the challenge today is to develop a standardized and repeatable *in vitro* system to study early embryo-maternal communication.

9. Conclusions

- Under our experimental conditions:
    - A large area of BOEC monolayer is not adequate to study a possible embryo signals.
    - Our limited evidence for an embryo-induced transcriptomic response in the cells of the oviduct (BOEC) suggests a local effect of the embryo.
- The improvement of BOECs *in vitro* culture may help to elucidate this maternal embryo-communication

10. Annex

Table 1. Maturation Media Composition

TCM 199, supplemented with:	
- Foetal Calf Serum (FCS)	10% (v/v)
- Epidermal Growth Factor (EGF)	10 ng/mL
- Gentamicin	0.5% (v/v)

Table 2. Fertilization Media Composition

Tyrode's Medium, supplemented with:	
- Bicarbonate	25 mM
- Lactate	22 mM
- Na-pyruvate	1 mM
- Fatty acid-free BSA	6 mg/mL
- Heparin Sodium Salt	10 mg/mL

Table 3. Culture Media Composition

SOF, supplemented with:	
- Sodium Lactate	4.2 mM
- Sodium Pyruvate	0.73 mM
- BME amino acids	30 µL/mL
- MEM amino acids	10 µL/mL
- Phenol-red	1 µg/mL
- BSA	3 mg/mL

Table 4. List of primers.

Gene symbol	Gene name	Putative function	Primer	Sequence 5' → 3'	Amplicon length (bp)
SLC26A3	Solute carrier family 26 (anion exchanger), member 3	Protein binding and transporter activity	Fw	AATCCCAGTCAGGAAGTAGAAGC	140
			Rv	GTGATGGATGAGGATCTCTCTGAA	
MCTP1	Multiple C2 domains, transmembrane 1	Calcium ion binding and protein binding	Fw	AGGGTAGGGACAACTCCAGA	232
			Rv	GAAGAGCCAGGGCTTGGTAA	
BMP5	Bone morphogenetic protein 5	Cytokine activity, protein binding and growth factor activity	Fw	TACAAGGATAGGAGCAACGGC	181
			Rv	TCACCCAATGATTGCTGGTCA	
ROCK1	Rho associated coiled-coil containing protein kinase 1	Nucleotide binding, protein kinase activity, protein binding and ATP binding	Fw	ACGTGACCTAGTGCCTTGTG	164
			Rv	CCTCAGTGTGCTTTTGTGCC	
ROCK2	Rho associated coiled-coil containing protein kinase 2	Nucleotide binding, protein kinase activity, protein binding and ATP binding	Fw	CTTGGCTGCTCAACTGGAGA	276
			Rv	TGCTCTTGGGCTTCCTTCAG	
SOCS3	Suppressor of cytokine signalling 3	Protein kinase inhibitor activity and protein binding	Fw	GCGAGAAGATCCCTCTGGTG	167
			Rv	CTAAAGCGGGGCATCGTACT	
GPX4	Glutathione peroxidase 4	Peroxidase activity and oxidoreductase activity	Fw	TGTGGTGAAGCGGTATGGTC	266
			Rv	TATTCCCACAAGGCAGCCAG	
SMAD6	SMAD family member 6	RNA polymerase II core promoter proximal region sequence-specific DNA binding	Fw	GGAGAAATTCGCTCCAAGTGC	242
			Rv	CCCTGCCTTTAAAACCCAAGC	
SCN9A	Sodium channel, voltage gated, type IX alpha subunit	Sodium ion binding	Fw	GTTGATAACCCTGTGCCTGGA	250
			Rv	CTTCAAAAGCCAGAGCACCAC	
EPSTI1	Epithelial stromal interaction 1	Tissue remodelling	Fw	AAACGACAGCAACAGGAGGAA	89
			Rv	CCTTGGAGTCGGTCCAGAAAA	
IGFBP3	Insulin-like growth factor binding protein 3	IGF-binding	Fw	GAGTCCAAGCGTGAGACAGAA	150
			Rv	GCGGCACTGCTTTTTCTTGTA	
H2AFZ	H2A histone family, member Z	RNA polymerase II core promoter sequence-specific DNA binding	Fw	AGGACGACTAGCCATGGACGTGTG	209
			Rv	CCACCACCAGCAATTGTAGCCTTG	
ACTG1	Actin gamma 1	Nucleotide binding, structural constituent of cytoskeleton, protein and ATP binding	Fw	GAGAAGCTCTGCTACGTCG	255
			Rv	CCAGACAGCACCGTGTTGG	
NFE2L2	Nuclear factor, erythroid 2 like 2	Transcriptional activator activity and RNA polymerase II distal enhancer sequence-specific binding	Fw	GCTCAGCATGATGGACTTGGAG	390
			Rv	GGGAATGTCTCTGCCAAAAGC	
PRELP	Proline/arginine-rich end leucine-rich repeat protein	Extracellular matrix structural constituent and protein binding	Fw	CAGCATCGAGAAAATCAATGGGA	158
			Rv	AGCACATCATGAGGTCCAGC	

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