Multiple-embryo transfer for studying very early maternal–embryo interactions in cattle

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Abstract

In the present paper, we highlight the need to study very early maternal–embryo interactions and discuss how these interactions can be addressed. Bovine species normally carry one or, less frequently, two embryos to term; there are very rare cases of triplets or higher-order multiple pregnancies in which all the offspring are born alive. Multiple-embryo transfer (MET) in cattle allows for the detection of endometrial responses in scenarios where single-embryo transfer would not. Although MET is non-physiological, the present study shows that at the very early embryonic stages, a uterus carrying zona-enclosed embryos does not exhibit non-physiological reactions. On the contrary, MET should be considered the sum of multiple individual effects triggered by developing embryos. We provide arguments to support our hypothesis that describe a rationale for current work with MET, and we discuss alternative hypotheses. Using cattle as a model, we describe how technical approaches to analyzing zona-enclosed early embryo–maternal interactions (i.e., transcriptomics, proteomics, and endometrial cell culture) can help identify molecular changes that may be difficult to observe when only a single embryo is present. We conclude that MET can be used for studying very early maternal–embryo interactions in vivo in monotocous species.

Introduction

During pre-hatching development, one or several zona-enclosed embryos develop in the monotocous and polytocous ungulate uterus respectively, as well as in the uterus of most, if not all, eutherian mammals. The small size of embryos during these early stages of preimplantation development makes it difficult to identify and isolate the endometrium adjacent to the embryo (i.e., the uterine microenvironment surrounding the embryo in vivo) (Hunter 1994). This technical limitation is one of the challenges that hinders the direct analysis of zona-enclosed embryo–endometrial interactions, especially in single-ovulating species. As a result, studies on very early zona-enclosed maternal–embryo interactions in single-ovulating species have been limited to the in vitro co-culture of embryos with endometrial cells. In cattle, endometrial cell cultures maintain ultrastructural characteristics and morphological hallmarks typical of the in vivo situation (Ullbrich et al. 2011). However, because an in vivo model with zona-enclosed embryos is lacking, there is no certitude that in vitro studies reliably reflect in vivo maternal–embryo interactions.

In vivo development up until day 7 is not necessary for pregnancy, but the oviduct and uterus confer improved traits to embryos, which makes them notably different from their in vitro counterparts (reviewed by Besenfelder et al. (2012)). Deprivation of oviductal and/or uterine factors leads to embryos with altered gene expression (Gad et al. 2012) and chromosomal abnormality (Viuff et al. 1999). Hence, embryos that develop in vivo are superior in quality to in vitro-produced embryos; they also survive cryopreservation much better and are less prone to developmental abnormalities (Rizos et al. 2002, Farin et al. 2006, 2010, Havlicek et al. 2010). Learning more about the oviductal and uterine environment is important, because this knowledge can help researchers improve in vitro embryo culture conditions. However, the information that has been obtained from the uterine medium and the endometrium up until now has scarcely had any real impact on the formulation of culture media, especially during the very early stages of embryo development. Thus, endometrial studies between cyclic and pregnant individuals during the elongation phase (i.e., after day 13) have provided valuable data in terms of molecules that can be added to in vitro culture media. However, because the uterine transcriptome does not differ between single-pregnant and cyclic cows, it could be that signals in the uterus are not detectable when a single embryo is present or that early embryos do not elicit such signals (Forde et al. 2011,
Bauersachs et al., 2012, Mansouri-Attia et al., 2012, Spencer et al., 2013). To our knowledge, there is no evidence demonstrating that the development of an individual zona-enclosed embryo is compromised by the simultaneous presence of other zona-enclosed embryos in the uterus. Therefore, exposure of the uterus to multiple embryos may represent a good strategy for increasing uterine signals up to detectable levels. Aside from polycylicous species, where the carriage of multiple embryos and pregnancies occurs in a physiological way, the superovulated cow may be a good example of the simultaneous development of multiple zona-enclosed embryos, in spite of the negative impact that superovulation exerts on the embryos and the uterine environment (Gad et al., 2011). Therefore, in the present study, the detection of local maternal–embryo interaction in the non-superovulated uterus was indirectly tested with an experimental model that included many embryos developing in the monotocous uterus of cows. Certainly, in the bovine species model, assisted reproductive technology, especially IVF and embryo development, performs better with regard to other domestic species. In addition, in a number of ways, cattle represent a better model for humans (Baumann et al., 2007) than does the usual mouse model (Vajta et al., 2010). Cows and women are normally monovulators; cattle and human blastocysts are about the same size (~140 µm in diameter), and their energy metabolisms, which are measured as oxygen and pyruvate, as well as their glucose consumption and lactate production, are quite similar (Thompson et al., 1996). Bovine embryos develop in vitro to the blastocyst stage at the same rate as human embryos do (20–40 and 20–50% respectively), and the major genome activation takes place at similar stages in both cases (4- to 8-cells in humans; 8- to 16-cells in cows).

