RESEARCH ARTICLE

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Viable and Morphologically Normal Boar Spermatozoa Alter the Expression of Heat-Shock Protein Genes in Oviductal Epithelial Cells During Co-Culture In Vitro

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SUMMARY

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

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INTRODUCTION

Oviductal epithelial cells (OECs) are involved in the transport of spermatozoa to the ampulla, the region of the oviduct where fertilization occurs (for review, see Hunter, 2005; Rodríguez-Martínez et al., 2005; Yeste, 2013). Various reports have also demonstrated that OECs can affect sperm viability, capacitation, and motility in boars (Fazeli et al., 1999; Yeste et al., 2009), humans (Ellington et al., 1998; Yao et al., 1999), and other mammalian species (Cortés et al., 2004; Lloyd et al., 2008). Spermatozoa can bind to OECs (Petrunkina et al., 2001), particularly within the isthmus segment of the oviduct where a sperm reservoir is formed during the estrous period (Suárez et al., 1991; Töpfer-Petersen et al., 2002), although not all spermatozoa have the same ability to attach to OECs. Selective binding to OECs 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), which are all preferred over capacitated spermatozoa, sperm with coiled tails or proximal droplets, or spermatozoa with damage to their chromatin structure. The effect of secreted OEC products has further been shown to influence sperm function in some mammalian species (McCauley et al., 2003; Quintero et al., 2005; Zhang et al., 2006). Co-incubation with apical plasma membranes isolated from OECs can also modulate sperm function in several mammalian species, for example, rabbits, pigs, horses, and cattle (see Holt et al., 2006 for references).

While OECs and OEC-derived proteins directly affect spermatozoa, the spermatozoa have been reported to influence the gene and protein expression of OECs. Indeed, in vitro studies 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 OECs both quantitatively and qualitatively. In vivo and in vitro studies also showed that the presence of spermatozoa in oviducts influenced the gene expression of OECs (Fazeli et al., 2004; Georgiou et al., 2007) and the composition of the proteins secreted by the same cells (Georgiou et al., 2005, 2007).

Previous studies have shown that a soluble apical plasma membrane fraction from OECs 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 soluble proteins that bind to boar spermatozoa was identified (Holt et al., 2005; Elliott et al., 2009), with several of the proteins identified as 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 if boar spermatozoa actively influence the gene expression of some chaperones/HSPs in OECs during in vitro co-culture. Specifically, we evaluated the expression of four selected genes in OECs following 3, 6, 9, 12, and 24 hr of co-culture with boar spermatozoa. These four chaperone/HSPs genes (clusterin, *CLU*; HSP 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-soluble apical plasma membrane fractions (Elliott et al., 2009).

We also tested the hypothesis that boar spermatozoa would influence the chaperone/HSP gene expression more effectively in cells of reproductive rather than nonreproductive origin (e.g., kidney; cultured LLC-PK1 cells), since the former influence more sperm-cell parameters, like viability, than the latter (Fazeli et al., 1999; Yeste et al., 2009). This preference would be expected if the sperm-oviduct interaction is of special functional significance. The conditionality of such an effect was first evaluated by testing if the reproductive cycle phase of the sow influenced the expression of the selected genes in OECs during in vitro co-culture. Secondly, to explore the importance of indirect and direct contact between the spermatozoa and epithelial cells (OECs and LLC-PK1 cells, respectively), (i) co-culture experiments were conducted both with and without diffusible membrane inserts, respectively, and (ii) the viability and morphology of sperm-bound and unbound populations were determined in relationship to the gene expression and sperm-binding indexes in co-cultures that did not contain inserts.

RESULTS

Comparison of *CLU, HSP90AA1, HSPA5,* and *HSPA8* Expression in OEC Follicular, OEC Luteal, and LLC-PK1 Monolayers

The relative transcript abundance of the four genes analyzed in follicular and luteal OECs and in LLC-PK1 cells in culture was measured (Fig. 1). LLC-PK1 cells presented significantly ($P < 0.05$) higher levels of *HSP90AA1* and *HSPA5* transcript compared with both follicular and luteal OECs. 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 OECs were observed when the expression of *CLU*, *HSP90AA1*, *HSPA5*, and *HSPA8* was evaluated after culture (Fig. 1). In addition, follicular and luteal OECs did not 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—e.g., adding

Abbreviations: CLU, clusterin; HSP, heat-shock protein; HSP90AA1, heat-shock protein 90 kDa alpha A1; HSPA5, glucose regulated protein 78 kDa; HSPA8, heat-shock cognate protein related 70 kDa; OEC, oviductal epithelial cell.

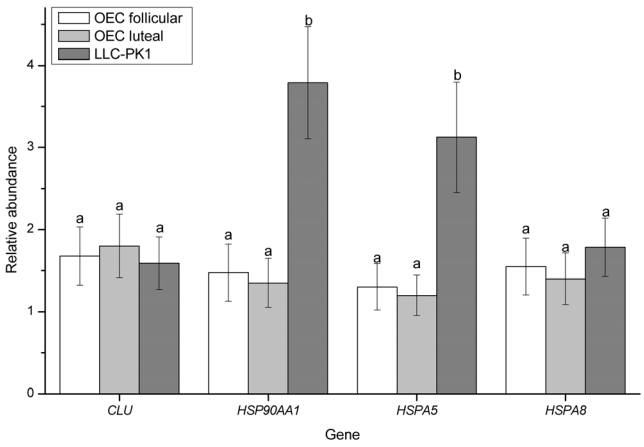

Figure 1. Relative abundances (mean ± standard error), of clusterin (*CLU*), *HSP90AA1*, *HSPA5*, and *HSPA8* in follicular OEC, luteal OEC, and LLC-PK1 cells. Results are from 16 independent experiments. Different superscripts (a and b) denote significant differences ($P < 0.05$) between the different epithelial cell types.

