Role of tetraspanin CD9 molecule in fertilization of mammals

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Summary

Fertilization process is a very clever and unique process comprising some essential steps resulting in formation of zygote. Tetraspanin CD9 is considered to be a serious candidate molecule participating in these events. The importance of CD9 has been discussed in relation to acrosome reaction, sperm-binding, sperm-penetration, sperm-egg fusion and eventually, egg activation. The abundant expression of CD9 oocyte plasma membrane and the presence of CD9-containing vesicles in the perivitelline space of intact oocytes have been confirmed**.** Despite the fact that majority of authors analyzed CD9 expressed on oocytes, several studies considered the function of sperm CD9, too. To understand CD9 involvement, various conditions of *in vitro* fertilization (IVF) assays using polyclonal as well as monoclonal antibodies or knockout mice were carried out. However, ambiguous data have been obtained about the importance of CD9 in sperm-egg binding or fusion. Although the current findings did not prove any hypothesis, the indispensable role of CD9 in fertilization process was not excluded and the precise role of CD9 remains unexplained.

Key words

Tetraspanin, Reproduction, CD antigen, Ova, Spermatozoa

Introduction

Fertilization process is a very clever, unique and sometimes also very "tricky" process comprising some essential steps resulting in formation of zygote. Gamete cells passing through the genital tract of both male and female lose some specific structural component to acquire a new one and finish fertilization successfully. Some molecules localized either on gamete cells or in the genital tract tissues involved in particular events of fertilization have been identified.

Although fertilization process has been described in general, the molecules and their exact role are not fully illustrated. Therefore, every molecule found in genital system or on the gamete surface is considered to be a potential candidate molecule involved in this process and significant effort is devoted to characterize and study it closer. A list of molecules known to be present on gametes is very broad and is still enlarging. Molecules known to be involved in mammalian sperm-egg fusion are also reviewed in Klinovska *et al.* (2014). For a better interpretation of fertilization process complexity, Fard Jahromi and Shamsir (2013) assembled the protein-protein interaction networks of human egg and human sperm. The egg protein map involves 1056 proteins and 1700 interactions between these proteins and sperm map comprises 6450 proteins and 34579 interactions which provide the entire possibilities of molecule functional connection. Some of the most studied molecules are also presented in Table 1.

CD9- a serious candidate molecule for involvement in fertilization process

CD9 protein belongs to the tetraspanin super family. Beside the CD9, several other differentiation antigens CD37, CD53, CD63, CD81/TAPA-1, CD82 and CD151 (Szala *et al.* 1990, Jankowski *et al.* 1994, Takagi *et al.* 1995) are classified in this family.

Concerning the cell expression, all cells seem to express several tetraspanins, with exceptions of erythrocytes (Boucheix *et al.* 1985, Shaw 1994, Sincock *et al.* 1997, Rubinstein and Boucheix 1999, Sincock *et al.* 1999) and tetraspanins were found in many species (Hemler 2003). Some tetraspanins have a wide distribution, whereas others have very restricted pattern (Boucheix and Rubinstein, 2001). On the mammalian oocyte membrane, the tetraspanins CD9, CD81 and CD151 (Ziyyat *et al.* 2006), CD63 (Sabetian *et al.* 2014) in human and CD81, CD98 (Takahashi *et al.* 2001) in mice have been detected. Chen *et al.* (1999) observed CD9 localization on the plasma membrane of oocytes in the ovary as well as on oocytes isolated from the oviduct and Ziyyat *et al.* (2006) and previously Kaji *et al.* (2000), Le Naour *et al.* (2000), Miyado *et al.* (2000) confirmed the abundant expression of CD9 egg plasma membrane in mice.

