Title

Viable and morphologically normal boar spermatozoa alter the expression of Heat Shock Protein genes in oviductal epithelial cells during co-culture *in vitro*

Running head

Spermatozoa and HSPs in oviductal cells

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Abbreviations: ANOVA, analysis of variance; HSPs, Heat Shock Proteins; OECs, oviductal epithelial cells; s.e.m, standard error of the mean; CLU, clusterin; HSP90AA1, heat shock protein 90 kDa alpha A1; HSPA5, glucose regulated protein 78 kDa, HSPA8, heat shock cognate protein related 70 kDa,; APM, apical plasma membranes; ACTB, β-actin; RT, Reverse Transcription; qPCR, Quantitative real-time PCR

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Abstract

The principal aim of this study was to determine whether boar spermatozoa influence the expression of four selected chaperone and Heat Shock Proteins (HSPs) genes, namely clusterin (*CLU*), *HSP90AA1*, *HSPA5*, and *HSPA8* in oviductal epithelial cells (OEC) during co-culture *in vitro*. All corresponding proteins of these genes were previously identified in a sperm-interacting 70 kDa soluble fraction derived from apical plasma membranes of OEC. The present study also sought to determine whether: a) spermatozoa must bind to OEC directly for an effect on gene expression to be elicited; b) reproductive and non-reproductive epithelial cell (LLC-PK1, pig kidney) types respond equivalently, in terms of alterations in chaperone and HSP gene expression, during sperm-OEC co-culture. Spermatozoa induced a significant upregulation ($P<0.05$) in *HSP90AA1* and *HSPA5* in OEC after 3 h, and in *HSPA8* after 6 h of co-culture when they were in direct contact with epithelial cells. Conversely, no upregulation of HSPs was observed when spermatozoa did not directly bind to OEC. Spermatozoa also induced a significant upregulation ($P<0.05$) of the same three genes when in direct contact with LLC-PK1 cells but later than OEC. Interestingly, the extent of HSPs upregulation induced by direct contact of spermatozoa with epithelial cells relied on sperm binding index, and bound sperm population was mainly viable and morphologically normal. In conclusion, the upregulation of HSP genes caused by direct contact between spermatozoa and OEC rather than non-reproductive epithelial cells, suggests HSPs could play an integral role in the modulation of sperm function in the oviductal reservoir.

1. Introduction

Oviductal epithelial cells (OEC) are involved in the transport of spermatozoa to the ampulla, the region of the oviduct where fertilisation occurs (for review: see Hunter, 2005; Rodríguez-Martínez et al., 2005; Yeste, 2013). Various reports have also demonstrated that OEC have an effect on sperm viability, capacitation and motility in boars (Fazeli et al., 1999; Yeste et al., 2009), in humans (Ellington et al., 1998; Yao et al., 1999) and in other mammalian species (Cortés et al., 2004; Lloyd et al., 2008). Spermatozoa have been shown to bind to OEC (Petrunkina et al., 2001), particularly within the isthmus segment of the oviduct where a sperm reservoir is formed during the oestrous period (Suárez et al., 1991; Töpfer-Petersen et al., 2002). It is worth noting that not all spermatozoa have the same ability to attach to OEC. Selective binding to OEC has been reported for acrosome-intact (Gualtieri and Talevi, 2000), uncapacitated (Fazeli et al., 1999), morphologically normal spermatozoa (Green et al., 2001; Yeste et al., 2012), and spermatozoa without chromatin fragmentation (Ardón et al., 2008), in preference to capacitated spermatozoa, sperm with coiled tails or proximal droplets, or spermatozoa with damage to their chromatin structure. In addition to this, the effect of the products secreted by OEC has been shown to affect sperm function in some mammalian species (McCauley et al., 2003; Quintero et al., 2005; Zhang et al., 2006).

Co-incubation with apical plasma membranes (APM) isolated from OEC has also been demonstrated to modulate sperm function in several mammalian species (e.g. rabbits, pigs, horses and cattle; see Holt et al. (2006) for references). In addition to OEC and OEC-derived proteins exerting an effect on spermatozoa, the spermatozoa in turn have been reported to influence the gene and protein expression of OEC. Indeed, studies *in vitro* in equine (Ellington et al., 1993) and bovine (Thomas et al., 1995) showed that co-culturing with spermatozoa altered the *de novo* protein synthesis of OEC both

quantitatively and qualitatively. Studies *in vivo* and *in vitro* also showed that the presence of spermatozoa in oviducts influenced the gene expression of OEC (Fazeli et al., 2004; Georgiou et al., 2007) and the composition of the proteins secreted by the same cells (Georgiou et al., 2005; Georgiou et al., 2007).

Previous studies have shown that a soluble apical plasma membrane (sAPM) fraction from OEC maintains boar (Fazeli et al., 2003) and ram (Lloyd et al. 2009) sperm viability and suppresses the motility of bicarbonate-sensitive sperm subpopulations within boar ejaculates (Satake et al. 2006). A subfraction of these sAPM proteins that bind to boar spermatozoa was identified (Holt et al., 2005; Elliott et al., 2009), and several of the proteins identified were chaperones and heat shock proteins (HSPs), prompting the suggestion that they play a role in the modulation of sperm function (Elliott et al. 2009).

Against this background, the present study sought to determine whether boar spermatozoa actively influence the gene expression of some chaperones/HSPs in OEC during co-culture *in vitro*. Specifically, we evaluated the expression of four selected genes in OEC following 3 h, 6 h, 9h, 12 h and 24 h of co-culture with boar spermatozoa. These four chaperone/HSPs genes (clusterin, *CLU*; heat shock protein 90 kDa, *HSP90AA1*; glucose regulated protein 78 kDa, *HSPA5*; and heat shock cognate protein 70 kDa, *HSPA8*) were selected because their corresponding proteins had been previously identified in porcine-sAPM (Elliot et al., 2009).

In addition, we also tested the hypothesis that boar spermatozoa would influence the chaperone/HSP gene expression more effectively in cells of reproductive rather than non-reproductive origin (kidney; LLC-PK1), since the former influence more sperm cell parameters, like viability, than the latter (Fazeli et al., 1999; Yeste et al., 2009). This would be expected if the sperm-oviduct interaction is of special functional significance.

On the other hand, the present study also assessed whether the reproductive cycle phase of the sow would influence the expression of the selected genes in OEC during *in vitro* co-culture.

Finally, to explore the importance of indirect and direct contact between the spermatozoa and epithelial cells (OEC and LLC-PK1): a) the co-culture experiments were conducted both with and without diffusible membrane inserts, respectively, and b) the viability and morphology of sperm bound and unbound populations were determined together with the relationship between gene expression and sperm binding indexes in those co-cultures that did not contain inserts.

2. Results

2.1. Comparison of CLU, HSP90AA1, HSPA5, and HSPA8 expression in OEC follicular, OEC luteal and LLC-PK1 monolayers

Figure 1 shows relative transcript abundances of the four genes analysed in follicular and luteal OEC and in LLC-PK1 cells in culture. LLC-PK1 cells presented significant ($P<0.05$) higher levels of *HSP90AA1* and *HSPA5* transcript abundances compared with both follicular and luteal OEC. Conversely, no significant differences in *CLU* and *HSPA8* were observed among the epithelial cell cultures.

