Fusion as the result of sperm–somatic cell interaction

M Mattioli, A Gloria, A Mauro, L Gioia and B Barboni

Department of Comparative Biomedical Sciences, University of Teramo, Piazza A. Moro, 45, 64100 Teramo, Italy

Correspondence should be addressed to A Gloria; Email: gloriaalessia@libero.it
B Barboni; Email: bbarboni@unite.it

Abstract

The research has been designed to investigate whether acrosome-reacted spermatozoa can fuse with somatic cells and to check whether this event may involve the molecular machinery implicated in the sperm–egg fusion. Boar spermatozoa were capacitated *in vitro* and then treated with A23187 to induce acrosome reaction and activate their fusogenic potential. Reacted spermatozoa, loaded with the membrane-permeant fluorescent dye calcein AM, were incubated with plated granulosa cells or cells derived from stable cell lines: CRFK, VERO, and ESK4. The fusion between spermatozoa and somatic cells was revealed by the diffusion of the fluorescent dye from the sperm to the cell as membrane fusion and cytoplasmic continuity between the two cells were established. The involvement of integrin $\alpha 6$ and tetraspanin CD9 in the process of fusion was assessed by carrying out the experiment in the presence of antibodies against these molecules. Moreover, the incidence of fusion displayed by the different cell types used was analyzed in relation to their content in the above molecules assessed by western blot and immunostaining. The role of CD9 was additionally investigated by using CD9-negative cells. The data presented demonstrate that boar spermatozoa can fuse with different somatic cell types derived from different species and the process requires the combined presence of both integrin and tetraspanin molecules on the cell plasma membrane.

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Introduction

Fertilization in mammals requires the completion of several steps that begin with the deposition of the sperm into the female genital tract and end with sperm–egg membrane fusion, the culminating event that brings together paternal and maternal genomes and triggers the activation of embryo development.

Cell fusion is generally believed to be a rare event, restricted to specific cell types, which consists in the fusion of the membrane lipid bilayers with consequent cytoplasm mixing of two different cells. Membrane fusion occurs in animal systems at three different levels: viral–host cell fusion, intracellular vesicles fusion, and cell–cell fusion. Of these three contexts, cell–cell fusion is comparatively the most poorly understood process and the membrane factors involved are still far from clear despite the physiological relevance of this phenomenon. In the case of sperm–egg interaction, membrane fusion occurs after a long and complex series of sequential approaching interactions that assume the meaning of a highly selective process at the end of which only the spermatozoa, which are more suitable for fertilization, can reach the oocyte. The sperm encounters two layers surrounding the oocyte, the expanded cumulus and the zona pellucida (ZP), which must be crossed before sperm–egg fusion. At the ZP level, a rather complex molecular interaction occurs with the proteins of the ZP

that triggers the acrosome reaction (AR) in the sperm cell (Podell *et al.* 1984, Florman & Wassarman 1985, Wassarman 1987, 1990, Hardy & Garbers 1994, Metz & Palumbi 1996, Oehninger 2001, McCartney & Lessios 2004). Reacted spermatozoa may then cross the ZP and enter the perivitelline space where the conclusive phase of the sperm–egg interaction is played, eventually ending in gamete membrane fusion and in the sperm components, including the nucleus, finally entering into the ooplasm (Yanagimachi 1994).

The process of cell–cell fusion involves three key events: 1) membrane recognition, mainly due to ligand–receptor binding; 2) membrane apposition, where membrane protein interactions bring the membranes close together; and 3) lipid bilayers mixing, involving the sequential fusion of the proximal and of the distal layers. At this point, cytoplasmic continuity between the two cells is established. The precise molecular mechanisms involved in this process have attracted the attention of many researchers, but the limitation of available material and the problems related to the study of protein–protein interactions have not allowed to definitively clarify this topic yet. However, experimental evidence indicates a list of potential candidates that may take part in cell fusion by acting on the two cell sides. Sperm proteins belonging to the ADAM family (a disintegrin and metalloprotease), particularly fertilin β (ADAM2) and cyritestin (ADAM3), present on the sperm membrane,