The uterine fluid composition is influenced by the interactions between embryos, the endometrium, and immune cells in utero, and it can affect conceptus and endometrial growth by increasing the concentration of cytokines (Leung et al., 2000, Oliveira & Hansen, 2008, Ideta et al., 2010, Muñoz et al., 2012). The uterine fluid contains not only live cells (immune, detached endometrial) but also secreted products and cell debris, such as epithelial cells that are in constant renewal and extrusion into the lumen (Welsh, 1993, Fahey et al., 2006). Because early embryos normally live in this environment, analyzing uterine fluid samples recovered from live animals with multiple embryos developing in the uterus increases the likelihood of detecting embryo-induced changes (Muñoz et al., 2012, Gómez et al., 2013, Trigal et al., 2014). We propose that uterine fluid recovery would be a better choice for analyzing endometrial changes induced by zona-enclosed embryos than a biopsy method would, seeing as biopsies do not allow for an accurate sampling of the areas surrounding a live embryo. Using a multiple-embryo transfer (MET) model, we found several differentially expressed proteins (DEP) in the uterine fluid on day 8 (Muñoz et al., 2012, Gómez et al., 2013). Some of these proteins in the uterine fluid are submitted to the different bilateral regulation that exists between horns (Trigal et al., 2014). We showed evidence that the metabolic and functional uterine fluid and blood plasma profiles obtained with MET are consistent with those observed when a single embryo develops (Muñoz et al., 2012, Gomez et al., 2015).

In the present work, we review the MET model as an efficient system for studying maternal–embryo interactions. Knowledge regarding in vitro bovine embryo–uterine interactions from day 5 to day 8 is essential if we wish to mimic the actual period during which embryos can develop in vitro before we transfer those embryos to recipients.

**Response models in the uterine fluid and endometrium**

In order to determine if MET triggers non-physiological reactions in the uterus, our previous studies analyzed specific protein profiles and hexoses in uterine fluid collected from MET, artificial insemination (AI), and sham-transferred animals (Muñoz et al., 2012, Gomez et al., 2015). In addition, we examined the prostaglandin F2α and E2 contents in the blood of these animals and the embryotrophic properties of the uterine fluid proteins in an in vitro embryo culture system. The observed changes fit in with the following response models (RM):

- **RM type 1:** endometrial response to embryos: (ET = AI) ≠ Sham.
- **RM type 2:** endometrial response not detectable with a single embryo: ET ≠ (AI = Sham).
- **RM type 3:** lack of endometrial response: ET = AI = Sham.
- **RM type 4:** continuous endometrial response variation resulting from the number of embryos, with significant differences as follows: ET > AI > Sham or Sham > AI > ET.

Responses that did not fit these criteria were considered non-validated (i.e., non-physiological; for example, AI > ET > Sham).