Tetraspanins are characterized by four transmembrane regions and two extracellular loops (Boucheix *et al.* 1991). These domains are of unequal size, small extracellular loop (EC1) contains 20-28 amino acids and the large extracellular loop (EC2) contains 76-131 amino acids (Boucheix and Rubinstein 2001), which both can interact with other luminal proteins including potential specific receptors. The transmembrane anchor may interact with other transmembrane segments; and the N-terminal and C-terminal cytoplasmic tails may interact with cytoskeletal elements including microfilaments, microtubules and molecules involved in signal transduction processes (Lefévre *et al.* 2010). Tetraspanins are enriched in cholesterol-rich microdomains of a unique lipid composition distinct from lipids rafts, where they organize other proteins such as integrins, kinases, human leukocyte antigens and growth factor receptors (Lazo 2007, Hemler 2003). These complex associations create tetraspanin webs involved in signaling, cell-cell and cell-extracellular matrix adhesion, and in processes as cell migration/motility, viral infection, immune responses, tumor metastasis and hematopoietic stem cell differentiation (Yunta and Lazo 2003). Association and organization

of these membrane proteins in different cellular membranes can be regulated through the covalent modification, palmitoylation (Charrin *et al.* 2002). Palmitoylation of membrane proximal cysteins is required for their association with the cholesterol complexes (Charrin *et al.* 2003a). Palmitoylated tetraspanins are thought to be important for assembly of webs, favoring association with other members of this superfamily and their associated proteins (Berditchevski *et al.* 2002, Yang *et al.* 2002).

It is known that molecular weight of CD9 molecule (approximately 24-25 kDa) is similar in all tissues and cell types in mice (Ito *et al.* 2010), pig (Kaewmala *et al.* 2011) and cattle (Martin-Alonso *et al.* 1992). Chemical structure, molecular organization and expression pattern designate CD9 molecule for implication in fertilization process. Various conditions of *in vitro* fertilization (IVF) assays using polyclonal as well as monoclonal antibodies were carried out to resolve this question. The second approach was through the gene-manipulated animals, where knockout mice have been usually used. It should be mentioned that achieved results differ largely.

Based on functional role of large extracellular loop (EC2) in many tetraspanins, this part of tetraspanin structure was supposed to be important for fusion (Zhu *et al.* 2002, Hemler 2003). Actually, Zhu *et al.* (2002) observed inhibited sperm-egg fusion when oocytes were preincubated with bacterially expressed mouse EC2 construct and demonstrated that constructs including the large extracellular loop of CD9 significantly inhibited gamete fusion only if were incubated with oocytes, but not with sperm. Higginbottom *et al.* (2003) reported a hypervariable region of CD9 large extracellular domain important for the sperm-egg fusion. The cytoplasmic tails of tetraspanin molecule can interact with cytoskeletal elements (Lefévre *et al.* 2010). Sutovsky *et al.* (1996) found out that disruption of actin microfilaments with microfilament disruptors during bovine or murine fertilization decreased sperm incorporation into the ooplasm whereas fusion and oocyte activation was affected in dependence of used

disruptor type. That is the other reason to consider the role of CD9 molecule in reproduction in connection with its association complexity.

CD9, integrins and disintegrins

Functional role of CD9 protein in fertilization process seems to be dependent on the assembling of tetraspanin web comprising different molecular actors. The group of integrins as well as members of ADAMs family (proteins with a disintegrin and metalloprotease domain) fertilin α (ADAM-1), fertilin β (ADAM-2), and cyritestin (ADAM-3), expressed on sperm, were thought to be important for sperm-binding or gamete fusion. With regard to the function in general, the integrin receptors can mediate cell adhesion, migration, proliferation, cell differentiation and apoptosis and connect the intracellular cytoskeleton and extracellular matrix (Schwartz *et al.* 1995). Due to the observed integrins expression on oocytes, they were studied in IVF assays, although controversial data about the importance for sperm-egg binding or fusion were obtained.

Shaw *et al.* (1995), Jones *et al.* (1996) and Hemler (1998) described interaction of tetraspanin CD9 protein with the integrins α3β1, α6β1, α4β1 and αIIbβ3 where CD9 mediates migration, signaling and adhesion to extracellular matrix substrates. Chen *et al.* (1999) reported an interaction between sperm fertilin β and α6β1 bound to egg cytoskeleton, which can be possibly mediated by CD9. Although β1 integrins are the major molecules identified in tetraspanin macromolecular complexes (Lozahic *et al.* 2000, Yauch *et al.* 2000), the interaction of CD9 with integrins seems to be indirect (through another tetraspanin) (Yauch *et al.* 1998, Serru *et al.* 1999). These suggestions were confirmed in co-localization experiments, where CD9 was not identified in complex with α6 which supports the hypothesis of indirect association of CD9 with α6β1 (Charrin *et al.* 2003b, Berditchevski *et al.* 2002, Ziyyat *et al.*

2006). Miller *et al.* (2000) found out that the lack of α6β1 integrin in total α6-integrin knockout mice has not affected oogenesis and maturation of eggs from ovaries cultivated *in vitro.* Moreover, no reduction in number of sperm bound or fused with eggs lacking α6 has been observed compared to wild-type control.