have been shown to play a central role in sperm–egg binding and fusion by interacting with specific ligands (integrins) on the egg membrane. Integrins are trans-membrane $\alpha\beta$ heterodimers that play crucial roles in cell–cell adhesion, cell–extracellular matrix adhesion, and multiple signaling pathways. The integrin $\alpha 6\beta 1$ was first proposed as the receptor for sperm on mouse egg by Almeida *et al.* (1995). Since then a number of reports have confirmed the expression of integrin $\alpha 6\beta 1$ on the membrane of both gametes and the involvement of this protein in sperm–egg interaction (Chen & Sampson 1999, Linfor & Berger 2000, Barraud-Lange *et al.* 2007). Nevertheless, the immunoneutralization experiments aimed at blocking the function of this integrin have provided disparate results, according to the technique, enzymatic, chemical, or mechanical, used to remove the ZP (Almeida *et al.* 1995, Evans *et al.* 1997, Evans 1999). Moreover, oocytes from integrin $\alpha 6\beta 1$ -deficient mice showed normal binding and fusion with sperm, suggesting that integrin is not essential for gamete fusion in mice (Miller *et al.* 2000).

More recently, another surface protein, CD9, has been discovered to play an essential role in sperm–egg fusion in mouse as well as in other species (Chen *et al.* 1999, Kaji *et al.* 2000, Miyado *et al.* 2000, Le Naour *et al.* 2000, Zhu *et al.* 2002, Li *et al.* 2004, Zhou *et al.* 2009). The involvement of CD9, a member of the tetraspanin family, has been documented by the inhibition of sperm–egg fusion recorded in the presence of anti-CD9-specific antibodies, as well as by using egg of CD9-knockout mice in IVF systems. Ziyyat *et al.* (2006) have proposed a functional link between these two molecules suggesting that CD9 controls the membrane redistribution of surface proteins, including integrins, into clusters that may be necessary for sperm–egg interaction.

Interestingly, the proteins involved in membrane fusion, such as integrins and CD9, are widely diffused on the plasma membranes and are highly conserved. Many cell types, like granulosa cells (GC), express CD9 as well as integrin $\alpha 6\beta 1$ associated on their membrane, just like the egg, and could sustain an interaction with sperm cells ending in cell–sperm fusion (Takao *et al.* 1999). The possibility that spermatozoa might interact with somatic cell types may provide new models to investigate the molecular basis of cell fusion. This might increase the investigation potential providing increased availability of biological material, different membrane protein arrangements, and the standardized membrane structure of specific cell lines. Moreover, an obvious potential exploitation of this sperm–cell interaction could be the development of reproducible and reliable tests to assess sperm–cell function and add further steps in the knowledge of the process of fertilization. The present research has therefore been designed to investigate whether acrosome-reacted spermatozoa can fuse with somatic cells of different origin. Moreover, the use of function-blocking antibodies and western blots'

analysis have been carried out to assess whether this spermatozoa–cell fusion involves the same molecular machinery that is involved, as far as we know, in the sperm–egg fusion.

The data presented do show that boar spermatozoa can fuse with different somatic cell types derived from different species and that fusion requires the combined presence of integrins and tetraspanins.

Results

Functional fusion assay

Boar spermatozoa were capacitated *in vitro* and then exposed to A23187 to induce AR and activate their fusogenic potential. Freshly ejaculated spermatozoa displayed a percentage of spontaneous AR of $3.1 \pm 1.5\%$ (3.1 ± 0.5 , $n=100$). The proportion of acrosome-reacted spermatozoa at the end of the capacitating incubation was $13.2 \pm 4\%$, while the percentage rose to $48.7 \pm 6.6\%$ in the spermatozoa exposed to A23187 ($P \leq 0.001$).

When A23187-reacted spermatozoa were added to cell preparations, a proportion of plated cells became intensely fluorescent (Fig. 1; right panel) due to the dye diffusion from fused sperm. When the functional fusion assay (FFA) was carried out by adding nonreacted spermatozoa (spermatozoa not exposed to A23187) to cultured cells, the percentage of fusion recorded never exceeded 2% and most of the cells were nonfluorescent even when fluorescent spermatozoa were close to them, as shown in Fig. 1 (left panel). The absence of fluorescence in these cells incubated together with acrosome-intact, calcein-loaded spermatozoa demonstrates that the dye does not leak from loaded spermatozoa and diffuse into plated cells, confirming that the simple presence or contact of loaded sperm with somatic cells does not result in cell fluorescence unless fusion takes place.