A summary of the analysis carried out for the purpose of validating MET as a means for studying early maternal–embryo interactions is shown in Table 1. Briefly, we were able to demonstrate that BSA, purine nucleoside phosphorylase (PNP), and heat shock 70 kDa protein 5 (HSPA5) showed a type 1, type 3, and type 3 validation profile respectively (Muñoz et al., 2012). The cow endometrium predominantly transcribes PNP (Forde et al., 2014). Unaltered PNP and HSP70 levels indicated no changes in purine metabolism and no stress resulting from cytotoxic damage. Other proteins, such as NFKB, clusterin, I-20S, and β-actin were recently validated.
Table 1  Summary of analysis performed in uterine fluid, endometrial cells, and blood for validation purposes in the presence of multiple embryos in the uterus vs artificial insemination or sham transfer.

<table>
<thead>
<tr>
<th>Element</th>
<th>Localization</th>
<th>Response</th>
<th>Validation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein expression</td>
<td></td>
<td></td>
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<tr>
<td>BSA</td>
<td>Uterine fluid</td>
<td>ET &gt; (AI = Sham)</td>
<td>Type 1</td>
<td>Muñoz et al. (2012)</td>
</tr>
<tr>
<td>Purine nucleoside phosphorlase (PNP)</td>
<td>Uterine fluid</td>
<td>ET = AI = Sham</td>
<td>Type 3</td>
<td>Muñoz et al. (2012)</td>
</tr>
<tr>
<td>Heat shock 70 kDa protein S (HSPAS)</td>
<td>Uterine fluid</td>
<td>ET = AI = Sham</td>
<td>Type 3</td>
<td>Muñoz et al. (2012)</td>
</tr>
<tr>
<td>Actin</td>
<td>Uterine fluid</td>
<td>ET = AI = Sham</td>
<td>Type 3</td>
<td>Gomez et al. (2015)</td>
</tr>
<tr>
<td>Clusterin</td>
<td>Uterine fluid</td>
<td>ET = AI = Sham</td>
<td>Type 3</td>
<td>Gomez et al. (2015)</td>
</tr>
<tr>
<td>i2O-S</td>
<td>Uterine fluid</td>
<td>ET = AI = Sham</td>
<td>Type 3</td>
<td>Gomez et al. (2015)</td>
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<tr>
<td>NFkB</td>
<td>Uterine fluid</td>
<td>ET = AI = Sham</td>
<td>Type 3</td>
<td>Gomez et al. (2015)</td>
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<tr>
<td>Total protein</td>
<td>Uterine fluid</td>
<td>ET &gt; AI &gt; Sham</td>
<td>Type 4</td>
<td>Muñoz et al. (2012)</td>
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<tr>
<td>Gene expression</td>
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<tr>
<td>TNF</td>
<td>Endometrial</td>
<td>ET = Sham</td>
<td>AI = cyclic</td>
<td>Mansouri-Attia et al. (2012) and Correa-Álvarez et al. (2015b)</td>
</tr>
<tr>
<td>IL1B</td>
<td>Endometrial</td>
<td>ET = Sham</td>
<td>Interleukin AI = cyclic</td>
<td>Oliveiraira et al. (2013) and Correa-Álvarez et al. (2015a)</td>
</tr>
<tr>
<td>Cells</td>
<td></td>
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<tr>
<td>CD45+ leukocytes</td>
<td>Endometrial (ICQ)</td>
<td>ET = Sham</td>
<td>AI = cyclic</td>
<td>Correa-Álvarez et al. (2015b) (ET vs sham)</td>
</tr>
<tr>
<td>(caruncular)</td>
<td></td>
<td></td>
<td></td>
<td>Groebner et al. (2011) (AI vs cyclic)</td>
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<tr>
<td>Functional studies</td>
<td></td>
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<td></td>
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<tr>
<td>Embryotrophic</td>
<td>Uterine fluid</td>
<td>ET* &gt; Sham</td>
<td>Shown; other species</td>
<td>Emond et al. (2004), Almiñana et al. (2012) and Chen et al. (2013)</td>
</tr>
<tr>
<td>HDGF</td>
<td>Uterine fluid</td>
<td>ET &gt; Sham</td>
<td>Improved in vitro embryo development</td>
<td>Gómez et al. (2014)</td>
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<tr>
<td>Other molecules</td>
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<tr>
<td>Glucose</td>
<td>Uterine fluid</td>
<td>ET = AI = Sham</td>
<td>Type 3</td>
<td>Gomez et al. (2015)</td>
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<tr>
<td>Fructose</td>
<td>Uterine fluid</td>
<td>ET = AI = Sham</td>
<td>Type 3</td>
<td>Gomez et al. (2015)</td>
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<tr>
<td>Prostaglandin F2α</td>
<td>Blood plasma (ET = AI) &gt; Sham</td>
<td>Type 1</td>
<td>Gomez et al. (2015)</td>
<td></td>
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<tr>
<td>Prostaglandin E2</td>
<td>Blood plasma</td>
<td>ET = AI = Sham</td>
<td>Type 3</td>
<td>Gomez et al. (2015)</td>
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</table>