No significant differences ($P>0.05$) between follicular and luteal OEC were observed when the expression of *CLU*, *HSP90AA1*, *HSPA5* and *HSPA8* was evaluated after culture (Figure 1). In addition, follicular and luteal OEC did neither differ in their response to sperm when co-cultures were evaluated at any of the relevant time points. For this reason, data from the two types of OEC co-cultures were combined for presentation in this section.

No significant differences ($P>0.05$) in gene expression were observed in the negative controls (consisted of adding spermatozoa to epithelial cells just before expression analyses) between the time points investigated (0, 3, 6, 9, 12 and 24 hours). This suggested that the changes in gene expression observed in OEC and LLC-PK1 (described below) were due to the presence of spermatozoa rather than to an artefact of removing and replenishing the cell culture media.

2.2. CLU expression during sperm co-culture with epithelial cells

No significant differences ($P>0.05$) in *CLU* gene expression (Figure 2) were observed between incubation times in either the sperm-OEC or the sperm-LLC-PK1 co-cultures (both with and without diffusible membrane inserts). Significant differences between boar ejaculates were not observed either. Furthermore, no significant differences ($P>0.05$) in *CLU* gene expression were observed between OEC and LLC-PK1 co-cultured with spermatozoa at any of the time points investigated.

2.3. HSP90AA1 expression during sperm co-culture with epithelial cells

HSP90AA1-relative abundance was significantly ($P<0.05$) affected by cell type, presence/absence of insert, boar ejaculate, and co-culturing time. *HSP90AA1* gene expression (Figure 3) increased progressively in sperm-OEC co-cultures without inserts throughout the 24 h incubation period. Indeed, *HSP90AA1* gene expression was significantly higher at 3 h compared to 0 h ($P<0.05$), 6 h compared to 3 h ($P<0.01$), 12 h compared to 6 h ($P<0.05$) and 24 h compared to 12 hours ($P<0.05$) in OEC without inserts. Conversely, a lower upregulation was observed in oviductal cells after 24 h when co-cultured with membrane inserts. In the case of sperm-LLC-PK1 co-cultures, *HSP90AA1* gene expression was only observed to increase significantly at 9 h, 12 h and

24 h (P<0.05) and in the absence of the inserts. At 0 h, *HSP90AA1* gene expression was significantly greater (P<0.05) in LLC-PK1 co-cultured with spermatozoa compared to OEC co-cultured with spermatozoa. At 3 h, 6 h, 12 h and 24 h, *HSP90AA1* gene expression was significantly greater (P<0.05) in OEC co-cultured with spermatozoa and without inserts compared to the other three co-cultures (OEC without inserts, and LLC-PK1 with and without inserts).

2.4. HSPA5 expression during sperm co-culture with epithelial cells

HSPA5 gene expression, similar to *HSP90AA1* gene expression, was significantly (P<0.05) affected by cell type, presence/absence of insert, boar ejaculate, and co-culturing time. The expression of this gene was observed to increase progressively over the first 12 h of sperm-OEC co-culture without membrane inserts (Figure 4). Indeed, *HSPA5* gene expression was significantly higher (P<0.05) at 6 h compared to 0 h, 12 h compared to 6 h and 24 h compared to 9 h but not significantly different at 24 h compared to 12 h in OECs following co-culture with spermatozoa. Conversely, no significant differences (P<0.05) were observed throughout the evaluation period in sperm-OEC co-culture with membrane inserts. On the other hand, *HSPA5* gene expression only reached significantly different levels (P<0.05), compared to 0 h, in LLC-PK1 following 12 and 24 hours of co-culture with spermatozoa and in the absence of membrane inserts. At 0 h, *HSPA5* gene expression was significantly higher (P<0.05) in LLC-PK1 co-cultured with spermatozoa compared to OEC co-cultured with spermatozoa. At 12 and 24 hours, this relationship between the two types of co-culture had reversed (P<0.05). No significant differences were either observed over co-culturing time when inserts separated sperm and LLC-PK1 cells.

193 *2.5. HSPA8 expression during sperm co-culture with epithelial cells*

194 Relative abundance of *HSPA8* was also affected by cell type, presence/absence of insert,
195 boar ejaculate, and co-culturing time ($P<0.05$). Indeed, *HSPA8* gene expression (Figure
196 5) was significantly upregulated ($P<0.05$) in OEC co-cultured with spermatozoa in the
197 absence of membrane inserts at 6 h, 9 h, 12 h and 24 h when compared to 0 h. In the
198 case of LLC-PK1 co-cultured with spermatozoa, *HSPA8* gene expression was
199 significantly upregulated ($P<0.05$) after 9 hours when compared to 0 hours. With the
200 exception of the 6 h and 24 h time points, where *HSPA8* gene expression was
201 significantly higher ($P<0.05$) in sperm-OEC co-cultures compared to sperm-LLC-PK1
202 co-cultures, *HSPA8* gene expression was similar in the two co-culture types. No
203 significant differences ($P>0.05$) in the expression of *HSPA8* were observed over the
204 evaluation period in either OEC or LLC-PK1 cells after co-culturing with spermatozoa
205 in the presence of membrane inserts.

206

207 *2.6. Analysis of sperm populations unbound and bound to epithelial cells, and*
208 *relationship between sperm binding indexes and gene expression*

209 Figure 6 shows the sperm binding indexes to epithelial cells in those 6-wells plates
210 without insert. Significant differences ($P<0.05$) were found between OEC and LLC-
211 PK1, co-culturing time, and boar ejaculate. A significant interaction ($P<0.05$) between
212 these three factors was also observed, thereby indicating that an individual boar effect
213 existed. The sperm binding indexes were significantly higher in OEC than in LLC-PK1
214 at all the co-culture periods, and started to decrease after 3 h of co-culture. When the
215 viability of spermatozoa bound to OEC/LLC-PK1 and unbound sperm populations were
216 examined (Figure 7), it was seen that the percentage of viable spermatozoa was higher
217 in those sperm populations bound to epithelial cells, both to OEC and LLC-PK1, than