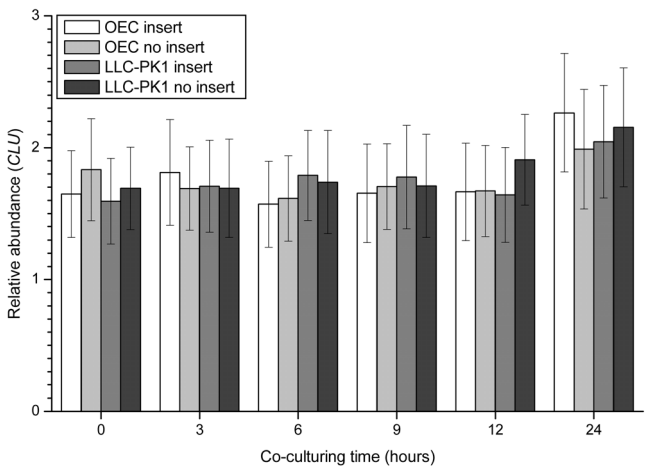

Figure 2. Clusterin (*CLU*)-relative abundance (mean ± standard error) in OECs and LLC-PK1 cells co-cultured with spermatozoa (with and without inserts). Results are from 16 independent experiments. No superscripts are shown as no significant differences ($P > 0.05$) were observed between the different epithelial cell types and the different incubation times investigated.

spermatozoa to epithelial cells just before expression analyses—between the time points investigated (0, 3, 6, 9, 12, and 24 hr). This suggested that the changes in gene expression observed in OECs and LLC-PK1 (described below) were due to the presence of spermatozoa rather than to an artifact of removing and replenishing the cell culture media.

CLU Expression During Sperm Co-Culture With Epithelial Cells

No significant differences ($P > 0.05$) in *CLU* gene expression (Fig. 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 OECs and LLC-PK1 cells co-cultured with spermatozoa at any of the time points investigated.

HSP90AA1 Expression During Sperm Co-Culture With Epithelial Cells

HSP90AA1-relative abundance was significantly ($P < 0.05$) affected by cell type, the presence/absence of insert, boar ejaculate, and co-culturing time. *HSP90AA1* gene expression (Fig. 3) increased progressively in sperm-OEC co-cultures without inserts throughout the 24-hr incubation period. Indeed, *HSP90AA1* gene expression was significantly higher at 3 hr compared to 0 hr ($P < 0.05$), 6 hr compared to 3 hr ($P < 0.01$), 12 hr compared to 6 hr ($P < 0.05$), and 24 hr compared to 12 hr ($P < 0.05$) in OECs without inserts. Conversely, a smaller change was observed in OECs after 24 hr when co-cultured with membrane inserts. In the case of sperm-LLC-PK1 co-cultures, *HSP90AA1* gene expression only increased significantly at 9, 12, and 24 hr ($P < 0.05$) and in the absence of the inserts. At 0 hr, *HSP90AA1* gene expression was significantly greater ($P < 0.05$) in LLC-PK1 cells co-cultured with spermatozoa compared to OECs co-cultured with spermatozoa. At 3, 6, 12, and 24 hr, *HSP90AA1* gene expression was significantly greater ($P < 0.05$) in OECs co-cultured with spermatozoa and without inserts compared to the other three co-culture conditions (OECs without inserts, and LLC-PK1 cells with and without inserts).

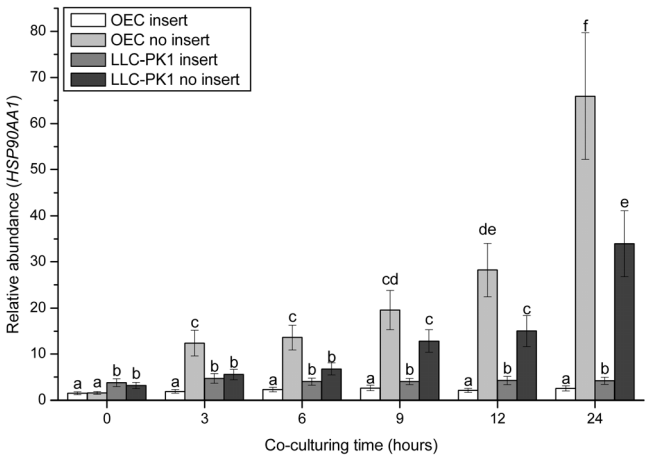

Figure 3. *HSP90AA1*-relative abundance (mean ± standard error) in OECs and LLC-PK1 cells co-cultured with spermatozoa (with and without inserts). Results are from 16 independent experiments. Different superscripts (a–f) denote significant differences ($P < 0.05$) between the different epithelial cell types and the different incubation times investigated.

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, the presence/absence of insert, boar ejaculate, and co-culture time. The expression of this gene was observed to increase progressively over the first 12 hr of sperm-OEC co-culture without membrane inserts (Fig. 4). Indeed, *HSPA5* gene expression was significantly higher ($P < 0.05$) at 6 hr compared to 0 hr, 12 hr compared to 6 hr, and 24 hr compared to 9 hr, but not significantly different at 24 hr compared to 12 hr in OECs following co-culture with spermatozoa. Conversely, no significant differences ($P < 0.05$) were observed throughout the evaluation period in sperm-OEC co-cultures with membrane inserts. In LLC-PK1 cultures, *HSPA5* gene expression only reached significantly different levels ($P < 0.05$), compared to 0 hr, following 12 and 24 hr of co-culture with spermatozoa and in the absence of membrane inserts. At 0 hr, *HSPA5* gene expression was significantly higher ($P < 0.05$) in LLC-PK1 cells co-cultured with spermatozoa compared to OECs co-cultured with spermatozoa; at 12 and 24 hr, however, this relationship between the two types of co-cultures reversed ($P < 0.05$). No significant differences were observed over the co-culturing period when inserts separated sperm and LLC-PK1 cells.

HSPA8 Expression During Sperm Co-Culture With Epithelial Cells

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

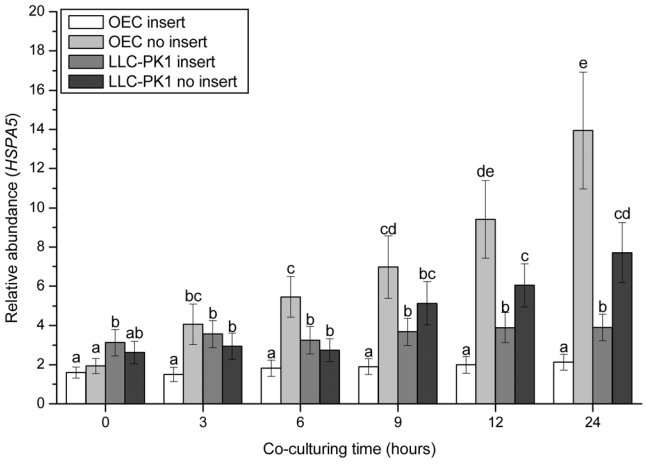

Figure 4. *HSPA5*-relative abundance (mean ± standard error) in OECs and LLC-PK1 cells co-cultured with spermatozoa (with and without inserts). Results are from 16 independent experiments. Different superscripts (a–e) denote significant differences ($P < 0.05$) between the different epithelial cell types and the different incubation times investigated.