*A dialysate of ET uterine fluid added to SOF medium improved in vitro blastocyst development and expansion in culture.

Successfully validated in the uterine fluid and showed no changes in the presence of one or more embryos; they therefore corresponded to a type 3 validation. The nuclear transcription factor NFkB is central in inflammatory response regulation. The protease clusterin has been shown to exert a role in hexose metabolism, to protect against oxidative activity, and to indicate immunoproteasome activity is also linked to interferon-γ (Bose et al. 2011), a close analog to the ruminant interferon-α, which suggests that embryonic recognition at these early stages does not involve interferons. In contrast, the recognition of embryonic sex did involve antigen processing and differential activation (Gómez et al. 2013). In one of our more recent studies, we analyzed IL1B (Correa-Álvarez et al. 2015a) and TNF (Correa-Álvarez et al. 2015b) protein and mRNA expression in the bovine uterus and embryos on day 8. Whereas the presence of multiple embryos decreased TNF and increased IL1B protein levels, TNF and IL1B gene expression did not change, which is consistent with data found on day 7 in single-embryo vs cyclic animals (Mansouri-Attia et al. 2012, Oliveira et al. 2013). In addition, we found that the presence of embryos in the uterus reduces the number of endometrial CD45 positive leukocytes, specifically in caruncular areas; this concurs with previous work carried out by Groebner et al. (2011), although their study involved a more advanced development stage and the presence of a single embryo in the uterus. We have also demonstrated that total uterine fluid protein decreases with the number of live embryos recovered, which fits in with a type 4 validation (Muñoz et al. 2012). Figure 1 shows how the endometrial effects of single and multiple embryos can be measured by analyzing uterine fluid.

Decreased uterine fluid protein represents reduced luminal uterine fluid, and it is associated with improved in vitro embryonic development. This type of improvement has been observed when proteins from uterine fluids that contain viable embryos are added to embryo culture medium in contrast with sham uterine fluid from the same cows (Muñoz et al. 2012). Substances that are capable of increasing uterine secretory activity negatively affect embryo development (Jordan et al. 1983). In addition, in non-ruminant species, the physiological reabsorption of uterine fluid facilitates luminal closure before implantation, thereby ensuring that the embryos are maintained in the correct locations for implantation (Eytan et al. 2001, 2004, Yaniv et al. 2003). Therefore, a reduction in secreted uterine fluid volume would operate by holding the floating embryo near the uterine wall during endometrial transport, which is a prerequisite for successful, finely tuned maternal–embryo interactions (Chen et al. 2013). Interestingly, the intensity of the
Figure 1. Outline hypothesis of embryo development and changes in signaling and secreted uterine fluid. Uterine fluid volume (light blue layer) is higher in the cyclic uterus (A; sham transfer), but it can be locally depressed in the physiologically pregnant uterus in specific areas surrounding the embryo, and the changes might not affect a large enough surface to detect maternal–embryo exchanges as measured by specific molecules (blue arrows). B: one embryo develops. In the presence of multiple viable embryos, embryonic signals lead to a stronger reduction in uterine fluid volume, as shown by the significantly reduced total protein contents being closely related to the number of viable embryos collected from the uterus (Munóz et al. 2012); at the same time, the increased number of embryos may lead to a higher amount of exchanges (blue arrows) whose molecular features can be measured because of a lower signal-to-noise ratio. The reduction in fluid volume may operate to keep the embryo closer to the uterine wall, which facilitates molecular exchanges and then embryo–maternal interactions. Obvious changes may not be detectable in all of the molecules analyzed, because some changes are dependent on the presence of one embryo and not on the number of the embryos.