Regarding the ADAMs family, fertilin α and β accrue from larger precursors at different stages of sperm maturation in the testes and epididymis (Blobel 2000). The fertilin β is present on the equatorial region of mouse sperm (Yuan *et al.* 1997) and in posterior head region of guinea pig sperm (Blobel 2000). The extracellular domain of fertilin α and fertilin β binds to microvillar region on mouse oocytes (Evans *et al.* 1997, Bigler *et al.* 2000). Cyritestin molecule expression has been found out to be restricted in testes (Evans 2002).

Chen *et al.* (1999) proved binding inhibition of fertilin-coated beads (and sperm) to eggs by monoclonal anti-CD9 antibody and suggested two possible models for explanation. In the first model, fertilin may contact integrin α6β1 or parts of CD9 and consequently monoclonal antibody either sterically blocks fertilin binding or causes dissociation between α6β1 and cytoskeleton and inhibits fertilin binding. In the second model Chen *et al.* (1999) suggest, CD9 is not physically associated with α6β1 and binding of monoclonal antibody to CD9 sends a signal into egg that weakens the association of α6β1 with cytoskeleton (thereby inhibiting fertilin binding). The same authors observed apical epithelial expression of CD9 on mouse tissues and α6 subunit in the utero-tubal junction and oviductal isthmus correlated with requirement of sperm to adhere to these sites and with the failure of fertilin β-lacking sperm to transit these regions. Sperm-egg fusion and binding were reduced in mAb CD9-pretreated zona-free eggs and fertilin $\beta^{-/-}$ eggs. Interestingly, Chen *et al.* (1999) showed that anti-CD9 antibodies influenced not only the sperm-egg binding and fusion, but also the binding of the fertilin β and cyritestin (Takahashi *et al.* 2001) disintegrin domain to the egg. Possibly, CD9-regulation of ADAM- integrin association should be taken into consideration. Moreover, they

studied involvement of the fertilin β, CD9, and α6β1 in a model of murine sperm migration. They supposed that sperm come to the epithelium and the affinity between sperm fertilin β and oviductal integrin α6β1, associated with CD9, is changed according to activation. Integrins can exist with low or high binding capacity for particular ligands and CD9 possibly influence the activation state of integrin (Xiang *et al.* 2002). Integrins, CD9 and fertilin are distributed in a way that indicate their participation in mediation the binucleate cell migration, adhesion, and/or fusion with uterine epithelium, processes connected with ruminant placentation (Xiang *et al.* 2002).

In spite of the gamete localization and different experimental studies performed, an essential role of mentioned integral membrane proteins in fusion has not been finally confirmed.

Reproduction process in CD9-deficient mice

Several authors (Kaji *et al.* 2000, Le Naour *et al.* 2000, Miyado *et al.* 2000) published studies where CD9-deleted mice were undertaken in experiments. Females exhibited normal oogenesis in comparison with the wild type mice, but many sperm "prisoned" in perivitelline space were detected due to the failure of fusion ova plasma membrane (Kaji *et al.* 2000, Miyado *et al.* 2000). Inoue *et al.* (2007) tested various gene-manipulated animals and observed normal zona-penetration when CD9-deficient ova have been used, but due to a lack of fusion, spermatozoa accumulated in the perivitelline space as there was no zona pellucida-based antipolyspermy defense facilitated. In the study of Miyado *et al.* (2000), $CD9^{-/-}$ females were infertile whereas $CD9^{-/-}$ males showed normal fertility when mated with wild type mice. The same authors observed failure of fusion and consequently decrease in a litter size in $CD9^{-/-}$ mouse females to less than 2% compared to wild-type mice. On the other hand,

fertilized eggs developed to term, when sperm were directly injected into oocytes. Kaji *et al.* (2002) showed that incompetence of sperm-egg fusion in CD9-deleted mice (despite normal binding and penetration through zona pellucida) can be rendered by mouse mRNA CD9 injection into these oocytes. Taken all together, CD9 seems to be directly involved in the events of sperm and egg membrane fusion, although, experiments using the knockout mice are very relevant for functional study of CD9 molecule but as all approaches, some limitations should be taken into account. If the results from IVF assays using knockout mice are evaluated, some differences in CD9-deficient eggs compared to wild type mouse eggs should be considered. Jégou *et al.* (2011) described changes in microvilli properties as well as in protein expression and distribution.