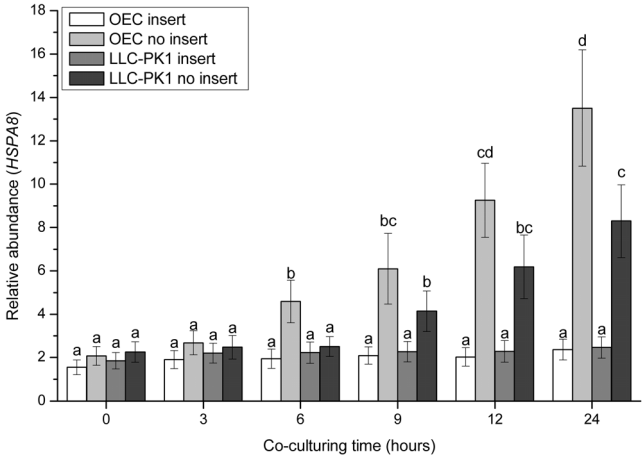

Figure 5. *HSPA8*-relative abundance (mean ± standard error) in OECs and LLC-PK1 cells co-cultured with spermatozoa (with and without inserts). Results are from 16 independent experiments. Different superscripts (a–d) denote significant differences ($P < 0.05$) between the different epithelial cell types and the different incubation times investigated.

Analysis of Sperm Populations Unbound and Bound to Epithelial Cells, and Relationship Between Sperm-Binding Indexes and Gene Expression

Significant differences ($P < 0.05$) were found in sperm-binding indexes between OECs and LLC-PK1 cells, co-culturing time, and boar ejaculate (Fig. 6). A significant interaction ($P < 0.05$) between these three factors was also observed, thereby indicating that an individual-boar effect existed. The sperm-binding indexes were significantly higher in OECs than in LLC-PK1 cells at all the co-culture periods, and started to decrease after 3 hr of co-culture.

When the viability of spermatozoa bound to OECs or LLC-PK1 cells and unbound sperm populations were examined (Fig. 7), it was seen that the percentage of viable spermatozoa was higher in those sperm populations bound to epithelial cells, both to OECs and LLC-PK1 cells, than those that remained unbound. In addition, after 12 and 24 hr

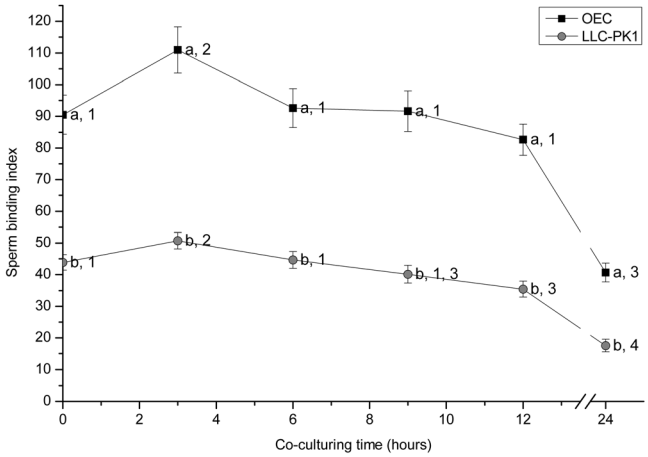

Figure 6. Sperm-binding index (mean ± standard error) in sperm populations bound to either OECs or LLC-PK1 cells, in co-cultures without insert. Results are from 16 independent experiments. Different letters (a and b) mean significant differences ($P < 0.05$) between cell types within a given time point, whereas different numbers (1–4) mean significant differences between time points within cell type (OEC or LLC-PK1).

of co-culture, the percentage of viable spermatozoa bound to epithelial cells was higher for OECs than in LLC-PK1 cells.

As far as morphology of the bound- and unbound-sperm population is concerned (Table 1), the percentage of morphologically normal spermatozoa was significantly ($P < 0.05$) higher in those spermatozoa that bound to epithelial cells than in unbound populations at all relevant time points. In addition, a significant increase ($P < 0.05$) of

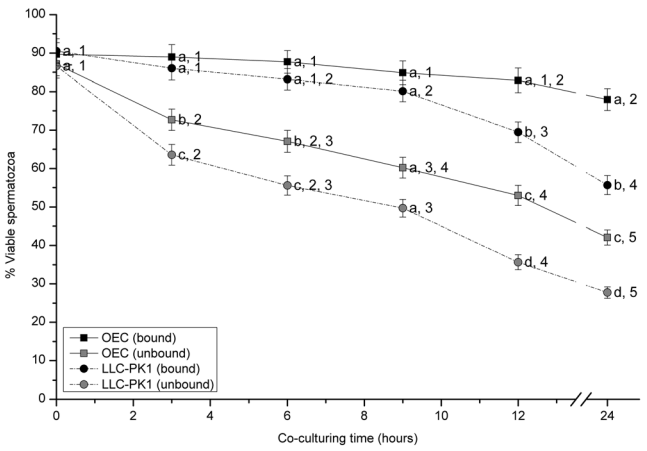

Figure 7. Sperm viability, as percentage of viable spermatozoa (mean ± standard error), in bound and unbound sperm populations in co-cultures without insert. Results are from 16 independent experiments. Different letters (a–d) mean significant differences ($P < 0.05$) between cell types and bound/unbound 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.