The blastocyst formation that was shown in vitro by three samples recovered from three ET females (Munóz et al. 2012) was consistent with the number of live embryos recovered in each uterine fluid sample, but it significantly differed from uterine fluid samples recovered from sham-transferred animals. This means that developing embryos are able to counteract the native proinflammatory conditions in the uterus (Munóz et al. 2012). The idea that the embryo modifies its environment to facilitate its own development has been suggested to also occur in other species (Emond et al. 2004, Almiñana et al. 2012, Chen et al. 2013).

Hepatoma-derived growth factor (HDGF), which had not been identified in the uterus, was recently detected in bovine uterine fluid using the reported MET model (Munóz et al. 2012, Gómez et al. 2014). In the present study, in vitro production (IVP) embryos were cultured with recombinant HDGF, and promoting effects on embryo development were observed. Such effects were highly stage-specific, because the development of day 6 morulae, unlike day 5 morulae, was promoted through blastulation (Gómez et al. 2014). In terms of uterine synchrony, this finding is quite relevant because of the fact that the precocious release of growth factors by the uterus and the progesterone-stimulated transport of serum-borne growth factors into the uterine lumen may act in a non-specific manner on embryo growth and may affect development up until the end of gestation (Barnes 2000). Therefore, stage-specific studies are needed in order to understand maternal–embryo interactions and to be able to apply this knowledge to the improvement of in vitro embryo culture technology. Because one single zona-enclosed embryo in the uterus does not permit the reliable identification of molecules, MET represents an obvious advantage, as shown by the example of HDGF.

**Uterine capacity: when do functional differences between monotocous and polytocous species arise?**

In cows, the superovulated uterus is considered to be an adverse environment for embryo development as compared to the non-superovulated uterus (Gad et al. 2011). Embryos that develop in a superovulated environment exhibit up-regulation of genes involved in metabolic processes, protein synthesis, and energy production as compared to embryos that develop in a non-stimulated uterus (Gad et al. 2011), as is the case in our procedure with MET. Despite their altered gene expression, embryos developed in superovulated cows are normally used as a ‘gold standard’ in experiments with species in which the supply of embryos is restricted because single-embryo development, such as that in cows, is the norm.

In order to determine whether the number of embryos present in the uterus affects their viability, we examined an independent dataset with the flushing output of superovulated-inseminated cows and heifers generously provided and compiled by Dr Claire Ponsart (UNCEIA, France). The variation of viable embryo production was analyzed with regard to total embryo recovery.