The incidence of cell fusion was clearly affected by the culture interval used to prepare plated cells (Fig. 2). After 4 h of culture, the cells, independently from the type, had just started to attach to the bottom of the coverglass chamber and retained a clear spherical shape, and low fusion rates were recorded when acrosome-reacted spermatozoa were added to these cells (VERO: 0.8 ± 0.2 ; ESK4: 0.73 ± 0.2 ; CRFK: 4.76 ± 0.68 ; GC: 3.73 ± 0.61).

When the culture of plated cells was carried out for 24 h, cells were attached to the bottom of coverglass displaying a polyedric shape and the incidence of cell fusion markedly increased after exposure to reacted spermatozoa (VERO: 3.2 ± 0.9 ; ESK4: 3.8 ± 0.81 ; CRFK: 20.6 ± 1.5 ; GC: 9.86 ± 2.9).

After a culture interval of 48 h, the cells were still attached but extremely flattened on the coverglass, and low fusion incidence was recorded after acrosome-reacted sperm addition (VERO: 0.58 ± 0.01 ; ESK4: 0.68 ± 0.1 ; CRFK: 0.83 ± 0.15 ; GC: 1.5 ± 0.5).

Figure 1 Representative epifluorescence images of sperm-cells fusion. Capacitated sperm cells, not exposed (left panel: A–E) or exposed (right panel: F–H) to A23187, were loaded with fluorescent calcein AM (green) and then co-incubated for 1 h with cell lines as described in Materials and Methods. Cell nuclei were identified with DAPI (C–E and J–H; blue). (B, D, and E) Absence of fusion between spermatozoa and CRFK cells. No somatic cell shows green fluorescence, and calcein is retained in the cytoplasm of intact sperm cells. (G, K, and H) Example of sperm–CRFK cells fusion (arrow). The green fluorescent dye has diffused into a somatic cell that fused with the sperm and entered into cytoplasmic continuity. The asterisk shows an example of the absence of sperm–cell fusion. The green fluorescence is visible only inside the intact spermatozoa even though they are close to the cell. (Magnification: 400 $\times$).

According to these preliminary results, only cells cultured for 24 h were used for the further FFA experiments.

Role of CD9 and integrin $\alpha 6$ in cell fusion process

The following experiments, aimed at analysing the molecular mechanism of sperm–cell fusion by using specific function-blocking antisera, were carried out exclusively with CRFK cells cultured for 24 h for their higher fusion potential. Zona-free metaphase II (MII) oocytes ($n=50$) were used as the reference cells naturally involved in cell fusion, and CD9 negative cells were used to further test the role of this specific molecule in the process of fusion.

As shown in Figs 3 and 4, anti-CD9 and anti-integrin $\alpha 6$ antibodies were able to significantly ($P \leq 0.001$)

reduce both sperm–egg and sperm–CRFK cell fusion. The percentage of sperm–egg fusion (i.e. the percentage of oocytes with at least one fused spermatozoon) was reduced from 83% in the absence of antibodies to 6.2% and to 10% in the presence of anti-CD9 and anti-integrin $\alpha 6$ antibodies respectively.

Similarly, the percentage of fusion was reduced in CRFK cells ($P \leq 0.001$) from values of 22% in the absence of antibodies to 6.3 and 2.7% when anti-CD9 and anti-integrin $\alpha 6$ were added respectively. By contrast, the addition of nonspecific rat anti-goat IgG did not affect the fusion rate in both oocytes and CRFK cells (79 and 20.8% respectively $P > 0.05$).

When FFA was carried out with Raji, the special cell type that does not express CD9, the incidence of fusion was as low as 0.5% ($P \leq 0.001$; Fig. 4).

CD9 and integrin $\alpha 6$ expression in somatic cells

Western blot analysis was carried out in the different cell types used for FFA in order to assess whether the different fusion capacity of these somatic cells was related to their integrin $\alpha 6$ and CD9 contents on the plasma membrane.

As shown in Fig. 5, CD9 protein was differently expressed in the cells types during 4, 24 and 48 h of culture. CD9, detected as a single 24 kDa protein band (Fig. 5A), was clearly expressed, at 4 h of culture, in CRFK cells (densitometric values (d.v.): 0.135 ± 0.03) and, to a lesser extent, in GC and VERO cells (d.v.: 0.077 ± 0.02 and 0.075 ± 0.01 respectively). On the contrary, in ESK4 and in negative control Raji cells, the detected levels were very low (d.v.: 0.025 ± 0.002 and 0.025 ± 0.001 respectively; Fig. 5A and B). CD9 protein expression levels strongly increased after 24 h of

Figure 2 Functional fusion assay analyses in (A) CRFK, (B) VERO, (C) ESK4, and (D) granulosa cells (GC) after 4, 24, and 48 h of culture. ^{a,b,c}Values statistically different ($P < 0.05$) during culture time within cell types.