218 those that remained unbound. In addition, after 12h and 24h of co-culture, the
219 percentage of viable spermatozoa bound to epithelial cells was higher in OEC than in
220 LLC-PK1.
221 As far as morphology of bound and unbound sperm population is concerned (Table 2),
222 the percentage of morphologically normal spermatozoa was significantly (P<0.05)
223 higher in those spermatozoa that bound to epithelial cells than in unbound populations
224 in all relevant time points. In addition, a significant increase (P<0.05) of
225 morphologically normal spermatozoa, with respect to 0 h, was seen in bound sperm
226 populations at 3h and up to the end of co-culture period. This increase was concomitant
227 with a significant decrease in the percentage of morphologically normal spermatozoa in
228 unbound sperm populations (both in OEC and LLC-PK1 co-cultures) from 3 h to 24 h
229 of co-culture, with respect to 0h. In general, those spermatozoa that were attached to
230 epithelial cells were viable and morphologically normal, whereas the percentages of
231 viable and morphologically normal spermatozoa were significantly (P<0.05) lower in
232 unbound sperm populations. As sperm binding indexes were significantly (P<0.05)
233 higher in OEC than in LLC-PK1 from the beginning of the experiment, the former were
234 more able than the latter to maintain sperm survival.
235 Finally, and given that an individual effect from boar ejaculate was seen both in sperm
236 binding indexes and HSPs expression, the relationship between sperm binding index
237 and expression of *CLU*, *HSP90AA1*, *HSPA5* and *HSPA8* was also investigated using
238 linear mixed models and Pearson correlation in co-cultures without insert. No effect
239 (P>0.05) of sperm binding index, epithelial cell type (OEC *vs.* LLC-PK1), or interaction
240 between type of epithelial cell and sperm binding index were observed in *CLU*
241 expression during all the co-culture period. Sperm binding indexes and *CLU*-transcript
242 abundance were found not to be correlated (P>0.05) at any of the time points

243 investigated. In contrast, relative abundances of *HSP90AA1*, *HSPA5* and *HSPA8* genes
244 were significantly (P<0.05) affected by sperm binding index and epithelial cell type
245 (OEC *vs.* LLC-PK1), as linear mixed model showed. A relationship between sperm
246 binding index and epithelial cell type (P<0.05) was also observed in all the three cases.
247 Significant correlation coefficients (P<0.05) were found between HSPs-transcript
248 abundances and sperm binding indexes after 3h (*HSP90AA1*, r=0.39), 6h (*HSP90AA1*,
249 r=0.45; *HSPA5*, r=0.41; *HSPA8*, r=0.39), 9h (*HSP90AA1*, r=0.48; *HSPA5*, r=0.43;
250 *HSPA8*, r=0.40) and 12h of co-culture (*HSP90AA1*, r=0.51; *HSPA5*, r=0.44; *HSPA8*,
251 r=0.37).
252
253 **3. Discussion**
254 The present study was undertaken to: 1) verify earlier reports that spermatozoa are
255 capable of upregulating OEC gene expression *in vitro* (Yeste et al., 2009) and 2) to
256 determine whether the presence of spermatozoa specifically alters the expression of four
257 genes in reproductive cells (OECs) rather than non-reproductive cells (LLC-PK1),
258 during co-culture *in vitro*.
259 The four genes investigated were chosen deliberately because the proteins they encode;
260 namely, CLU, HSP90AA1, HSPA5 and HSPA8, are found within a subfraction of
261 sAPM proteins known to bind to boar spermatozoa (Elliott et al., 2009) and may have
262 potential roles as modulators of sperm function (e.g. viability; Elliott et al., 2009; Lloyd
263 et al., 2008, 2009) .
264 Expression of HSP90AA1, HSPA5 and HSPA8, but not CLU, genes was upregulated in
265 OECs progressively over the co-culture period. When epithelial cells and spermatozoa
266 were in direct contact, this outcome may be attributable to various roles of heat shock
267 proteins, but not clusterin, in modulating sperm function. In support of this hypothesis,

there is some evidence suggesting that both HSP90AA1 (Ecroyd et al., 2003; Hou et al., 2008) and HSPA5 are involved with the modulation of sperm capacitation (Lachance et al., 2007). In addition, HSPA5 also modulates sperm-ZP binding (Marín-Briggiler et al., 2010), whereas HSPA8 appears to be involved with maintaining sperm viability and has the ability to repair the sperm membrane damage through an increase of plasmalemma fluidity (Elliott et al., 2009; Lloyd et al., 2009; Moein-Vaziri et al., 2014). The observation that individuals HSPs may not modulate sperm function in exactly the same way might explain why *HSP90AA1* and *HSPA5* were upregulated in OECs earlier than *HSPA8* in the co-culture period with spermatozoa.

In contrast to the sperm-OEC co-cultures, the upregulation of *HSP90AA1, HSPA5* and *HSPA8* gene expression in sperm-LLC-PK1 co-cultures was slower, reaching significantly elevated levels later than OEC and when spermatozoa were directly bound to the epithelial cells. Our own previous results show that spermatozoa that bind to OEC remain viable for longer than those which bind to non-reproductive epithelial cell types (Lloyd et al., 2008; Yeste et al., 2009). The ability of OECs to upregulate *HSP90AA1*, *HSPA5* and *HSPA8* gene expression more rapidly than LLC-PK1 in response to the presence of spermatozoa might explain the differential ability of the two epithelial cell types to support sperm survival. In addition, the sperm viability was better maintained when spermatozoa were co-cultured with LLC-PK1 without inserts than when they were incubated in the presence of membrane inserts. This finding, which again matches with our own previous reports (Fazeli et al., 1999; Green et al., 2001; Yeste et al., 2009; Yeste et al., 2012), indicates that the non-specific induction of HSPs expression in LLC-PK1, observed at a lower extent than OEC, could be related with the observed prolonging-effect on sperm viability.

On the other hand, the lack of differences in gene expression between follicular and luteal OECs probably explains why previous investigators found that OEC (Suárez et al., 1991; Fazeli et al., 1999; Petrunkina et al., 2001) and APM (Fazeli et al., 2003) derived from sows in different phases of the reproductive cycle support sperm viability equivalently *in vitro*. However, it is not clear whether the lack of the differences between the follicular and luteal stages is due to the nature of oviduct epithelium in respect of the endometrium, or is the result of the cell dedifferentiation and manipulation during in vitro culturing, since this has been documented in other reports about in vitro culture of cells from reproductive origin (Bassols et al., 2004; 2007). Apart from this, specific environmental conditions, as heat stress, have been found to upregulate *HSP90AA1* expression in bovine OEC (Kobayashi et al., 2013).

In the present study, we did not investigate the effects of other biological (e.g. oocytes, embryos, bacteria, etc) or non-biological (e.g. glass-beads) entities on chaperone/HSP gene expression by OEC. Notwithstanding, existing evidence suggests that OEC do respond to the presence of both spermatozoa and oocytes, by altering the abundance of the proteins they secrete, although the exact alterations are cell-type-specific (Georgiou et al., 2005; 2007; Kodithuwakku et al., 2007; Aldarmahi et al., 2012; 2014). The observation in the present paper, which shows that HSP gene expression is only markedly upregulated when spermatozoa are in direct contact with OEC rather than when they are kept separate using diffusible membrane inserts, tends to support this view. We thus propose, in agreement with other studies (Aldarmahi et al., 2012; 2014) that when spermatozoa bind directly to OEC in co-culture a specific signal transduction pathway within the OEC is activated which then results in the upregulation of HSP gene expression. HSPs synthesised *de novo* in response to sperm appear to translocate from within OEC to the oviductal lumen (Georgiou et al., 2007), and interact directly with the