TABLE 1. Sperm Morphology, as Percentage of Morphologically Normal Spermatozoa, in Bound- and Unbound-Sperm Populations in Co-Cultures Without Insert

	0 hr	3 hr	24 hr
Bound sperm population			
OECs	$84.0 \pm 3.2^{a,1}$	$93.1 \pm 3.4^{a,2}$	$93.8 \pm 3.5^{a,2}$
LLC-PK1 cells	$83.4 \pm 3.1^{a,1}$	$90.2 \pm 3.3^{a,2}$	$92.6 \pm 3.2^{a,2}$
Unbound sperm population			
OECs	$70.3 \pm 2.7^{b,1}$	$64.8 \pm 2.4^{b,2}$	$65.0 \pm 2.5^{b,2}$
LLC-PK1 cells	$72.1 \pm 2.6^{b,1}$	$63.5 \pm 2.3^{b,2}$	$64.2 \pm 2.5^{b,2}$
Negative control	$75.2 \pm 3.0^{c,1}$	$72.8 \pm 2.9^{c,1}$	$73.5 \pm 2.7^{c,1}$

Results are from 16 independent experiments. Different letters (a–c) indicate significant differences ($P < 0.05$) between cell types and negative control, and bound/unbound populations within 0, 3, or 24 hr of co-culture, whereas different numbers (1–2) indicate significant differences between 0, 3, or 24 hr of co-culture within a given combination of bound/unbound population and cell type, or negative control. Mean ± standard error shown.

morphologically normal spermatozoa, with respect to 0 hr, was seen in bound sperm populations at 3 hr and continued to the end of co-culture period. This increase was concomitant with a significant decrease in the percentage of morphologically normal spermatozoa in unbound sperm populations (both in OEC and LLC-PK1 co-cultures) from 3 to 24 hr of co-culture, with respect to 0 hr. In general, those spermatozoa that attached to epithelial cells were viable and morphologically normal, whereas the percentages of similar spermatozoa were significantly ($P < 0.05$) lower in unbound sperm populations. As sperm-binding indexes were significantly ($P < 0.05$) higher in OECs than in LLC-PK1 cells from the beginning of the experiment, the cells of oviductal origin were more able to maintain sperm survival than those of renal origin.

Given that an individual effect from boar ejaculate was seen both in sperm-binding indexes and HSPs expression, the relationship between sperm-binding index and expression of *CLU*, *HSP90AA1*, *HSPA5*, and *HSPA8* was also investigated using linear-mixed models and Pearson correlation in co-cultures without insert. No effect ($P > 0.05$) of sperm-binding index, epithelial cell type (OEC vs. LLC-PK1), or interaction between type of epithelial cell and sperm-binding index were observed in *CLU* expression during the entire co-culture period. Sperm-binding indexes and *CLU*-transcript abundance were found not to be correlated ($P > 0.05$) at any of the time points investigated. In contrast, the relative abundances of *HSP90AA1*, *HSPA5*, and *HSPA8* transcripts were significantly ($P < 0.05$) affected by sperm-binding index and epithelial cell type (OEC vs. LLC-PK1), based on the linear-mixed model. A relationship between sperm-binding index and epithelial cell type was also observed in all the three cases ($P < 0.05$). Significant correlation coefficients ($P < 0.05$) were found between HSP-transcript abundances and sperm-binding indexes after 3 hr (*HSP90AA1*, $r = 0.39$), 6 hr (*HSP90AA1*, $r = 0.45$; *HSPA5*, $r = 0.41$; *HSPA8*, $r = 0.39$), 9 hr (*HSP90AA1*, $r = 0.48$; *HSPA5*, $r = 0.43$; *HSPA8*, $r = 0.40$), and 12 hr of co-culture (*HSP90AA1*, $r = 0.51$; *HSPA5*, $r = 0.44$; *HSPA8*, $r = 0.37$).

DISCUSSION

The present study was undertaken to verify earlier reports that spermatozoa are capable of upregulating OEC gene expression in vitro (Yeste et al., 2009) and to determine if the presence of spermatozoa specifically alters the expression of four genes in reproductive cells (OEC) rather than nonreproductive cells (LLC-PK1), during in vitro co-culture. The four genes investigated were deliberately chosen because the proteins they encode—namely, CLU, HSP90AA1, HSPA5, and HSPA8—are found within a subfraction of soluble apical plasma membrane proteins known to bind to boar spermatozoa (Elliott et al., 2009) and may have potential roles as modulators of sperm function (e.g., viability) (Lloyd et al., 2008, 2009; Elliott et al., 2009).

Expression of *HSP90AA1*, *HSPA5*, and *HSPA8*, but not *CLU*, genes was progressively upregulated in OECs over the co-culture period when epithelial cells and spermatozoa were in direct contact. This outcome under specific interacting conditions may be attributable to various roles of HSPs, 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-zona pellucida binding (Marín-Briggiler et al., 2010), whereas HSPA8 appears to be involved in maintaining sperm viability and has the ability to repair 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 individual HSPs may not modulate sperm function in exactly the same way might explain why *HSP90AA1* and *HSPA5* transcript levels were upregulated in OECs earlier than *HSPA8* in the co-culture period with spermatozoa.

In contrast to 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 OECs and only when spermatozoa could directly bind the epithelial cells. Our previous results showed that spermatozoa that bind to OECs remain viable for longer than those that bind to nonreproductive 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 cells in response to the presence of spermatozoa might explain the differential ability of the two epithelial cell types to support sperm survival. In addition, maintenance of sperm viability was better when spermatozoa were co-cultured with LLC-PK1 cells 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, 2012), indicates that the nonspecific induction of HSP expression in LLC-PK1 cells, which was lower than that from OECs, could be related to the observed prolonging effect on sperm viability.

The similar gene expression profiles between follicular and luteal OECs, on the other hand, probably explains why previous investigators found that OECs (Suárez et al., 1991; Fazeli et al., 1999; Petrunkina et al., 2001) and apical plasma membranes (Fazeli et al., 2003) derived from sows in different phases of the reproductive cycle support sperm viability equally in vitro. It is not clear whether the lack of the differences between the follicular and luteal stages is due to the nature of the oviduct epithelium with respect to the endometrium or is the result of cellular dedifferentiation and manipulation during in vitro culturing, as has been documented in other reports describing the in vitro culture of cells of reproductive origin (Bassols et al., 2004, 2007). Specific environmental conditions, such as heat stress, have also been found to upregulate *HSP90AA1* expression in bovine OECs (Kobayashi et al., 2013).