Figure 3 Percentage values of zona-free mature oocytes fertilized (IVF) in the absence (CTR) and in the presence of nonspecific IgGs or after the pre-incubation with specific anti-CD9 or anti-integrin $\alpha 6$ antibodies. ^{a,b}Values statistically different ($P < 0.05$) between treatments.

culture only in CRFK (d.v.: 0.39 ± 0.01) and in GC cells (d.v.: 0.20 ± 0.02), but the expression was slightly down-regulated after 48 h (d.v.: CRFK 0.19 ± 0.01 and GC 0.13 ± 0.01). Differently, in the remaining cell types after 24 and 48 h of culture, the CD9 protein expression was not modulated and their quantitative levels were similar (d.v.: VERO 0.1 ± 0.005 and 0.06 ± 0.001 ; ESK4 0.025 ± 0.001 and 0.025 ± 0.002 ; Raji 0.025 ± 0.001 and 0.03 ± 0.02) to those observed after 4 h of culture (Fig. 5A and B).

Conversely to the increased CD9 expression recorded after 24 h of culture in CRFK and GC, the results of immunoblotting obtained about integrin $\alpha 6$ expression demonstrated that this protein, in all the cell types cultured for 24 h, was expressed at similar quantitative levels (d.v.: 0.25 ± 0.05 ; Fig. 6). Unchanged levels of the integrin $\alpha 6$ protein in these cells were observed at 4 and 48 h (data not shown).

Immunostaining using anti-CD9 and anti-human integrin $\alpha 6$ antibodies allowed to identify the distribution of the above molecules on the plasma membranes of the different cell types analyzed. While integrin $\alpha 6$ was similarly distributed on the membrane of all the cell types tested (data not shown), CD9 membrane

Figure 4 Percentage values of sperm-cell fusion recorded in CRFK after 24 h of culture in the absence (CTR) and in the presence of nonspecific γ -globulins (IgGs) or after the pre-incubation with anti-CD9 or anti-integrin $\alpha 6$ antibodies. The incidence of fusion was also evaluated with the CD9-negative cells (Raji) cultured for 24 h. ^{a,b,c,d}Values statistically different ($P < 0.05$) between treatments.

distribution were dependent on the cell type analyzed. In fact, as shown in Fig. 7, CD9 was clearly detectable on the membrane of CRFK cells, was revealed by a faint signal in GC, while it was nearly absent in the remaining cell types as well as in the CD9 negative cells (Raji).

Discussion

The results of this research show that calcein-loaded spermatozoa can transfer the dye to somatic cells once the fusogenic machinery is activated by AR. Spermatozoa can express their fusogenic potential only after AR. The requirement of AR for the activation of the

Figure 5 Analysis of CD9 protein expression. (A) Representative images of immunoblotting of CD9 and tubulin proteins expression on the different cell types after 4, 24, and 48 h of culture. Raji cells were used as negative control for the CD9 protein expression. The experiments were repeated three times with similar results. (B) Densitometric evaluation of proteins expression levels normalized on the basis of tubulin expression. ^{a,b,c}Values statistically different ($P < 0.05$) between the cell types.

Figure 6 Expression of integrin $\alpha 6$ and CD9. (A) Representative images of immunoblotting of CD9, integrin $\alpha 6$, and tubulin proteins expression on the different cell types after 24 h of culture. The experiments were repeated three times with similar results. (B) Densitometric evaluation of proteins expression levels normalized on the basis of tubulin expression. ^{a,b,c}Values statistically different ($P < 0.05$) between the cell types.

fusogenic part of the equatorial segment of the sperm head is well known (Flechon *et al.* 1986, Baldi *et al.* 1996), and here we show that AR conditions the fusion with other cell types too. Specific structural and spatial rearrangements of fertilin β at the equatorial segment occur after AR, thus activating the fusogenic potential of the spermatozoa. Dye transfer, in fact, potentially does not occur when nonreacted sperm (sperm not treated with A23187) are added to cell preparations. In this case, in fact, somatic-plated cells closely contacting fully fluorescent sperm do not acquire any detectable fluorescence. Moreover, this finding allows to exclude that fluorescent dye may leak out of loaded sperm cells and then diffuse into the cells.