Zona-enclosed embryos recovered from the superovulated cow uterus are able to develop normally upon transfer to cyclically synchronized recipients. Among 23 097 French superovulated cows and heifers from 13 breeds in UNCEIA, ~3% of the flushings produced ≥ 20 viable embryos, including top records of 71 total and 41 viable embryos (C Ponsart, UNCEIA, personal communication). The rate of viable embryos significantly (P < 0.0001) correlated with the total number of embryos recovered (r = +0.74), which indicated that the uterine capacity was not compromised by the number of embryos present on day 7 ± 0.5. This positive correlation was unaffected by parity (i.e., it was similar between cows and heifers; data not shown). In the present meta-analysis, we observed that the number of total embryos in the uterus did not compromise the number of embryos judged as viable, which indicates that the uterine capacity at very early stages is not affected when dozens of embryos develop. Importantly, restrictions to
multiple-embryo development arise from the capacity of hormonally stimulated ovaries to release a high number of fertilizable oocytes and not from the uterine capacity to support embryonic development at early stages, as proven by the normal pregnancy rates obtained when such embryos are transferred. In addition, there is some evidence in twinner females of superior maternal uterine environments for fetal development associated with a quantitative trait locus (QTL) that is linked to twinning rate and is separate from the ovulation rate (Echternkamp et al. 2007). In fact, in ruminants and particularly in cattle, the capacity of the uterus and placenta to provide support for fetuses generally appears to exceed the usual number of fetuses present (Echternkamp 1992, Echternkamp et al. 2007). In contrast, the capacity of the uterus and placenta to maintain fetuses is often exceeded in litter-bearing species, such as pigs, and this leads to embryonic losses (Vonnahme et al. 2002, Town et al. 2005) and late (days 30–40) conceptus losses.

At the uterine level, evidence provided at zona-enclosed embryonic stages suggests the existence of changes that can be included within the framework of cell-to-cell interactions. Such changes are governed by the same rules as those for either one or many embryos in the uterus in spite of potential species-specific mechanistic differences. Thus, maternal–embryo interactions seem to exist at an individual level (i.e., each embryo has its own environment in its endometrial surroundings). Therefore, the relationship between a zona-enclosed embryo and the uterus can be circumscribed to an area surrounding the embryo (referred to as the ‘micro-environment’ in the reference literature). Such paracrine interactions are difficult to analyze directly in single-ovulating species because of the small size of the embryos at early stages, which impedes the identification and isolation of the endometrium adjacent to the embryo. However, the endometrium adjacent to zona-enclosed embryos has not been analyzed in multiple-ovulating species either. Therefore, at these very early zona-enclosed stages, the uterus can sustain multiple embryos independent of its monotocous or polytocous nature, and as we will discuss later in the present report, it is not until later in the development process that the embryos significantly relate to each other and competition starts to restrict the number of developing embryos within the typical range of each species and individual.

**Conceptus migration as the first indicator of embryonic overload in the uterus**

Regardless of the species, the number of offspring at birth depends on the number of oocytes ovulated, fertilization rate, embryonic viability, and uterine capacity (Bennett & Leymaster 1989). In contrast, differences in uterine length do not necessarily translate into differences in the functional capacity of the uterus. In an excellent recent study, Vallet et al. (2013) suggested that in sheep and cattle, the uterus is usually capable of supporting more than a single fetus, although the two species differ in the consequences of multiple births. Thus, when two conceptuses are present, the placentas of cattle often anastomose, which results in a shared blood supply between the placentas (Plante et al. 1992); therefore, if one fetus is lost for any reason, the other fetus is put at risk (Echternkamp et al. 2007). In sheep, it is likely that such losses do not take place, because sheep embryos, like pig embryos, undergo intrauterine migration. On the contrary, in cattle, conceptus migration is a rare event that appears to be influenced by the number of conceptuses present. In cows, consistent migration has been observed when many conceptuses were introduced into the uterine horns using embryo transfer; however, such migration has not been observed before day 12 (McMillan & Peterson 1999, Berg et al. 2010, Spencer et al. 2013). The lack of migration in cattle could be associated with reduced estrogen secretion by bovine conceptuses, which synthesize and release estrogen at much lower levels than the conceptuses of pigs do (Gadsby et al. 1980, Wilson et al. 1992), because pig embryos migrate physiologically. Thus, migration may require multiple conceptuses in order to secrete enough estrogen to stimulate uterine motility, which is not physiological in cattle. In ungulates, their characteristically delayed implantation suggests that blastocyst competition is also delayed as comparison to species that lack the elongation period and direct implantation of the hatched blastocysts.