Study of CD9 in fertilization process by monoclonal antibodies

CD9 role can be also studied by additional approach, in experimental assay using monoclonal antibodies (mAbs) directed to CD9 or integrin molecules and valuable data can be achieved regarding the importance of various epitopes in particular steps of fertilization process. In agreement with Chen *et al.* (1999) and Takahashi *et al.* (2001) who showed that antibodies against CD9 blocked sperm-egg binding and fusion in mice, Zhou *et al.* (2009) observed a decrease in number of bound sperm as well as penetrated eggs in IVF assay in cattle. On the contrary, Miyado *et al.* (2000) pre-treated mouse zona pellucida-free eggs with anti-CD9 antibody, no effect on rate of sperm-binding to eggs has been determined. However, when the insemination time was shortened from 60 to 15 minutes, reduction of sperm-binding to 53% has been detected. Regardless the valuable results, these cases point out also to an importance of experimental condition settings for consistent outstanding achievement. When Miller *et al.* (2000) assessed fertilization rate in wild cumulus-intact type eggs, it was nearly

completely inhibited using anti-CD9 antibody (KMC8.8) but not anti-α6 antibody (GoH3). The antibody did not affect passage of sperm through zona pellucida and cumular mass. As we mentioned previously, it seems CD9 acts independently to α6β1 integrin but probably in association with other molecules. On the contrary, Almeida *et al.* (1995) observed inhibition in sperm-binding to zona-free eggs by GoH3. These findings are in agreement with conclusion of Evans et al. (1997) who described dependence of antibody effect on oocytes preparation. The results can be influenced by methods of zona pellucida removal or whether zona pellucida-intact eggs were applied. Particularly, pronase treatment but not chymotrypsin or acid Tyrode eliminated CD9 from the membrane and caused infertility of eggs (Komorowski *et al.* 2003).

CD9 as a part of membranous vesicles

Production and acceptance of membranous vesicles derived from different cells is thought to be a mechanism ensuring communication between cells (Thery *et al.* 2009).

Epididymosomes are referred to as a membranous vesicles secreted by cells into intraluminal fluid of epididymis. Fertilizing ability acquirement of spermatozoa is associated with transit through the epididymal caput and corpus and epididymosomes are responsible for protein transfer and modification of sperm membrane composition (Sullivan *et al.* 2007). Wubbolts *et al.* (2003) and Trajkovic *et al.* (2008) described CD9 as a component of membrane vesicles released from a wide range of cells. Miyado *et al.* (2008) incubated the CD9-deleted mouse epididymal spermatozoa ($CD9^{-/-}$) with $CD9^{-/-}$ zona pellucida-removed eggs in medium containing the vesicles from $CD9^{+/+}$ eggs. Consequently, the spermatozoa were capable of fusion with $CD9^{-/-}$ eggs that indicate rendering of fusibility $CD9^{-/-}$ eggs. The same authors showed localization of CD9-containing vesicles in the perivitelline space and