Interestingly, the incidence of sperm–cell fusion was markedly affected by the cell preparations. In fact, besides the different fusion capacity of the cell types tested, attributable to their membrane's biochemical composition, as discussed later, a higher fusion efficiency was recorded in cells cultured for 24 h compared with cell preparations cultured for 4 or 48 h. Fusion is likely to result from the combination of several factors, from the presence of the molecules involved in cell–cell interaction, to the shape of the cells and therefore the amount of surface exposed to the sperm, to the vigor of sperm motility that may condition the first attachment phases of sperm on the plated cells.

Western blot analysis shows low levels of CD9 in cells cultured for 4 h, thus providing a potential explanation of the low fusion incidence recorded in these preparations. Similarly, the levels of these molecules in cells cultured for 48 h are lower compared with that recorded in 24 h cultured cells. The drop of fusion efficiency

recorded in 48 h cultured cells may also depend on the tridimensional conformation of the cells in culture, which, at this time, are markedly flattened on the bottom of the culture chamber and may be less exposed to wandering spermatozoa than 24 h cultured cells. However, little doubt exists that physical impediment exerted by spermatozoa conditions the potential interaction between sperm and plated cells. Even in the best experimental conditions adopted, the incidence of somatic cell fused with spermatozoa never exceeded 22%. Preliminary experiments (data not shown) demonstrated that such a level could not be increased using higher sperm concentrations, since sperm themselves with their tendency to agglutinate creates with their tails a rather dense network that limits the access to plated cells.

The present investigation demonstrates that boar sperm can fuse with somatic cells belonging to the same species as well as with cells derived from other species.

Figure 7 Representative confocal laser microscope images of CD9 protein expression on different cell types cultured for 24 h. Immunolabelings were performed as described in Materials and Methods. (Magnification: 400 $\times$).

A number of reports have described sperm interaction with somatic cells, from the nonspecific binding with tissue that is not naturally interacting with the sperm, such as the sperm fusion with leucocytes (Ashida & Scotfield 1987) and the case reported by Almeida *et al.* (1995), where F9 mouse embryonal carcinoma cells bound spermatozoa with an intensity proportional to the amount of integrins on the plasma membrane, to the well-known binding of spermatozoa to the oviduct cells, which is likely to play a physiological role in the selection and/or capacitation of spermatozoa that are retained in the oviduct reservoir, waiting for the ovulated oocytes (Hunter 1990).

However, in none of these cases, the binding was consistently followed by sperm–cell fusion. This is not surprising as the fusogenic potential of spermatozoa, naturally activated by AR, had not been previously switched on in the above situations. In our investigation, such a condition was achieved by treating *in vitro* capacitated spermatozoa with the Ca ionophore A23187, which resulted in proportion of reacted sperm close to 50%.

If cell–cell binding is a pre-requisite for fusion, the simple cell–cell contact is not a condition sufficient to drive fusion that, in fact, can occur only when apposing cell membranes have the proper molecular machinery. The present work has been designed to assess whether the process of sperm fusion with somatic cells involves the same molecular machinery involved in sperm–egg fusion. Sperm–egg fusion is mediated by a series of molecular interactions between sperm fertilin, egg integrins, and members of the tetraspanin family (Wassarman *et al.* 2001, Primakoff & Myles 2002). In the work of Almeida *et al.* (1995) and in more recent investigations (Ziyyat *et al.* 2006), sperm–egg binding and fusion are considered as two sequential phases, the first being a pre-requisite but nondeterminant for final fusion. $\alpha 6 \beta 1$ integrin has been suggested to mediate the binding of fertilin β (ADAM2) and cyritestin (ADAM3), two members of the ADAM family (a disintegrin and metalloprotease) expressed on sperm (Chen & Sampson 1999, Chen *et al.* 1999, Takahashi *et al.* 2001, Tomczuk *et al.* 2003), and fusion might occur following a second protein interaction or simply as a result of the former adhesion. However, the role of integrin in the process of sperm–egg fusion is controversial. The use of function-blocking antibodies has given conflicting results, mostly conditioned by the technique adopted to prepare zona-free oocytes (Almeida *et al.* 1995, Evans *et al.* 1997, Evans 1999). The inhibitory effects of the anti-integrin antibody on sperm–egg interaction has, in fact, supposed to derive on the protease treatment used to prepare zona-free eggs responsible for the loss of function and/or the alteration of some egg membrane proteins involved in gamete interaction.