To our knowledge, migration can be considered a first sign of uterine overloading in cattle; therefore, in the case of MET on day 7 and multiple-embryo recovery at elongation, migration is a non-physiological event. Certainly, the evident uterine crowding that has been shown to occur on days 12 and 13 after day 7 MET (Spencer et al. 2013) contrasts with MET and day 8 embryo recovery in the present work, where non-physiological effects were not detected. Embryonic migration is truly difficult to achieve by or before day 8, because of the fact that the quantity of estrogen released by the tiny blastocysts (150–200 μM) is very small in comparison with embryos that are more developed and larger in size. Because elongation is what gives increased volume and size to early embryos, embryonic migration results from reduced uterine space, which in turn limits embryo access to uterine resources. For this reason, it would be advisable to recover MET embryos prior to elongation and migration periods.

**Dissecting why early embryos favor translational changes vs transcriptomic changes in the endometrium**

The presence of early embryos in the uterus does not induce detectable changes in endometrial gene
expression (Forde et al. 2011, Bauersachs et al. 2012, Mansouri-Attia et al. 2012, Spencer et al. 2013). Yet, failure to detect changes in endometrial mRNA abundance may be an artifact because samples for RT-PCR usually contain tissues from multiple regions, which would mask local differences in mRNA levels. However, it is worth considering whether or not embryos are able to take advantage of activating endometrial gene expression at the time of their uterine passage.

A plausible answer is that it appears to be unlikely that embryos activate endometrial transcription as a way of changing their protein or metabolite environments. In the uterus, embryo displacement is not linear through the uterine horn axis; rather, it moves in loops (Chen et al. 2013). This means that at the time of its daily journey down the uterine horn, a cattle zona-enclosed embryo would move a matter of centimeters. The very small size of the embryo (~150 μm) and the high density in uterine fluid stabilized by glycoproteins, which avoids the dispersal of compounds (Hunter 1994), may result in a more reduced area for paracrine embryo-endometrial exchanges in real time. Under these conditions, the embryo-influencing area is probably very limited, perhaps measuring no more than a few millimeters. The signaling circuit that is triggered by an embryo upon endometrial gene expression until changes occur in proteins or in transcribed microRNA (miRNA) or until metabolites become exposed to the lumen or are secreted into the uterine fluid could take more than 1 day. Although such effects have not been reliably measured in vivo, protein responses to stimuli that imply mRNA expression within in vitro endometrial cells can take days to occur (Horn et al. 1998, Xiao & Goff 1999, Ulbrich et al. 2009). Therefore, at the end of this period, an embryo would be far (cm) from the endometrial microenvironment (perhaps mm in size) whose gene expression had been stimulated, and the embryo would therefore not be dependent on the endometrial responses induced at the transcriptional level. We propose that embryonic-induced transcription is not essential for supporting very early embryo development, and the molecular influence on zona-enclosed embryos, which is exerted by the uterus, therefore has to be managed in a more dynamic manner. We postulate that the embryo would not benefit from changes induced at the gene expression level(s), and there are therefore no obvious reasons for locally relevant gene expression changes at very early maternal–embryo interactions.

**Exploring faster endometrial responses to the embryo**

Responses through post-transcriptional and post-translational (PTS) regulatory mechanisms are faster than those that depend on de novo mRNA synthesis are, which makes PTS changes better suited for supporting rapid and dynamic dialogue between the embryo and the mother. Fast responses may also involve miRNA release from the uterus, seeing as miRNA secretion is regulated through different, but coordinated, pathways (reviewed by Mittelbrunn & Sánchez-Madrid (2012)). In humans, the uterine fluid contains miRNA exosomes, which are also released by endometrial cells cultured in vitro (Ng et al. 2013). The ovine uterine fluid contains exosomes that show differential mRNA expression between pregnant and cyclic animals (Burns et al. 2014); such differences in uterine fluid are not obvious in endometrial cells. Nevertheless, in cows, the endometrium contains miRNAs that are associated with receptivity (Ponsaksili et al. 2014), and the bovine blastocysts also release miRNAs (Kropp et al. 2014). Therefore, a number of endometrial mechanisms are potentially able to provide fast, non-transcriptional, endometrial responses to early developing embryos.