317 sperm membrane, probably through cholesterol molecules and/or lipid rafts that are
318 present/accessible in uncapacitated but not in capacitated spermatozoa (Moein-Vaziri et
319 al., 2014). Interestingly, the importance of direct contact between OEC and spermatozoa
320 has been highlighted previously in the bovine, where indirect contact stimulated only a
321 fraction of the protein production changes observed when spermatozoa and OEC were
322 in direct contact (Ellington et al., 1993). It remains to be investigated whether the
323 protein abundances of the selected genes investigated mirror the mRNA abundances
324 observed in the present study, although this possibility seems likely (Georgiou et al.,
325 2007). Should this be the case, additional work would be required to elucidate whether
326 such proteins are destined for the OEC surface (Sostaric et al., 2006) or secretion
327 (Georgiou et al., 2005; 2007).
328 Previous reports have already determined that cells from reproductive origin maintain
329 better sperm function and survival than those from non-reproductive origin (Fazeli et
330 al., 1999; Yeste et al., 2009; 2012), and that direct contact between OEC and sperm is
331 needed for maintaining sperm survival in vitro. In addition, the selective binding ability
332 previously reported (Yeste et al., 2012) is confirmed by the present work, as we have
333 observed that those sperm populations that remained bound to OEC and LLC-PK1 were
334 viable and morphologically normal. As the sperm binding indexes were higher in OEC
335 than in LLC-PK1 co-cultures, this also confirms the superior ability of the former
336 compared to the latter in maintaining sperm survival of viable and morphologically
337 normal spermatozoa.
338 One of the most interesting findings of this study is that not all spermatozoa from all
339 boars had the same ability to bind and therefore alter the expression of HSPs when in
340 direct contact with epithelial cells. It is worth noting that in this work, the sperm binding
341 index and the viability and morphology of sperm populations bound to and unbound

342 from epithelial cells were determined in separate well-plates but using spermatozoa
343 derived from the same ejaculates and OEC/LLC-PK1 monolayers from the same batch.
344 From our data, we thus suggest that boar sperm quality from each individual ejaculate
345 contributes to the differences observed in sperm binding and on the sperm ability to
346 alter HSPs expression.
347 Previous reports have failed to show differences between breeds in the sperm ability to
348 modulate the expression of different relevant genes (adrenomedullin, HSPA8, and
349 prostaglandin E synthase; Aldarmahi et al., 2012). From our data, it seems that
350 individual rather than breed differences is a factor, as all our boars came from the same
351 breed. This is similar to that observed by others; even though in some cases
352 cryopreserved rather than fresh spermatozoa was used (Waterhouse et al., 2006; Pinart
353 and Puigmulé, 2013). In addition, HSPs expression was found to be correlated and
354 influenced by sperm binding indexes throughout co-culture, thereby indicating that not
355 only direct contact, i.e. without diffusible membrane insert, but also the number of
356 sperm that bind to epithelial cells is relevant when upregulating gene expression. In this
357 context, we should bear in mind that those spermatozoa bound to epithelial cells were
358 mainly viable and morphologically normal. This could partially explain why individual
359 ejaculate differences were seen in the ability to upregulate HSP expression, and would
360 suggest that the initial sperm quality of a given ejaculate may influence the ability of
361 such ejaculates to alter HSPs expression. This hypothesis along with our findings
362 warrants more research on this topic.
363 In conclusion, the present study shows for the first time that boar spermatozoa alter the
364 expression of HSP genes directly, quickly and markedly in OEC *in vitro*, and that an
365 individual boar ejaculate effect exists. Our results confirm previous reports assessing
366 protein expression (Ellington et al., 1993) *in vitro*, and lead us to accept that oviductal

monolayers present a useful model for studying sperm physiology within the oviduct, in agreement with Aldarmahi et al. (2012; 2014). However, more research is warranted to evaluate whether the absence of in vivo oviductal milieu (stroma and steroid hormones) has any impact on the ability of OEC to be influenced by sperm presence. Moreover, our findings suggest, once again, that spermatozoa are capable of directly eliciting changes in their own microenvironment upon arrival in the oviduct and that HSP proteins have an important functional role within the oviductal sperm reservoir.

4. Materials and methods

4.1. Growth medium

Oviductal epithelial cells were cultured in medium 199 (TCM199 containing Earle's, L–glutamine (0.1 g/l), and Hepes (25 mM)); Sigma-Aldrich®, St. Louis, MO, USA) supplemented with 10% (v/v) heat inactivated foetal calf serum (FCS; Sigma-Aldrich®), 1% (v/v) penicillin-streptomycin solution (stock solution: 10,000 units penicillin-G and 10 mg streptomycin per ml; Sigma-Aldrich® id.: P-4333), and 0.5% (v/v) Fungizone® Antimycotic (stock solution: 250 µg of amphotericin B and 205 µg of sodium deoxycholate per mL; Gibco, Invitrogen Corp., UK). The growth medium was stored at 4 ºC and filtered with 0.22 µm filters (Pall-Gelman Laboratory, Ann Arbor, USA) before use.

4.2. Co-culture medium

Oviductal epithelial cell and LLC-PK1 co-culturing was carried out using a modified TALP medium (2 mM $CaCl_2$, 3.1 mM KCl, 0.4 mM $MgCl_2 \cdot 6\ h_2O$, 100 mM NaCl, 25 mM $NaHCO_3$, 0.3 mM $NaH_2PO_4 \cdot 2H_2O$, 21.6 mM sodium lactate, 10 mM Hepes, 1 mM

sodium pyruvate and 6 $mg \cdot ml^{-1}$ Bovine serum albumin (BSA; Sigma-Aldrich®), pH=7.2; Parrish et al., 1988; Fazeli et al., 1999).

4.3. OEC culture

Oviductal epithelial cells were cultured following the method described by Fazeli and colleagues (1999). Briefly, oviducts with ovaries were collected at a local abattoir from cycling gilts (8-10 months of age), and the follicular phase oviducts separated from the luteal phase oviducts after judging the appearance of the ovaries. They were then washed with PBS (Gibco), stored at 27–30°C and transported within 3 hours *post-mortem* to the laboratory. After that, the oviducts were trimmed, connective tissue removed, and subsequently flushed with PBS lacking calcium and magnesium (PBS – $CaCl_2$ – $MgCl_2$, Gibco). Following this, they were filled with collagenase (Sigma-Aldrich®) 0.25% (w/v) in Hanks' Balanced Salt Solution (HBSS, Life Technologies, Paisley, UK) on a Petri dish, the extremes closed and they were incubated at 37.5ºC and 5% CO_2 for 2 hours.

The oviducts were then milked and the cells recovered and centrifuged (5 minutes at 100×*g* 20ºC) three times. The pellet was resuspended twice with 2 ml of red blood cells lysing buffer (Sigma-Aldrich®), and once with HBSS (10×). Finally, the cells were resuspended in growth medium, subsequently seeded at 10^6 $cells \cdot ml^{-1}$ into flasks, and cultured at 37.5ºC and 5% CO_2 until confluence (after approximately 6-7 days). The growth medium was refreshed every 48 hours.