Moreover, the finding that oocytes derived from integrin-negative mice can be successfully fertilized (Miller *et al.* 2000) suggests that this molecule does not

play an essential role in the process of sperm–egg fusion in the mouse, although its participation in the process cannot be excluded. At present, available data suggest that the integrins may take part in a membrane protein web that marks the fertilization site in the egg membrane. The molecular redundancy that characterizes many fundamental processes, such as fertilization, is probably responsible for the difficult interpretation of the experimental results. Similarly, an appropriate distribution of interacting molecules may be required on the plasma membrane of the apposing cells for fusion to occur (Tarone *et al.* 1993, Takahashi *et al.* 2001, Ziyyat *et al.* 2006).

Immunoneutralization experiments carried out with a specific function-blocking antibody anti-integrin $\alpha 6$ suggest that this protein is involved in sperm–egg fusion as well as in spermatozoa–cell fusion. In this last particular case, the inhibitory effect exerted by anti-integrin antibody could not be attributed to any enzymatic treatment since cell preparation did not involve the use of any enzyme.

Even in the case of sperm–cell fusion, integrins may represent an important anchoring site for the sperm disintegrins, thus creating the pre-requisite for fusion or, in addition, they could directly take part in the following phases of fusion, but this is presently unclear.

A marked inhibitory influence on cell fusion was recorded when anti-CD9 antibody was added to the FFA system, in agreement with the finding of Zhu *et al.* (2002). Our results confirm these data and extend the involvement of this factor to sperm fusion with other cell types. Interestingly enough, the levels of expression of this protein and its presence on the cell plasma membrane were positively related to fusion capacity of the cells tested, and the absence of fusion recorded in the negative control CD9-negative cells confirms the central role played by this molecule.

The combination of the results of immunoneutralization, immunoblotting, and immunolocalization suggests that both integrins and CD9 are required for somatic cells fusion. Both molecules are most likely involved in clustering membrane proteins involved in fusion, thus affecting their efficacy, but their relative influence turns out to be different. Integrins are, in fact, present at similar levels in all the cell types tested, while CD9, with levels of expression changing markedly from one cell type to the other, assumes the meaning of the fusion-conditioning factor. CRFK and GC, for their high CD9 concentration in the plasma membrane, are in fact superior to the other cell types for studying sperm–cell interaction.

The finding that sperm–cell fusion seems to involve two of the major molecules that support sperm–egg fusion suggests that sperm–cell fusion may provide an useful model for the understanding of the mechanisms of fertilization. Moreover, specific cell lines, with good fusogenic activity, such as CRFK, may be used to develop

reliable test for assessing sperm function and to replace the hamster zona-free oocytes test with more standardized cell assays.

Understanding the molecular machinery residing beyond membrane fusion is a relevant issue also with respect to cell therapy, gene targeting, and, more in general, to the identification of the more suitable route to gain access to the cell and to condition or to correct its functions.

Materials and Methods

Chemical agents and antibodies

Chemicals were of the purest analytical grade. FCS was purchased from Gibco BRL. Calcein AM was purchased from Molecular Probes Inc (Eugene, OR, USA). Rat anti-CD9 (KMC8.8) MAB and peroxidase-conjugated anti-rat IgG were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA), whereas rat anti-human integrin $\alpha 6$ (GoH3) MAB was from Chemicon International Inc., Temecula, CA, USA, anti-goat IgG and monoclonal anti- α -tubulin clone B-5-1-2 were from Sigma. Nitrocellulose was purchased from Hybon C Extra, Amersham Pharmacia.

All the other reagents and hormones used for these experiments were purchased from Sigma Chemical Co.