In the present study, we did not investigate the effects of other biological (e.g., oocytes, embryos, bacteria, etc.) or nonbiological (e.g., glass-beads) entities on chaperone/HSP gene expression by OECs. Notwithstanding, existing evidence suggests that OECs 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 made herein, that HSP gene expression is only markedly upregulated when spermatozoa are in direct contact with OECs rather than when they are kept separate using diffusible membrane inserts, tends to support this view. In agreement with other studies (Aldarmahi et al., 2012, 2014), we thus propose that spermatozoa binding directly to OECs in co-culture activates a specific signal transduction pathway within the OECs that results in the upregulation of HSP gene expression. HSPs synthesized de novo in response to sperm appear to translocate from within OECs to the oviductal lumen (Georgiou et al., 2007) and interact directly with the sperm membrane, probably through cholesterol molecules and/or lipid rafts that are present/accessible in uncapacitated but not in capacitated spermatozoa (Moein-Vaziri et al., 2014). Interestingly, the importance of direct contact between OECs and spermatozoa has been highlighted previously in the bovine, where indirect contact stimulated only a fraction of the protein production changes observed when spermatozoa and OECs were in direct contact (Ellington et al., 1993). It remains to be determined if the protein abundance of the genes investigated previously mirrors the mRNA abundance observed in the present study, although this correlation seems likely (Georgiou et al., 2007). Should this be the case, additional work would be required to elucidate whether such proteins are destined for the OEC surface (Sostaric et al., 2006) or secretion (Georgiou et al., 2005, 2007).

Previous reports have already determined that cells of reproductive origin maintain better sperm function and survival than those from nonreproductive origin (Fazeli et al., 1999; Yeste et al., 2009, 2012), and that direct contact between OECs and sperm is needed to maintain sperm survival in vitro. In addition, the selective binding capacity previously reported (Yeste et al., 2012) is confirmed by the present work, wherein the sperm populations that

remained bound to OECs and LLC-PK1 cells were viable and morphologically normal. As the sperm-binding indexes were higher in OEC than in LLC-PK1 co-cultures, these results also confirm the superior ability of OECs to maintaining sperm survival of viable and morphologically normal spermatozoa.

One of the most interesting findings of this study is that not all spermatozoa from all boars had the same ability to bind to cells, and therefore to alter the contact-dependent expression of HSPs by these epithelial cells. It is worth noting that in this work, the sperm-binding index and the viability and morphology of sperm populations bound-to and unbound-from epithelial cells were determined in separate well plates, but the spermatozoa were derived from the same ejaculates and the OEC/LLC-PK1 monolayers were from the same split. From our data, we thus suggest that boar sperm quality from individual ejaculates contributes to the differences observed in sperm binding and on the ability of sperm to alter HSPs expression.

Previous reports failed to show differences between breeds in the ability of sperm to modulate the expression of different relevant genes (adrenomedullin, HSPA8, and prostaglandin E synthase) (Aldarmahi et al., 2012). From our data, it seems that individual rather than breed differences are contributing factors, as all our boars came from the same breed. This finding is similar to what has been observed by others, even though in some cases cryopreserved rather than fresh spermatozoa were used (Waterhouse et al., 2006; Pinart and Puigmulé, 2013). As HSP expression was also found to be correlated with and influenced by sperm-binding indexes throughout co-culture, direct contact (i.e., without a diffusible membrane insert) and the number of sperm that bind to epithelial cells are relevant for upregulating gene expression. Further, we should bear in mind that the spermatozoa bound to the epithelial cells were mainly viable and morphologically normal. Together, these observations could partially explain why individual ejaculate differences in the ability to upregulate HSP expression were seen, and would suggest that the initial sperm quality of a given ejaculate may influence the ability of such ejaculates to alter HSP expression. This hypothesis, along with our findings, warrants more research on this topic.

In conclusion, the present study shows for the first time that boar spermatozoa alter the expression of HSP genes directly, quickly, and markedly in co-cultured OECs, and that an individual boar ejaculate effect exists. Our results confirm previous reports assessing protein expression in vitro (Ellington et al., 1993), 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). More research is warranted to evaluate if the absence of in vivo oviductal milieu (stroma and steroid hormones) has any impact on the ability of OECs to be influenced by the presence of sperm. 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.

MATERIALS AND METHODS

Growth Medium

OECs were cultured in medium 199 (TCM199 containing Earle's, L-glutamine [0.1 g/L], and HEPES [25 mM]; Sigma–Aldrich®, St. Louis, MO) supplemented with 10% (v/v) heat-inactivated fetal 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., Paisley, UK). The growth medium was stored at 4°C and filtered with 0.22-μm filters (Pall-Gelman Laboratory, Ann Arbor, MI) before use.

Co-Culture Medium

OEC 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/ml bovine serum albumin [BSA] [Sigma–Aldrich®], pH = 7.2; Parrish et al., 1988; Fazeli et al., 1999).

OEC Culture

OECs were cultured following the method described by Fazeli et al. (1999). Briefly, oviducts were collected with ovaries at a local abattoir from cycling gilts (8–10 months of age), and the follicular-phase oviducts were separated from the luteal-phase oviducts after judging the appearance of the ovaries. They were then washed with phosphate-buffered saline (PBS) (Gibco), stored at 27–30°C, and transported within 3 hr post mortem to the laboratory. The oviducts were then 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®) diluted to 0.25% (w/v) in Hanks' Balanced Salt Solution (HBSS) (Life Technologies, Paisley, UK) on a Petri dish, the extremes closed, and then incubated at 37.5°C and 5% CO_2 for 2 hr.

The oviducts were then milked, and the cells recovered and centrifuged (5 min at 100*g* 20°C) 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/ml into flasks, and cultured at 37.5°C and 5% CO_2 until confluent (after approximately 6–7 days). The growth medium was refreshed every 48 hr.

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 37.5°C and 5% CO_2 for 30 min. Digestion stopped by adding 10 ml of supplemented TCM199. The TCM199 (containing the cells) was centrifuged for 5 min at 100*g* 20°C. The pellet was resuspended in one of two ways,

either for RNA extraction or for cryopreservation, depending on the next objective.