When confluence was reached, the cells were washed with pre-warmed PBS – $CaCl_2$ – $MgCl_2$ (Gibco) and digested with 3 ml trypsin-EDTA solution (0.5 g porcine trypsin and 0.2 EDTA 4 Na/L; Sigma-Aldrich®) per flask at 37.5ºC and 5% CO_2 for 30 minutes. Digestion was then stopped by adding 10 ml of supplemented TCM199. The

TCM199 (containing the cells) was centrifuged for 5 minutes at 100×*g* 20ºC. The pellet was resuspended in one of two ways, either for RNA extraction or cryopreservation, depending on the next objective.

4.4. Verification of epithelial cell nature in OEC cultures

Cytokeratins are characteristic protein components of epithelial cells (Dobrinski et al., 1999), so the epithelial cell nature of the OECs was verified by immunocytochemistry using a cytokeratin primary antibody. OECs grown for 20 hours in 24-well culture dishes were fixed at 4ºC in 3% formaldehyde (Merck, Darmstadt, Germany) and 60 mM saccharose (Merck) in 0.1 M PBS (Gibco) for 30 minutes. Next, the cells were washed three times with 10 mM PBS and permeabilised for 10 minutes in a solution of 10 mM PBS containing 0.1% Triton X-100. Following another 10 mM PBS 5-minute wash, the cells were incubated for 10 minutes in a blocking solution (10 mM PBS supplemented with 20 mM glycine (Serva, Innogenetics, Gent, Belgium) and 1% BSA). The cells were then incubated with a mouse monoclonal anti-cytokeratin pan antibody recognizing cytokeratins 4, 5, 6, 8, 10, 13 and 18 (1:20 dilution; Chemicon, Pacisa-Giralt, Barcelona, Spain) for 45 minutes at 37.5ºC. They were then rinsed twice in PBS for 10 minutes and incubated with a rabbit anti-mouse IgG FITC-conjugated secondary antibody (1:50 dilution; Dako Diagnostics S.A., Barcelona, Spain) at 37.5ºC for 30 minutes. After washing twice for 10 minutes in PBS, the nuclei of the cells were counter-stained with 5 µM bisbenzimide Hoechst 33258 (Sigma-Aldrich®; Sigma code: 861405, empirical formula: $C_{25}H_{24}N_6O{\cdot}3HCl{\cdot}xH_2O$) for 7 minutes. Finally, the cells were mounted with mounting medium (Sigma-Aldrich®, code: M1289; EC Number 245-690-6) containing sodium phosphate and citric acid in glycerol, and observed under a fluorescence microscope (Axio Imager.Z1; Zeiss, Germany). A total of 200 cells were

counted and classified as either epithelial (positive cytokeratin immunoreactivity) or non-epithelial (cytokeratin immunoreactivity absent). 95.61% ± 1.30 (mean ± SEM) of OECs showed positive cytokeratin immunoreactivity, confirming their epithelial nature.

4.5. OEC cryopreservation and thawing

Following trypsinisation, approximately 10^6 OECs were harvested for cryopreservation in freezing medium that consisted of 10% (dimethyl sulphoxide (DMSO; Sigma-Aldrich®) in FCS (Sigma-Aldrich®). Discarding the supernatant, the cell pellet was resuspended in 1 ml of freezing medium, and transferred to a labelled cryogenic vial (Nalgene, Rochester, MI, USA) before immediate transfer to a -20ºC freezer for 20 minutes and then to another at -80ºC.

When needed, the cryovials were taken from the freezer at -80ºC and incubated in a water bath for 2 minutes at 37ºC until thawed. The content of the vial was added to a tube containing 10 ml of pre-equilibrated growth medium. Samples were centrifuged at $100 \times g$ and 20ºC for 5 minutes and the supernatant discarded. The pellet was resuspended with growth medium and cells seeded into a flask containing 10 ml of growth medium.

4.6. LLC-PK1 culture

Pig kidney epithelial cells (LLC-PK1; American Tissue Type Culture Collection (ATCC)-LGC Promochem, UK) were seeded into a flask at a concentration of 10^6 cells·mL^{-1}. They were cultured in TCM199 (Sigma-Aldrich®) supplemented with 3% (v/v) of FCS, 1% (v/v) Penicillin G/Streptomycin and 5% (v/v) Fungizone amphotericin B (Gibco), at 37.5ºC in 100% humidity and 5% CO_2, until confluence was reached.

Prior to co-culture with spermatozoa, the LLC-PK1 growth medium was replaced with TALP co-culture medium.

4.7. Experimental design

Figure 8 shows a representative scheme of the experimental design performed in the present work. Confluent monolayers of follicular OEC, luteal OEC and LLC-PK1 were established in 6-well plates, in triplicate. To prevent contaminations, a total of nine plates were set for evaluation at each relevant time point. Three plates contained follicular OEC, three contained luteal OEC and the other three contained LLC-PK1 cultures. For each epithelial cell type (i.e. follicular OEC, luteal OEC or LLC-PK1), two set of plates did not contain a diffusible membrane insert (0.4 μm; Millipore Corp., Spain), while the other set did. The purpose of this insert was to keep the spermatozoa and epithelial cells separate, whilst at the same time allowing the medium between the two cell types to be shared. Two other negative controls were also included in the experiment. One consisted of TALP medium containing spermatozoa but without epithelial cells (OECs or LLC-PK1), while the other consisted of epithelial cells (i.e. OEC follicular, OEC luteal or LLC-PK1) without spermatozoa.

In all cases, growth media were removed from the confluent monolayers and replaced either with 3 ml of washed spermatozoa (final concentration of 1.87×10^6 spermatozoa·ml^{-1}) in TALP medium or with TALP medium without spermatozoa (negative controls with cells but without spermatozoa). The co-cultures and negative controls were incubated at 37.5ºC, 100% humidity and 5% CO_2. Just before the relevant time points (0, 3, 6, 9, 12 and 24 hours of incubation), the TALP medium was removed from those controls that did not contain spermatozoa and was replaced with 3 ml of spermatozoa (final concentration of 1.87×10^6 spermatozoa·ml^{-1}) in TALP medium. As

there were two 6-well plates without insert per each time point, one was used to evaluate bound and unbound sperm populations to OEC, and the other was used to evaluate gene expression of OEC and LLC-PK1. Those 6-well plates that contained the insert were only used for evaluating gene expression. The TALP medium (containing spermatozoa) was removed from the co-cultures (both containing and not containing the diffusible membrane insert) and from the controls. The monolayers were then either trypsinised for RNA extraction, or used for evaluating bound and unbound sperm populations as described. In the case of trypsinisation for RNA extractions, the expression of *ACTB*, *CLU*, *HSP90AA1*, *HSPA5* and *HSPA8* was determined in both the co-cultures and negative controls using real-time quantitative PCR.

The experimental design was replicated sixteen times using spermatozoa from sixteen different boars and different vials derived from four different batches of each epithelial cell type (in the case of OECs, each vial contained cells from a mixture of females at the same oviductal cycle stage).

4.8. Sperm preparation

Sixteen ejaculates from 16 healthy and sexually mature Piétrain boars served as biological replicates for all the experiments and were collected using the gloved hand technique. These boars were housed in climatic buildings under stable conditions of relative humidity and controlled temperature. They were fed an adjusted commercial diet twice a day and the rate of semen collection was twice a week. No fertility problems were recorded by the AI station (JSR Healthbred Ltd.; Thorpe Willoughby, Yorkshire, UK).