Sperm preparation

Semen samples were collected by the gloved-hand technique from five boars of proven fertility, 3 years old, of Landrace genetic lines. The seminal plasma was removed by centrifugation through a two-step discontinuous gradient of 35 and 70% isotonic Percoll (Harrison *et al.* 1996) to select motile cells. After removal of the supernatant layers, the resultant loose pellet was resuspended in residual 70% Percoll and washed by centrifugation at 800 *g* for 10 min in Dulbecco's phosphate buffer with 0.4% BSA. The pellet was then diluted in capacitation medium (tissue culture medium (TCM) 199 supplemented with 10% FCS, calcium lactate 2.25 mM, sodium pyruvate 1.25 mM, and glucose 13.9 mM) and incubated for 2 h at a final concentration of 1×10^8 spermatozoa (spz)/ml at 38.5 °C in 5% CO₂ humidified atmosphere. At the end of capacitation, spermatozoa were incubated for 20 min in the above-described medium supplemented with A23187 (1 μ M) and calcein AM (4 μ M). The ionophore A23187 was used to trigger AR and activate sperm fusogenic potential. The incidence of AR before and after A23187 treatment was evaluated in each sperm sample by FITC–PSA staining performed according to Barboni *et al.* (1995) and Mattioli *et al.* (1996). The membrane-permeant calcein AM was used to load the sperm cells. This dye can freely diffuse inside the cells, where it is transformed in a polar molecule that cannot cross back the intact membrane.

Following calcein loading, spermatozoa were thoroughly washed by centrifugation before addition to zona-free MII oocytes (IVF: positive control) or to the different cell preparations.

Oocytes collection, in vitro maturation

Ovaries of pre-pubertal gilts were collected at a local slaughterhouse and transported to the laboratory within 1 h, kept at a temperature of 25 °C. After washing in saline, the ovaries were mechanically dissected under sterile conditions in Dulbecco's phosphate buffer with 0.4% BSA and 70 mg/l kanamycin. Ovarian healthy follicles, 4–5 mm in diameter, selected on the basis of their translucent appearance, good vascularization, and compactness of their granulosa layer, were opened and cultured as everted follicles according to Mattioli *et al.* (1989). In brief, the follicles were put in Petri dishes containing 2 ml of TCM 199 medium supplemented with 10% FCS, 70 mg/l kanamycin, insulin–transferrin–selenium 10 μ l/ml, 1 μ g/ml porcine-LH, and 1 μ g/ml FSH (USDA-pLH-B1 and USDA-pFSH-B1, kindly provided by the National Pituitary Hormone Distribution Program, NIDDK-NIH, Bethesda, MD, USA), opened, and turned inside out. Everted follicles were then placed on a stainless grid to avoid contact with the Petri dish bottom. After 44 h of culture of the cumulus–oocytes complexes collected, the oocyte was denuded of expanded cumulus mass in Hepes–TCM 199 with hyaluronidase 0.1% (w/v), and only the oocytes with extruded polar body (MII stage) were selected; ZP was removed with pronase 0.25% (PBS) using a fine glass pipette on a warmed stage at 38.5 °C before fusion assay experiments.

Cells preparation

Cell fusion experiments were carried out using standard cell lines or cells isolated from ovarian follicles. CRFK (cat kidney cells), VERO (African green monkey cells), ESK4 (embryonic swine kidney; BS CL 42), and Raji (CD9-negative B cells: Raji Human Burkitt lymphoma) cell lines were purchased from IZS of Brescia. GC were collected from pig ovarian follicles (4–5 mm in diameter) according to Mattioli *et al.* (1989).

All cell samples were washed twice by centrifugation (800 *g* for 5 min) in Dulbecco's phosphate buffer with 0.4% BSA and then resuspended in minimal essential medium supplemented with 10% FCS, 1 mmol glutamine/l, and 70 μ g gentamycin/ml. Cell suspension (1×10^4 cells/2 ml) was then plated in a chambered coverglass (a special chamber with a fine coverglass at the bottom purchased from NUNC, Naperville, IL, USA) and cultured at 37.5 °C in 5% CO₂.

To define the best experimental conditions for the study of sperm–cell interaction, the different cell types were cultured for different intervals (4, 24, and 48 h) and, at the end of these culture intervals, the chambers were washed to remove cells in suspension or loosely attached and the remaining plated cells were used for the FFA as following described.

Functional fusion assay

FFA is based on the hypothesis that when a loaded spermatozoon fuses with another cell the dye diffuses and this cell becomes fluorescent.

AR-induced spermatozoa were added to plated cells to a final concentration of 1×10^7 spz/ml. After 1 h of co-incubation, plated cells were washed twice in Hepes–TCM

199 and stained with 4',6-diamidino-2-phenylindole (DAPI; 10 µg/ml) to count the total cell number. The fusion capacity of spermatozoa, expressed as percentage of calcein-positive cells/total cells (DAPI positive cells), was evaluated by using an epifluorescence microscope (Nikon Eclipse 600) equipped for u.v. and FITC (EX 330–380; DM 400; BA 420 and EX 465–495; DM 505; BA 515–555 respectively). Each fusion assay also included a negative control: calcein-loaded, acrosome-intact sperm (sperm capacitated but not treated with A23187), to exclude the possibility of dye leakage from nonfused sperm. Moreover, experiments of fusion were also carried out with zona-free mature oocytes (IVF: positive control).