Verification of Epithelial Cell Nature in OEC Cultures

Cytokeratins are characteristic protein components of epithelial cells (Dobrinski et al., 1999), so the epithelial nature of the OECs was verified by immunocytochemistry using a cytokeratin primary antibody. OECs grown for 20 hr in 24-well culture dishes were fixed at 4°C in 3% formaldehyde (Merck, Darmstadt, Germany) and 60 mM saccharose (Merck) in 0.1 M PBS (Gibco) for 30 min. Next, the cells were washed three times with 10 mM PBS and permeabilized for 10 min in a solution of 10 mM PBS containing 0.1% Triton X-100. Following another 5-min, 10 mM PBS wash, the cells were incubated for 10 min in 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 min at 37.5°C. They were then rinsed twice in PBS for 10 min and incubated with a rabbit anti-mouse IgG FITC-conjugated secondary antibody (1:50 dilution; Dako Diagnostics S.A., Barcelona, Spain) at 37.5°C for 30 min. After washing twice in PBS for 10 min, the nuclei of the cells were counterstained with 5 μM bisbenzimide Hoechst 33258 (Sigma–Aldrich®; id. 861405, empirical formula: $C_{25}H_{24}N_6O \cdot 3HCl \cdot xH_2O$) for 7 min. Finally, the cells were mounted with mounting medium (Sigma–Aldrich®, id. 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 nonepithelial (cytokeratin immunoreactivity absent). Mean ± standard error (95.61 ± 1.30%) of OECs showed positive cytokeratin immunoreactivity, confirming their epithelial nature.

OEC Cryopreservation and Thawing

Following trypsinization, approximately 10^6 OECs were harvested for cryopreservation in freezing medium that consisted of 10% dimethyl sulfoxide (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 labeled cryogenic vial (Nalgene, Rochester, MI) before immediate transfer to a −20°C freezer for 20 min and then to −80°C.

When needed, the cryovials were taken from the freezer at −80°C and incubated in a water bath for 2 min at 37°C 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*g* and 20°C for 5 min and then the supernatant were discarded. The pellet was resuspended with growth medium, and cells seeded into a flask containing 10 ml of growth medium.

LLC-PK1 Culture

Pig kidney epithelial cells (LLC-PK1; American Tissue Type Culture Collection-LGC Promochem, UK) were seeded into a flask at a concentration of 10^6 cells/ml. 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 37.5°C 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.

Experimental Design

Figure 8 shows a representative scheme of the experimental design performed in the present work. Confluent monolayers of follicular OECs, luteal OECs, and LLC-PK1 cells were established in six-well plates, in triplicate. To prevent contamination, a total of nine plates were set for evaluation at each relevant time point. Three plates contained follicular OECs, three contained luteal OECs, and the remaining three contained LLC-PK1 cell cultures. For each epithelial cell type (i.e., follicular OEC, luteal OEC, or LLC-PK1), two sets of plates did not contain a diffusible membrane insert (0.4 μm; Millipore Corp., Bedford, MA, USA), while the other set did. The purpose of this insert was to keep the spermatozoa and epithelial cells separate, while 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 with either 3 ml of washed spermatozoa (final concentration of 1.87×10^6 spermatozoa/ml) in TALP medium or with TALP medium without spermatozoa (negative controls of cells without spermatozoa). The co-cultures and negative controls were incubated at 37.5°C, 100% humidity, and 5% CO_2. Just before the relevant time points (0, 3, 6, 9, 12, and 24 hr 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) 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, while the other was used to evaluate gene expression of OECs and LLC-PK1 cells. Those six-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 trypsinized for RNA extraction, or used for evaluating bound and unbound sperm populations, as described. In the case of trypsinization for RNA extractions, the expression of beta actin (*ACTB*), *CLU*, *HSP90AA1*, *HSPA5*, and *HSPA8* was determined in both the co-cultures and negative controls using real-time quantitative PCR.