their accumulation here during the germinal vesicle and metaphase II- arrested stages of oocytes. Moreover, CD9 deficiency led not only to impaired microvilli formation on ooplasm (microvilli improve anchor of penetrating spermatozoa) (Runge *et al.* 2007), but also decreased accumulation of vesicles in perivitelline space. Miyado *et al.* (2008) found out that anti-CD9 antibody inhibited the association of sperm with CD9-containing vesicles as well as sperm-egg fusion. Interestingly, Kaji *et al.* (2000) observed CD9 distribution over the entire oocyte surface except the region overlying the mitotic spindle, which is the only area lacking the microvilli on the oocyte surface and where sperm inefficiently fuse with oocytes. Zhu *et al.* (2002) observed CD9 absent region over the metaphase plate and CD9 expression has been identical to microvilli expression in mouse oocytes. However, Li *et al.* (2004) detected no CD9-deficient area when porcine oocytes were tested. Differences in CD9 distribution between mouse and pig oocytes were the same as cortical granule (CG) distribution. There is not CG-free domain in mature porcine oocytes (Wang *et al.* 1997). After co-incubation, Kaji *et al.* (2000) demonstrated, that hamster egg ($CD9^{+/+}$) facilitates the fusion of sperm with $CD9^{-/-}$ eggs which indicates a mechanism, similar with mouse, through egg-released material-vesiculosomes. On the contrary, Gupta *et al.* (2009) and Barraud-Lange *et al.* (2012) obtained completely different data in similar experiments in mice. Former detected only 1.5% fusion of CD9-deficient oocytes in three experiments where CD9-lacking oocytes were inseminated in the presence of wild type oocytes. On the other hand, Barraud- Lange *et al.* (2007) have observed staining of anti-CD9 antibody on the head of $CD9^{-/-}$ males sperm in perivitelline space after IVF assay. These results support the hypothesis that transfer of oocyte membrane fragments containing CD9 on sperm has occurred. This suggestion was also confirmed in experiments, where no transfer of fluorescent dye between oocytes has been detected, but 10% of recovered spermatozoa showed oocyte-bound stain particles (represented oocyte plasma membrane fragments). Barraud-Lange *et al.* (2012) observed that fertilization ability

of $CD9^{-/-}$ mouse eggs cannot be reconstituted by sperm with surface bound CD9 oocyte membrane fragments.

CD9 on spermatozoa

For the first time, CD9 was detected in mouse and rat spermatogonia (Kanatsu-Shinohara et al. 2004, Kierszenbaum et al. 2006). An appearance of CD9 during the spermatogenesis and its expression on sperm was reported by Ito *et al.* (2010) in mice. Presence of CD9 mRNA has been detected on developing germ cells up to round spermatids and CD9 protein was found in primary spermatocytes up to nearly secondary spermatocytes but later the signal was getting weak or completely lost and again appeared in elongated spermatids. Generally, the signal was detected in spermatogonia, spermatocytes and round spermatids in all stages and no positive signal in Sertoli cells and in elongating and elongated spermatids. In electron microscopy of acrosome-reacted mouse spermatozoa using anti-CD9 antibody, immunogold particles have been predominantly concerned on the inner acrosomal membrane but many gold particles were found on the released vesicles (formed by fusion of plasma and outer acrosomal membrane) and on the surface of the equatorial segment (Ito *et al.* 2010). Barraud-Launge *et al.* (2012) observed the presence of CD9 molecule on 10% of cauda epididymal mature sperm and on 60-75% of acrosome-reacted murine sperm.

CD9 in non-rodent species

Only poor information is available in relation to CD9 in man and non-rodent species of animals. As for human reproduction, CD9 has been found in germinal vesicle oocytes as well as metaphase I and metaphase II oocytes (Coskun *et al.* 2003; Ziyyat *et al.* 2006).

Recently, Salvolini *et al*. (2013) described lower expression of CD9 in asthenozoospermic human sperm compared to normospermic samples.

For the first time, porcine CD9 molecule localization on gametes has been determined by Li *et al.* (2004). CD9 was observed after immunohistochemical staining on granulosa cell membrane as well as on oocyte plasma membrane in preantral follicles and in the fully grown follicles. Moreover, the immunofluorescence signal detected on the membrane of oocytes was enhanced as nuclear stage proceeded from germinal vesicle through metaphase I to metaphase II. Consistently, the density of 24 kDa protein representing CD9 oocyte molecule detected in immunoblotting, significantly increased during oocyte maturation. Porcine sperm was also subjected to immunofluorescence assay but no positive signal was observed. Both, sperm-binding and sperm-oocyte fusion were significantly reduced in the ZP- free oocytes, when the oocytes were pre-incubated with anti-CD9 antibody (Li *et al.* 2004). Based on results in mice, similar system involved in fertilization process could be assumed. Only one study concerning the expression of CD9 in reproductive and non- reproductive tissues in porcine is available (Kaewmala *et al.* 2011). The semi-quantitative reverse transcription PCR showed CD9 expression in Leidig cells, Sertoli cells and germ cells within the testis, in the epithelial cells of epididymis, vas deferens, prostate gland and spermatozoa in the lumen of epididymis. Moreover, using the immunofluorescence assay, Kaewmala *et al.* (2011) detected CD9 in the acrosomal region and acrosomal membrane of epididymal spermatozoa although Li *et al.* (2004) have not been previously successful. Kaewmala *et al.* (2011) used spermatozoa from the different regions of reproductive boar tract whereas Li *et al*. (2004) treated ejaculated frozen-thawed spermatozoa what was considered as the reason of this discrepancy.