After collection, the sperm rich fraction of each ejaculation was filtered through gauze and subsequently diluted 1:9 (v/v) in Beltsville thawing solution (BTS; Johnson et al.,

1988) and transported to the laboratory by Royal Mail Special Delivery (UK) for arrival the day after semen collection. Upon arrival, sperm viability and morphology of each ejaculate was evaluated following the protocols described in Section 4.11 (% Viable spermatozoa, mean ± SEM: 89.7 ± 3.2, % Morphologically normal spermatozoa: 80.1 ± 2.8). Following this, sperm was washed thrice, through centrifugation at 600×g and resuspension with PBS, to remove diluent traces. Final resuspension was in TALP medium.

4.9. Assessment of cell viability during co-culture

Epithelial cell viability was assessed during the entire co-culture period in a separated well that contained the epithelial cells (OEC follicular, OEC luteal, and LLC-PK1) without spermatozoa. Cell viability was evaluated as a percentage of confluence under a phase contrast inverted microscope (Nikon Ti-U Eclipse), as well as using a commercial staining kit purchased from Molecular Probes® (Live/Dead® viability/cytotoxicity kit for mammalian cells), which consisted of a dual staining with calcein acetoxymethyl (Calcein AM, final concentration: 2 μM) and ethidium homodimer-1 (final concentration: 4 μM). Stained cells were monitored using fluorescence and a B2A filter. For each sample, three counts of 100 were carried out, prior to calculating the corresponding mean ± standard error of the mean (SEM).

4.10. Analysis of viability, morphology and binding index of sperm population bound to epithelial cells

At each relevant time point, and in those 6-well plates set up to evaluate unbound and bound sperm populations, TALP medium containing freely swimming spermatozoa (unbound population) was removed and evaluated. Wells were then washed carefully

with 3 mL PBS to remove any traces of unbound sperm. After washing, 3 mL of fresh TALP medium was added, and three of these wells (bound population) were used to assess the ability of spermatozoa to bind the epithelial cells and the sperm viability, while the other three were used to evaluate the sperm morphology.

For evaluation of sperm viability and binding indexes in bound population, spermatozoa were stained using a dual-staining procedure with SYBR-14 (Molecular Probes Inc., Eugene, OR) and ethidium homodimer (EthD-1; Molecular Probes Inc.) as described in Fazeli et al. (1999), prior to examination using an inverted microscope (Nikon Eclipse Ti-U) equipped with differential interference contrast (DIC, Nomarski), epifluorescence and phase-contrast. Briefly, 30 µL of a 20 µM SYBR-14 stock solution (final concentration: 200 nM) and 3 µL of a 2.2 mM EthD-1 stock solution (final concentration: 2.2 µM) were added to different three wells, each containing 3 mL of fresh TALP medium. After incubation at 37.5ºC and 5% CO_2 for 15 minutes, samples were examined under an inverted epifluorescence microscope (Nikon Eclipse Ti-U) with Nikon S-Plan Fluor ELWD 20x/0.45 and Nikon S-Plan Fluor ELWD 40x/0.60 objective lens. In each of the three wells, one hundred spermatozoa each were counted and classified as viable (green) or non-viable (red), using B2-A (excitation filter: BP 450–490 nm; dichromatic mirror: 505 nm; suppression filter: LP 520 nm) and G2A (excitation filter: BP 510-560 nm; dichromatic mirror: 575 nm; suppression filter: LP 590 nm) filter cubes. The mean and standard error of the mean (SEM) were calculated per plate, each one corresponding to a relevant time and a given epithelial cell type.

On the other hand, the number of spermatozoa bound to epithelial cells (OEC follicular, OEC luteal, and LLC-PK1) was assessed in the same wells where sperm viability was evaluated, by counting 20 random fields of 0.625 mm of diameter and 0.307 mm^2 of area. A stage micrometer was used to determine the diameter of view field. The sperm

565 binding index was calculated as number of sperm bound to epithelial cells in a
566 monolayer surface of 0.05 mm^2, as described in Yeste et al. (2012). Three parallel co-
567 cultures (serving as replicates) were performed for each case within each relevant time
568 point, and the means ± SEM were calculated.
569 In the other three wells, the morphology of the spermatozoa bound to epithelial cells
570 was evaluated using an inverted phase-contrast microscope (Zeiss Axiovert 45)
571 equipped with a warming plate (set at 37.5ºC) and a digital camera (Axiocam ICc1).
572 Sperm morphology was assessed after fixing co-cultures with a 0.1 M PBS solution
573 containing 2% paraformaldehyde for 20 minutes at 4ºC. Next, samples were examined
574 at a magnification of 200x (Zeiss Achrostigmat 20x 0.30 objective lens), and
575 differences between morphologically normal spermatozoa, spermatozoa with proximal
576 and cytoplasmic droplets, and aberrant spermatozoa were noted. Within aberrant
577 spermatozoa, the following distinctions were made: aberrant head morphologies, coiled
578 tails, tails folded at the connecting or intermediate piece, and those tailed at Jensen's
579 ring (Yeste et al., 2008). Fields were randomly selected and 100 spermatozoa were
580 analysed for each well. Only those spermatozoa clearly attached to the epithelial cells
581 were taken into account. Three replicates were done and means ± SEM were then
582 calculated.
583
584 *4.11. Analysis of viability and morphology of unbound sperm population*
585 Sperm viability, morphology and concentration of unbound spermatozoa that were
586 freely swimming in the co-culture wells, were also evaluated following a similar
587 procedure than that described for bound sperm population with some minor
588 modifications. Again, three wells were used for evaluation of sperm viability, while the
589 other three were utilised for determination of sperm morphology. Although 3 mL of

590 TALP medium containing the unbound spermatozoa were taken from all wells, only 1
591 mL of each was used to evaluate either sperm viability or sperm morphology. In the
592 case of sperm viability, spermatozoa were also stained with SYBR-14 and EthD-1,
593 which is why 10 µL of a 20 µM SYBR-14 at stock solution and 1 µL of a 2.2 mM
594 EthD-1 stock solution were added to 1 mL of TALP medium containing the unbound
595 spermatozoa. Samples were then incubated at 37.5ºC for 15 minutes, prior to
596 examination under a conventional epifluorescence microscope (Leica DMLR-XA;
597 Leica, Germany) with Leica 40X 1.32 HCX PL APO objective lens, and two filter
598 cubes: I3 (excitation filter: BP 450-490 nm; dichromatic mirror: 510 nm; suppression
599 filter: LP 515 nm) for the observation of green (SYBR-14) fluorescence and N2.1
600 (excitation filter: BP 515-560 nm; dichromatic mirror: 580 nm; suppression filter: LP
601 590 nm) for the assessment of red (Eth-D1) fluorescence. One hundred spermatozoa
602 were counted per well, and three wells were examined prior to calculating the
603 corresponding means ± SEM. The criterion for considering a spermatozoon as viable
604 was the same as that explained for the bound sperm population.
605 Sperm concentration and morphology were assessed using a phase contrast microscope
606 (Olympus BX41) at a magnification of 100x (Olympus 10x 0.30 PLAN objective lens,
607 negative phase-contrast field), and using a Makler counting chamber (Sefi-Medical
608 Instruments, Haifa, Israel). A total of three samples, each coming from a different well,
609 were examined, prior to calculating the means ± SEM.
610 Sperm morphology was evaluated in accordance with the same criteria used for the
611 bound sperm population. Prior to evaluation of their morphology, spermatozoa were
612 fixed with 3% formaldehyde saline solution to immobilise the spermatozoa. Thereafter,
613 5µL of each sample were subsequently placed on a slide and mounted with a cover slip.
614 Preparations were evaluated at a magnification of 200x (Olympus 20x 0.40 PLAN

objective lens, positive phase-contrast field) and 100 spermatozoa were analysed in each sample, coming from a different well. Three individual samples (technical triplicates) were examined, and the corresponding means ± SEM were then calculated.