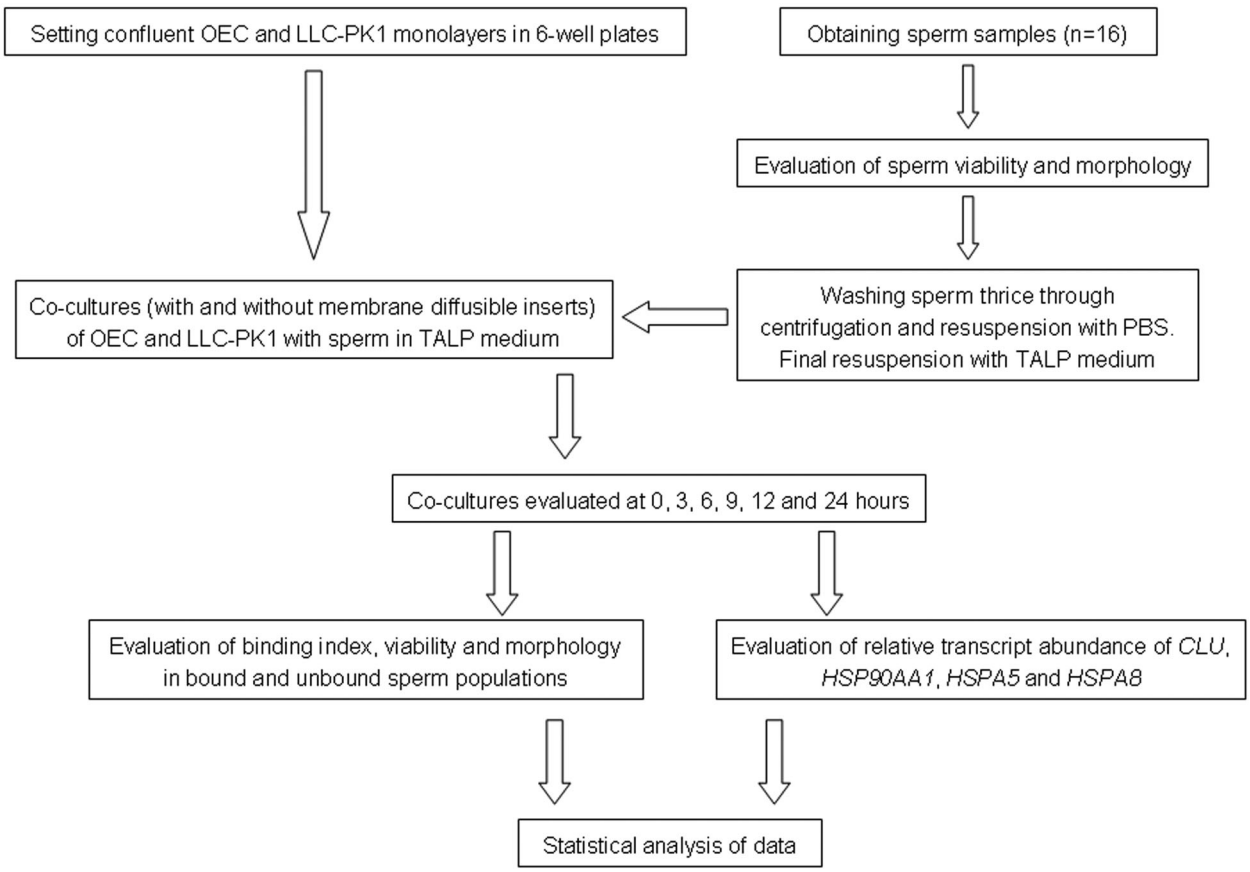

Figure 8. Representative scheme about the experimental design conducted in this work. This experimental design was repeated 16 times using sperm samples coming from 16 different boars.

The experimental design was replicated 16 times using spermatozoa from 16 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).

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. The rate of semen collection was twice a week. No fertility problems were recorded by the artificial insemination 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 (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 were evaluated following the protocols described in the following sections (percentage of viable spermatozoa, mean $\pm$ standard error: $89.7 \pm 3.2\%$; percentage of morphologically normal spermatozoa: $80.1 \pm 2.8\%$). Following this, sperm was washed three times with PBS by centrifugation collection at 600*g*. Final resuspension was in TALP medium.

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 cells) 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 $\pm$ standard error.

Analysis of Viability, Morphology, and Binding Index of Sperm Population Bound to Epithelial Cells

At each relevant time point, and in those six-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 the cell-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 37.5°C and 5% CO_2 for 15 min, samples were examined under an inverted epifluorescence microscope (Nikon Eclipse Ti-U) with Nikon S-Plan Fluor ELWD 20×/0.45 and Nikon S-Plan Fluor ELWD 40×/0.60 objective lens. In each of the three wells, 100 spermatozoa were counted and classified as viable (green) or nonviable (red), using B2A (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 were calculated per plate, each one corresponding to a relevant time and a given epithelial cell type.

The number of spermatozoa bound to epithelial cells (OEC follicular, OEC luteal, and LLC-PK1 cells) 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 fields. The sperm-binding index was calculated as the number of sperm bound to epithelial cells in a monolayer surface of 0.05 mm^2, as described in Yeste et al. (2012). Three parallel co-cultures (serving as replicates) were performed for each case within each relevant time point, and the means ± standard error were calculated.

The morphology of the spermatozoa bound to epithelial cells was evaluated in the other three wells using an inverted phase-contrast microscope (Zeiss Axiovert 45) equipped with a warming plate (set at 37.5°C) and a digital camera (Axiocam ICc1). Sperm morphology was assessed after fixing co-cultures with a 0.1 M PBS solution containing 2% paraformaldehyde for 20 min at 4°C. Next, samples were examined at a magnification of 200× (Zeiss Achrostigmat 20 × 0.30 objective lens), and differences between morphologically normal spermatozoa, spermatozoa with proximal and cytoplasmic droplets, and aberrant spermatozoa were noted. Within the group of aberrant spermatozoa, the following distinctions were made: aberrant head morphologies, coiled tails, tails folded at the connecting or intermediate piece, and those tailed at Jensen's ring (Yeste et al., 2008). Fields were randomly selected and 100 spermatozoa were analyzed for each well. Only those spermatozoa clearly attached to the epithelial ells were taken into account. Three replicates were done, and means ± standard error were calculated.

Analysis of Viability and Morphology of Unbound Sperm Population

Sperm viability, morphology, and concentration of unbound spermatozoa that were freely swimming in the co-culture wells were evaluated following a similar procedure to that described for the bound sperm population, with some minor modifications. Again, three wells were used for evaluation of sperm viability, while the other three were utilized for determination of sperm morphology. Although 3 ml of TALP medium containing the unbound spermatozoa were taken from all wells, only 1 ml of each was used to evaluate either sperm viability or sperm morphology. In the case of sperm viability, spermatozoa were also stained with SYBR-14 and EthD-1, which is why 10 μl of a 20 μM SYBR-14 at stock solution and 1 μl of a 2.2 mM EthD-1 stock solution were added to 1 ml of TALP medium containing the unbound spermatozoa. Samples were then incubated at 37.5°C for 15 min prior to examination under a conventional epifluorescence microscope (Leica DMLR-XA; Leica, Germany) with Leica 40 × 1.32 HCX PL APO objective lens, and two filter cubes: I3 (excitation filter: BP 450−490 nm; dichromatic mirror: 510 nm; suppression filter: LP 515 nm) for the observation of green (SYBR-14) fluorescence and N2.1 (excitation filter: BP 515−560 nm; dichromatic mirror: 580 nm; suppression filter: LP 590 nm) for the assessment of red (Eth-D1) fluorescence. One hundred spermatozoa were counted per well, and three wells were examined prior to calculating the corresponding means ± standard error. The criteria for considering a spermatozoon as viable were the same as that explained for the bound sperm population.

Sperm concentration and morphology were assessed using a phase-contrast microscope (Olympus BX41) at a magnification of 100× (Olympus 10 × 0.30 PLAN objective lens, negative phase-contrast field) and using a Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel). A total of three samples, each coming from a different well, were examined, prior to calculating the means ± standard error.

Sperm morphology was evaluated in accordance with the same criteria used for the bound sperm population. Prior to evaluation of their morphology, spermatozoa were fixed with 3% formaldehyde saline solution to immobilize the spermatozoa. Thereafter, 5 μl of each sample were subsequently placed on a slide and mounted with

cover glass. Preparations were evaluated at a magnification of 200× (Olympus 20 × 0.40 PLAN objective lens, positive phase-contrast field), and 100 spermatozoa were analyzed in each sample, which came from a different well. Three individual samples (technical triplicates) were examined, and the corresponding means ± standard error were calculated.