In 2009, presence of CD9 on plasma membrane of matured bovine oocytes specifically has been confirmed (Zhou *et al.* 2009). In IVF assay where CD9 on oocytes was blocked by anti-CD9 antibody (IVA-50), significant reduction in either sperm bound to

oocytes or penetrated spermatozoa was denoted. Recent study of Cupperová *et al.* (2014) detected CD9 expression on bull spermatozoa and described the distribution within the whole male genital tract. Regarding the tissue expression, the bovine CD9 was expressed constitutively on the apical surface of uterine epithelium, consistently to human uterine epithelium (Park *et al.* 2000, Yanez-Mo *et al.* 2001). Chen *et al.* (1999) were not able to detect murine CD9 in the uterine epithelium but CD9 was expressed on the apical surface of the uterine-oviduct junction. However, the group of Xiang *et al.* (2002) detected CD9 presence in subpopulation of binucleate cells in bovine trophoblast and they speculated that this surface expression of CD9 can be a prerequisite for their migration. Recently, Caballero *et al.* (2013) examined the particular parts of bovine epididymis for CD9 presence. They observed vesicles expressing CD9 predominantly in the epididymal fluid, gained from cauda region, whereas epithelial cells of epididymis, caput, corpus and cauda were positive for CD9. Based on proteomic analysis, Girouard *et al.* (2011) confirmed CD9 association with cauda epididymosomes. According to Caballero *et al*. (2013), CD9-positive microvesicles (30-120 nm) took about one third of total vesicle protein amount in epididymal fluid and only microvesicles secreted from the corpus distal to the cauda regions were CD9-positive. Moreover, transfer of CD9 molecules from membranous vesicles to acrosome and midpiece of epididymal spermatozoa has been detected. However, transfer of CD9 to epididymal corpus distal spermatozoa decreased only moderately (10-15%) when CD9 has been blocked by specific antibodies. Therefore, CD9 involvement in tetraspanin web on sperm membrane should be considered rather than CD9 molecule can act itself.

What is the role of CD9 in reproduction?

In terms of fertilization process, the particular mechanisms of the most acting "players" are only assumptive and there are only few molecules which function is fully understood. Although a great deal of information and experimental data are available regarding CD9, significant effort is permanently devoted to solve the precise function of this protein at fertilization. Majority of authors assigned CD9 expressed on oocytes but several studies identified CD9 on sperm, too. In general, as Stein *et al.* (2004) suggested, the role of CD9 should be considered in interaction with other/s molecules on oolema ("in cis") in egg-spermatozoa fusion and furthermore, CD9 could act as a receptor for sperm ("in trans") due to its association with integrins. It is therefore possible; that CD9 is involved in fertilization process not only through the expression on oocytes but an additional function in transfer of egg membrane vesicles to spermatozoa has been proposed by several reports. Rubinstein *et al.* (2006) and consequently, Barraud-Lange *et al.* (2007) and Miyado *et al.* (2008) found out that spermatozoa acquire CD9 from oocyte membrane in a process similar to trogocytosis, shortly described as a cell to cell contact-dependent transfer of membrane fragments. CD9 is transferred from oocyte membrane to the spermatozoa in the perivitelline space. According to Barraud-Lange *et al*. (2007), before the fusion, the sperm membrane reorganization comprising transfer of egg membrane fragments containing CD9 on sperm is needed. The possibility that oocytes themselves induced sperm CD9 expression was excluded as murine sperm from CD9-deficient males were applied in these experiments. In 2008 Myiado *et al.* published that CD9-containing vesicles (able to facilitate sperm-egg fusion) are released from eggs before fertilization in hamster and mice. Zuccotti *et al.* (1991) observed fluorescent stain transfer appertained to egg membrane CD9 from growing oocytes to binding sperm. Later when Miyado *et al.* (2000) examined oocytes in the same stage, CD9 presence on cell

membrane but absence of CD9-containing vesicles has been observed. These authors supposed that cell membrane CD9 is responsible for transfer of fluorescent signal.