4.12. RNA extraction and quantification

The extraction of total RNA from cells was carried out using an RNAqueous®-4PCR kit (DNA-free RNA isolation for RT-PCR) (Ambion Inc., Austin, TX, USA), following the manufacturer's instructions. After extraction, the RNA was incubated with DNaseI to ensure the removal of contaminating DNA and subsequently the amount of RNA and its purity was determined by spectroscopy (GENESYS™ 10 UV/Vis Spectrophotometer, Thermo Spectronics, UK) at wavelengths of 260 nm and 280 nm. The purity of the RNA was estimated by taking the quotient of the absorptions at 260 nm and 280 nm (Abs_{260}/Abs_{280}) and the extracted RNA was only used when this ratio was higher than 1.8 and the absorbance reading was higher than 0.15.

4.13. Reverse transcription – conventional polymerase chain reaction (RT-PCR)

RNA extracted from the cells was reverse transcribed to produce cDNA using the Reverse Transcription System kit (Promega, Madison, USA), according to the manufacturer's instructions. Briefly, each reaction contained 800 ng RNA, 4 µl $MgCl_2$, 2 µl buffer, 2 µl dNTPs, 0.5 µl RNase inhibitor, 0.5 µl OligodT and 0.6 µl of Reverse Transcriptase enzyme and made up to a total volume of 20 µl using nuclease-free water (Promega UK, Southampton, UK). Additionally, parallel reactions containing all the components above with the exception of the enzyme (no enzyme control) were set up to screen each RNA sample for the presence of contaminating DNA. When any no enzyme control was found positive, RT products were discarded and not analysed further.

Furthermore, a reaction containing all the reagents above but no RNA (no template control) was set up to screen the RT reagents for contaminants. The reactions were carried out at 42°C for 2 h using a GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, CA, USA). The resulting cDNA was then diluted 1:5 in sterile nuclease-free water (Promega) and stored at -20°C until use.

To design the primers for PCR, coding DNA sequences (CDS) for pigs (*Sus scrofa)*, sheep (*Ovis aries*), rats (*Rattus norvegicus*), mice (*Mus musculus*), cattle (*Bos taurus*) and humans (*Homo sapiens*) for each of the four selected genes and β-actin (*ACTB*) were obtained from Genbank (Entrez Nucleotide database; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) where possible and aligned using ClustalW software (http://www.ebi.ac.uk/clustalw.htm) to identify the regions of the CDS that were conserved across species. These conserved CDS regions were then used to design two primers for each of the selected genes (one forward and one reverse). The specificity of each primer to its target gene was then verified using a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) prior to purchase from MWG Biotech AG (UK). All primers were diluted to 50 $pmol \cdot \mu l^{-1}$ before being used. The sequences, the expected product sizes for each primer pair and the accession numbers of CDS used to design the primers are summarized in Table 1.

In preliminary experiments, expression of *ACTB*, *CLU*, *HSP90AA1*, *HSPA5* and *HSPA8* was examined and sequenced by conventional RT-PCR, to confirm the expected amplicon sizes and annealing temperature (60ºC) of primers. With this purpose, RT-PCR was performed on each cDNA sample using a GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, CA, USA). Each reaction contained: 200 ng cDNA, 5 µl 1X PCR buffer, 2.5 mM $MgCl_2$, 0.8 mM dNTPs, 500 nM of each forward and reverse primer (see Table 1 for details) and 0.5 U BioTAQ DNA polymerase. All these

reagents, except for the primers, were provided by Bioline® (London, UK). Each reaction was made up to a total volume of 50 µl using sterile nuclease-free water. Furthermore, a reaction containing all the reagents above but no cDNA (no template control) was set up to screen the PCR reagents for contaminants. Each reaction had an initial denaturation step of 95ºC for 5 minutes, followed by the optimal number of cycles determined separately for each primer pair of: denaturation at 94°C for 30 sec, 60ºC for 30 sec and elongation at 72°C for 30 sec. Each reaction also had a final elongation step of 72ºC for 7 minutes. The optimal number of cycles was determined separately for each primer pair using saturation curves, to ensure the end-point analysis (i.e. the densiometric analysis) of RT-PCR products was conducted during the exponential phase of the PCR cycle. RT-PCR products were separated using 2% (w/v) agarose gel (Bioline, London, UK) electrophoresis. Gels were stained with 0.6 µg ml^{-1} ethidium bromide in 1× TAE (TrisAcetat-EDTA; 90 mM Tris, 90 mM acetate, 2 mM EDTA) and RT-PCR products were visualised using UV light (SynGene Gene Genius system, Synoptics Ltd., Cambridge, UK).

Finally, the identity of the RT-PCR products generated was confirmed via DNA sequencing and BLAST search. Briefly, the RT-PCR products were purified using the QIAquick® PCR purification kit (Qiagen Ltd., Crawley, UK), according to the manufacturer's instructions. The purified RT-PCR products were then sequenced using the same forward and reverse primers (0.8 $pmol \cdot \mu l^{-1}$) used in the PCR, the ABI BigDye® Terminator cycle sequencing kit v3.1 (Applied Biosystems, Warrington, Cheshire, UK) and the Abi Prism® 3100-Avant Genetic Analyser (Applied Biosystems).

4.14. Real Time Polymerase Chain Reaction (qPCR)

Quantitative PCR was performed on each cDNA sample using a 7500 Real Time PCR system® (Applied Biosystems, Foster City, California, USA) and Power SYBR® Green PCR Master Mix (Applied Biosystems) for the detection of qPCR products. After optimising primer concentrations for two-step RT-qPCR, following the standard protocol recommended by the manufacturer, PCR reactions were run by using MicroAmp® 96-well reaction plates. The final volume per reaction was 20 µl, each reaction containing 10 ng cDNA, 10 µl 1× Power SYBR® Green PCR Master Mix (purchased from Applied Biosystems), 0.5 µl of forward and 0.5 µl of reverse primers (100 nM of each primer), and nuclease-free water up to 20 µl. Prior to evaluate the samples, the efficiency and specificity of all primers were determined by analysing melting curves, according with Power SYBR® Green PCR Master Mix's instructions. On the other hand, serial dilutions of cDNA samples were used as template with the appropriate concentration of primers and 1× Power SYBR® Green PCR Master Mix in a total volume of 25 µl.