In our proteomic experiments, we found that in uterine fluid, the expression of HDGF, TNF, and IL1B was regulated by the presence of zona-enclosed embryos (Muñoz et al. 2012). Within endometrial proteins, significant changes induced by embryos were observed by western blotting in specific isoforms and within ICQ localization for TNF and IL1B although not for HDGF. In contrast, endometrial mRNA levels for HDGF (Gómez et al. 2014), TNF and TNFRSF1B (Correia-Alvarez et al. 2015b), and IL1B and IL1R1 (Correia-Alvarez et al. 2015a) did not change. Although we used MET and sampling on several endometrial areas to increase the likelihood of obtaining a response, caution is needed because local and/or tissue-specific mRNA changes could be diluted in the whole endometrial analysis. In contrast, in the embryos, down-regulation transcription for HDGF, TNF, and TNFRSF1B was detected by RT-PCR during uterine passage, which may indicate a reaction to cognate proteins in the uterine fluid.

The present data is consistent with reports that indicate that only some embryo-induced changes in endometrial mRNA expression in cows give rise to detectable changes in protein levels (Forde et al. 2013). Conversely, at the time of pregnancy recognition, the conceptus induces changes in the levels of several uterine proteins involved in uterine remodeling, such as legumain, TIMP-2, HGF, and interleukin-1α (Hirata et al. 2003, Ledgard et al. 2009, 2011), without changing the levels of the corresponding mRNAs.

Dige analysis of proteins in uterine fluid at early stages revealed the presence of isoforms with different sizes and/or charges, which is consistent with PTS changes in protein glycosylation or phosphorylation (Muñoz et al. 2012, Gómez et al. 2013). Indeed, such differences have been consistently reported in human uterine fluid (reviewed by Salamonsen et al. (2013)), which suggests that PTS regulation is an important feature of endometrial remodeling that is then used to provide fast embryonic responses. The relationship between the expression of mRNA and protein varies among genes, and the correlation becomes weaker in
complicated biological processes, such as a continuous remodeling endometrium. Each gene is capable of producing heterogeneous protein molecules that can be further modified after translation (Katz-Jaffe & Gardner 2007). In fact, on days 12–13, although protein levels in uterine fluid or endometrial tissue may vary, embryo-induced changes in endometrial mRNA are scarce or nonexistent when only a single embryo is present (Forde et al. 2011, Groebner et al. 2011).

Conclusion
The present article reviews various independent datasets that support the normality of embryo-triggered responses that are dependent on METs (i.e., total protein, embryotrophic effects, and three validated proteins). Another recent study that we carried out deals with the validation of more proteins (n = 4), hexoses, and prostaglandins. At very early embryo stages, there is no equal, better, or contradictory in vivo model available for studying maternal–embryo interactions in live cattle. In addition, the MET model could be used with other monotocous and/or twinner domestic species, such as equine and large and small ruminants. We believe that uterine fluid recovery would be a better choice for analyzing endometrial changes induced by embryos than a biopsy method would. Biopsies would not allow an accurate sampling of the areas surrounding a live embryo, and even if conducting one was possible, there would be no way of verifying it. In addition, gene expression approaches at very early development stages in the uterus need to be further analyzed in order to determine if they can actually provide benefits to embryo development.

The evidence we have provided in the present review supports the hypothesis of a local microenvironment that surrounds the embryo in the uterus. These findings may contribute useful information that can lead to the improvement of in vitro embryo development technology not only in cattle but also in other mammalian domestic species and in humans, where studies are unfeasible because of ethical and technical challenges that require modeling in another species (Macklon & Brosens 2014).

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

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