In order to test whether sperm–cell fusion involves both CD9 and integrin $\alpha 6$, two major molecules supposed to be involved in the process of sperm–egg fusion (Podell *et al.* 1984, Wassarman 1987), the cell preparations as well as the oocytes, were pre-incubated for 1 h with specific antibodies anti-human integrin $\alpha 6$ (50 µg/ml; according to Ziyat *et al.* 2006) and anti-CD9 (50 µg/ml; according to Miller *et al.* 2000), and then washed before exposure to calcein-loaded spermatozoa. Plated cells and eggs were also challenged for fusion in the presence of nonspecific γ -globulins (anti-goat IgG 50 µg/ml, γ -globulin) or without any γ -globulins (control, CTR).

Finally, FFA was also carried out using Raji cells that do not express CD9 (Ikeyama *et al.* 1993, Shaw *et al.* 1995), in order to further investigate the role played by the tetraspanin in the process of cell fusion.

Western blot analysis

CRFK, VERO, ESK4, GC, and Raji cells were plated and cultured as previously described for 4, 24, and 48 h. Raji cells were used in the western blot experiments as negative control of CD9 protein expression.

The cells were then lysed in 300 µl of 2% SDS containing 2 mM phenylmethylsulphonyl fluoride, 2 mM sodium orthovanadate, 10 mM sodium fluoride, 10 µg/ml leupeptin, 10 µg/ml antipain, 100 units/ml aprotinin as phosphatase and protease inhibitors and sonicated for 30 s. Protein content was evaluated by Lowry method (Lowry *et al.* 1951). Equal amount of proteins (50 µg/sample) was then separated by 8 and 12% SDS-PAGE (Laemmly 1970) and electrophoretically transferred onto nitrocellulose (Hybond C Extra, Amersham Pharmacia Biotech, Inc.) according to standard procedures (Towbin *et al.* 1979). After staining with Ponceau S, the membranes were blocked for 1 h at room temperature (RT) with Tris buffer saline (TBS: 20 mM Tris pH 7.6, 150 mM) plus 5% (w/v) nonfat dry milk, and following primary antibodies were used according to the manufacturer's instructions: anti-CD9 (1:500); anti-integrin $\alpha 6$ (1:5000); monoclonal anti- α -tubulin clone B-5-1-2 (1:3000). Membranes were washed with TBS containing 0.05% (v/v) Tween 20 and incubated for 1 h with peroxidase-conjugated anti-mouse IgG or anti-rat IgG (1:3000). The signals were detected by using the ECL Western Blot analysis system (Amersham Bioscience). The quantitative densitometric analysis of proteins, normalized on α -tubulin expression, was carried out with the Advanced Image Data Analyzer (Rai Test; GMBH, Straubenhardt, Germany).

Immunofluorescence

GC, CRFK, VERO, ESK4, and Raji cells cultured for 24 h were fixed in 4% buffered paraformaldehyde (PBS pH 7.4) at 4 °C for 1 h. After washing for 5 min with PBS on oscillating plate, the samples were incubated for 1 h at RT with normal goat serum (1:20), and then treated one sample with a affinity-purified rat MAB KMC8.8 raised against CD9 and another sample with a affinity rat MAB GoH3 against human integrin $\alpha 6$, 200 µg/ml, for 1 h at RT. Control specimens were prepared by omitting the primary antibody. After two washing, cells were incubated with FITC-conjugated goat anti-rat IgG (1:200) for 1 h at RT in darkness, washed twice, and then analyzed by Bio-Rad laser-scanning confocal microscope (Radiance 2000 IK-2) equipped with an excitation wavelength of 488 nm and the detector set for an emission wavelength of 525 nm (magnification: 400×).

Statistical analysis

All experiments were carried out at least five times in independent trials (5 replicates/group per experiment). All data were expressed as mean ($\pm$ s.d). The results of treatments were compared by one-way ANOVA followed, when required, by Tukey's test (Orion Software Development, Version 2.0, Longmont, CO, USA). Differences were considered significant for $P < 0.05$ values.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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