Quantitative PCR reactions were performed as follows: one cycle of denaturation at 95ºC for 5 min, 40 cycles of amplification with denaturation step at 94ºC for 15 sec, annealing step for 30 sec at the appropriate annealing temperature of primers, and extension step at 72ºC for 40 sec. Fluorescence data were acquired during the 72ºC extension steps. The melting protocol consisted of heating samples from 50 to 94ºC, holding at each temperature for 5 sec, while monitoring the fluorescence. The comparative cycle threshold (C_T) method was used to quantify relative gene expression levels and quantification was normalized to an endogenous control, *ACTB*. Fluorescence data were acquired after each elongation step to determine the threshold cycle for each sample. The comparative Livak C_T method ($\Delta\Delta C_T$ method) was used by calculating the formula $\Delta C_T = C_{T,\text{ gen of interest}} - C_{T,\ ACTB}$. Fold increase in the expression of

specific mRNA in oviductal cells in contact with spermatozoa and without contact with them was calculated using the relative quantification method $2^{-(\Delta\Delta C_T)}$ (Livak and Schmittgen, 2001). In all cases, calculation of $\Delta\Delta C_T$ involved using the highest sample ΔC_T value (i.e. the sample with the lowest target gene expression) as an arbitrary constant to subtract from all other ΔC_T sample values. Fold differences in relative transcript abundance were calculated for target genes assuming an amplification efficiency of 100% and using the formula $2^{-(\Delta\Delta C_T)}$. Data were calculated as the fold change in gene expression normalized to *ACTB* and relative to epithelial cultured cells (either OEC or LLC-PK1) without spermatozoa (negative controls). Thus, for these controls $\Delta\Delta C_T$ equalled to zero. The housekeeping gene *ACTB* was chosen following a previous report (Pedersen et al., 2005; Nygard et al., 2007), where *ACTB* is considered as a good reference gene for high abundant transcripts. This internal standard gene was found to be constantly expressed in OECs and LLC-PK1, during all the co-culturing period.

4.15. Statistical analyses

Data (x) from mRNA relative abundances using the $2^{-(\Delta\Delta C_T)}$ method, each replicate was considered as a statistical case (n=16), were analysed with SPSS 19.0 for Windows (SPSS Inc., Chicago, IL, USA). Relative abundance of *CLU*, *HSP90AA1*, *HSPA5* and *HSPA8*, together with sperm parameters (sperm binding index, sperm viability and morphology) were tested for normality (the Shapiro-Wilk test) and for equal variance (Levene's test). When necessary, as in the case of non-normal data, an arcsine square root (x) transformation was carried out prior to analysis in order to stabilise the error variance.

First, relative abundances of all gene transcripts were compared between monolayers (OEC follicular, OEC luteal and LLC-PK1) with a one-way analysis of variance (ANOVA) followed by a *t*-test with Bonferroni adjustment.

A linear mixed model was used for comparing the effects of sperm's presence on gene expression in OEC and LLC-PK1 cultures. Fixed-effect factors were the type of cultured cells (OEC, LLC-PK1 and negative control) and the presence or absence of diffusible membrane inserts, random-effect factors were culture batch and ejaculate, and the incubation time was the intra-subject factor. The variable was the relative abundance of each gene of interest, and the model was followed by multiple pair-wise comparisons using a Bonferroni test. To evaluate differences between bound and unbound sperm populations in sperm binding indexes, sperm viability and morphology, another linear mixed model with Bonferroni's post-hoc test was run, with type of cultured cells as fixed-effect factor and culture batch and ejaculate as random-effect factors. The incubation time was the intra-subject factor.

Another linear mixed model was run to investigate the relationship between sperm binding index and gene-relative abundances. In this case, the type of cultured cells was the fixed-effect factor, culture batch and ejaculate were the random-effect factors, the sperm binding index was the covariate, and the incubation time was the intra-subject factor. The variable was again the relative abundance of each gene of interest, and Bonferroni test was used for multiple comparisons. Pearson correlation coefficient was also calculated between sperm binding indexes and relative transcript abundances of the four genes throughout the co-culture period.

In all statistical analyses, the significant level was set at 5%. Results are expressed as means ± standard error of the mean (SEM).

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper

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Figure Legends

Figure 1 Relative abundances, as mean ± SEM, of clusterin (*CLU*), *HSP90AA1*, *HSPA5* and *HSPA8* in follicular OEC, luteal OEC and LLC-PK1. The Figure represents the results obtained in sixteen independent experiments. Different superscripts (*a* and *b*) denote significant differences ($P<0.05$) between the different epithelial cell types.

Figure 2 Clusterin (*CLU*)-relative abundance (mean ± SEM) in OEC and LLC-PK1 co-cultured with spermatozoa (with and without inserts). The Figure represents the results obtained in sixteen independent experiments. No superscripts are shown as no significant differences ($P>0.05$) were observed between the different epithelial cell types and the different incubation times investigated.

Figure 3 *HSP90AA1*-relative abundance, as mean ± SEM, in OEC and LLC-PK1 co-cultured with spermatozoa (with and without inserts). The Figure represents the results obtained in sixteen independent experiments. Different superscripts (*a-f*) denote significant differences ($P<0.05$) between the different epithelial cell types and the different incubation times investigated.

Figure 4 *HSPA5*-relative abundance (mean ± SEM) in OEC and LLC-PK1 co-cultured with spermatozoa (with and without inserts). The Figure represents the results obtained in sixteen independent experiments. Different superscripts (*a-e*) denote significant differences ($P<0.05$) between the different epithelial cell types and the different incubation times investigated.

Figure 5 *HSPA8*-relative abundance (mean ± SEM) in OEC and LLC-PK1 co-cultured with spermatozoa (with and without inserts). The Figure represents the results obtained in sixteen independent experiments. Different superscripts (*a-d*) denote significant differences ($P<0.05$) between the different epithelial cell types and the different incubation times investigated.

Figure 6 Sperm binding index, as mean ± SEM, in sperm populations bound to either OEC or LLC-PK1, in co-cultures without insert. The Figure represents the results obtained in sixteen independent experiments. Different letters (*a-b*) mean significant differences ($P<0.05$) between cell types within a given time point, whereas different numbers (*1-4*) mean significant differences between time points within cell type (OEC or LLC-PK1).

Figure 7 Sperm viability, as percentage of viable spermatozoa (mean ± SEM), in bound and unbound sperm populations in co-cultures without insert. The Figure represents the results obtained in sixteen independent experiments. Different letters (*a-d*) mean significant differences ($P<0.05$) between cell types and bound/unbound populations within a given time point, whereas different numbers (*1-5*) mean significant differences between time points within a given combination of bound/unbound population and cell type.

Figure 8 Representative scheme about the experimental design conducted in this work. This experimental design was repeated sixteen times using sperm samples coming from sixteen different boars.