RNA Extraction and Quantification

The extraction of total RNA from cells was carried out using an RNAqueous®-4PCR kit (DNA-free RNA isolation for reverse-transcriptase PCR) (Ambion, Inc., Austin, TX), following the manufacturer's instructions. After extraction, the RNA was incubated with DNaseI to ensure the removal of contaminating DNA. The amount of RNA and its purity was determined by spectroscopy (GENESYS™ 10 UV/Vis Spectrophotometer, Thermo Scientific, Loughborough, UK) at wavelengths of 260 and 280 nm. The purity of the RNA was estimated by taking the quotient of the absorptions at 260 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.

Reverse Transcription: Conventional Polymerase Chain Reaction

RNA extracted from the cells was reverse transcribed to produce cDNA using the Reverse Transcription System kit (Promega, Madison, WI), according to the manufacturer's instructions. Briefly, each 20-μl 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 with a balance of 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, reverse-transcriptase products were discarded and not analyzed further. Furthermore, a reaction containing all the reagents above but no RNA (no template control) was set up to screen the reverse-transcription reagents for contaminants. The reactions were carried out at 42°C for 2 hr using a GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, CA). The resulting cDNA was then diluted 1:5 in sterile, nuclease-free water (Promega) and stored at −20°C until needed.

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 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 verified using a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) prior to purchase from MWG Biotech AG (Milton Keynes, UK). All primers were diluted to 50 pmol/μl 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 2.

In preliminary experiments, expression of *ACTB*, *CLU*, *HSP90AA1*, *HSPA5*, and *HSPA8* was examined and sequenced by conventional reverse-transcriptase PCR, to confirm the expected amplicon sizes and annealing temperature (60°C) of primers. Endpoint reverse-transcriptase PCR was performed on each cDNA sample using a GeneAmp® PCR system 9700 (Applied Biosystems, Foster City). Each reaction contained: 200 ng cDNA, 5 μl 1× PCR buffer, 2.5 mM $MgCl_2$, 0.8 mM dNTPs, 500 nM of each forward and reverse primer (see Table 2 for details),

TABLE 2. Primers Used

Gene	Primer	Primer sequence	Length (bp)	EMBL accession number
ACTB	Forward	5′-TTCGAGACCTTCAACACGCCG	345	NM_001009784
	Reverse	5′-GTTGCCGATGGTGATGACCTG		AJ312183
				M10277
CLU	Forward	5′-ATCACTGCTCAGCTCCCTGGA	279	NM_001831
	Reverse	5′-ATGTCCATCACGTGGCTCTGC		NM_013492
				NM_053021
				NM_213971
HSP90AA1	Forward	5′-TTCAGCCTAGATGCCCGAGGAAA	219	NM_213973
	Reverse	5′-ATGTGCAGCTCTTTCCCGGAGTC		U94395
				X15183
HSPA5	Forward	5′-GAAGGAGAAGACTTCTCGGAGAC	574	NM_005347
	Reverse	5′-TGCCTGTACCTTTGTCTTCAGC		NM_013083
				NM_022310
				DQ_029323
				XM867578
HSPA8	Forward	5′-GGACCTGCAGTTGGCATTGATCT	362	NM_174345
	Reverse	5′-TAGCCTGACGCTGAGAGTCGTTA		CX062900
				CX061052

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 95°C for 5 min, followed by the optimal number of cycles determined separately for each primer pair of: denaturation at 94°C for 30 sec, 60°C for 30 sec, and elongation at 72°C for 30 sec. Each reaction also had a final elongation step of 72°C for 7 min. 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) was conducted during the exponential phase of the PCR cycle. Reverse-transcriptase PCR products were separated using 2% (w/v) agarose gel (Bioline, London, UK) electrophoresis. Gels were stained with 0.6 μg/ml ethidium bromide in 1× TAE (TrisAcetat-EDTA; 90 mM Tris, 90 mM acetate, 2 mM EDTA) and products were visualized using ultraviolet light (SynGene Gene Genius system, Synoptics Ltd., Cambridge, UK).

The identity of the reverse-transcriptase PCR products generated was confirmed via DNA sequencing and BLAST search. Briefly, the products were purified using the QIAquick® PCR purification kit (Qiagen Ltd., Crawley, UK), according to the manufacturer's instructions. The purified products were then sequenced using the same forward and reverse primers (0.8 pmol/μl) 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, Warrington).

Real-Time Quantitative PCR

Quantitative PCR (qPCR) was performed on each cDNA sample using a 7500 Real-Time PCR system® (Applied Biosystems, Foster City) and Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City). After optimizing primer concentrations for two-step reverse-transcriptase qPCR, following the standard protocol recommended by the manufacturer, PCR reactions were run 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, Foster City), 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 evaluating the samples, the efficiency and specificity of all primers were determined by analyzing melting curves, according with Power SYBR® Green PCR Master Mix's instructions. Serial dilutions of cDNA samples were also used as template with the appropriate concentration of primers and 1× Power SYBR® Green PCR Master Mix in a total volume of 25 μl.

qPCR reactions were performed as follows: one cycle of denaturation at 95°C for 5 min, 40 cycles of amplification with denaturation step at 94°C for 15 sec, annealing step for 30 sec at the appropriate annealing temperature of primers, and extension step at 72°C for 40 sec. Fluorescence data were acquired during the 72°C extension steps. The melting protocol consisted of heating samples from 50 to 94°C, 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 OECs or LLC-PK1 cells) without spermatozoa (negative controls). Thus, f $\Delta\Delta C_T$ equaled to zero or these controls. 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 was found to be constantly expressed in OECs and LLC-PK1 cells during all the co-culturing period.

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 analyzed with SPSS 19.0 for Windows (SPSS, Inc., Chicago, IL). 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 nonnormal data, an arcsine square root (x) transformation was carried out prior to analysis in order to stabilize the error variance.

First, relative abundances of all gene transcripts were compared between monolayers (OEC follicular, OEC luteal, and LLC-PK1 cells) 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 included the type of cultured cells (OECs, LLC-PK1 cells, 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 the type of cultured cells as the 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.

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