Recently, Barraud-Lange *et al*. (2012) examined mouse eggs for ability to pass the membrane material to sperm via trogocytosis and exosome and both pathways have been confirmed to coexist. However, only low portion (<10%) of sperm positive for transferred membrane fragments or captured vesicles have been detected. Moreover, despite of no fertilization observed in CD9-null mouse oocytes, trogocytosis and vesicle transfer were not disturbed and loss of CD9 did not change the synthesis and secretion of vesicles localised in perivitelline space of CD9-deficient oocytes. Therefore the hypothesis of CD9 dependency of gamete exchange has been excluded. Ito *et al.* (2010) detected CD9 being unmasked after the acrosome-reaction in mice spermatozoa but no significant role in fertilization was concluded in $CD9^{-/-}$ male mice; fertilizing ability was not influenced when pregnancy rates or a litter size was compared. In the study of Barraud-Lange *et al.* (2012), exosome exchange and trogocytosis of membrane material from egg plasma membrane occurs predominantly on acrosome-reacted sperm.

Based on achieved results, in spite of large discrepancies, several hypotheses about possible mechanisms of CD9 functioning could be formulated. CD9 on the inner acrosomal membrane is relocated to the equatorial region of sperm head and can associate with the environment around the vesicles (originated from fusion of plasma and outer acrosomal membrane). Sperm CD9 on the inner acrosomal membrane can act as an organizer for the other inner acrosomal membrane proteins due to tetraspanin character of CD9 (Ito *et al.* 2010). CD9 can play a role in secondary attachment of sperm to the zona pellucida. After acrosome reaction, fusion of plasma membrane and outer acrosomal membrane occurs and inner acrosomal membrane receptors are exhibited (Ito *et al.* 2010). During the fusion, CD9 released from the oocyte surface could attach the sperm surface (Miyado *et al.* 2008). It can

be speculated that CD9 can act as a binding molecule on sperm inner acrosomal membrane or after transfer also on plasma membrane. Subsequently, CD9 could facilitate the fusion by the interaction between egg and sperm CD9 molecules. Sutovsky (2009) suggested IZUMO protein could be a binding ligand acquiring CD9 from the oocyte-released vesicles and further triggering fusion by interaction with CD9 on oolema. CD9 can act as a linker between the molecules involved in sperm binding or molecules facilitating fusion as Kaji and Kudo (2004) early considered. These hypotheses are supported by Vicens and Roldan (2014), they found correlated evolution between extracellular domain of IZUMO1 and CD9, suggesting that these proteins could undergo some form of specific molecular interaction.

Recently, the first essential receptor couple, the sperm IZUMO1 and the egg Juno (folate receptor 4), involved in fertilization process of mammals has been identified (Bianchi *et al.* 2014). According Chalbi *et al.* (2014), along with IZUMO1, egg CD9 concomitantly accumulates in the adhesion area. Based on these findings, CD9 as a partner of Juno in adhesion-induced membrane organization before the fusion has been suggested.

The tetraspanin nature of CD9 molecule must not to be omitted when potential role of CD9 in fertilization process is taken into consideration. Expression of CD9 in microvillar area of oocyte indicates its involvement in fertilization through the interaction with integrin and consequently disintegrin molecules. In general, integrins pose the junction between extracellular matrix and cytoskeleton and integrins expressed on egg. Miyado *et al.* (2000) proved physical association of integrin α6β1 with CD9 on mouse egg membrane by co-immunoprecipitation using α6 antibody. The same authors suggested that integrin α6β1 may transduce signal to CD9 and initiate or otherwise promote fusion. Takahasi *et al.* (2001) presumed that egg surface tetraspanin web involving β1-integrin-associated proteins including CD9 may constitute and save a site for fusing sperm. In study of Ziyyat *et al.* (2006), CD9 mAb prevented α6β1 integrin clustering and sperm-egg fusion when added solely before zona

pellucida removement in human IVF assay. CD9 can be responsible for reorganization of some membrane proteins including α6β1 integrin. Glazar and Evans (2009) observed the inhibition of sperm-binding to anti-CD9-blocked eggs only up to 60 minutes after insemination. They proposed the role of CD9 at the beginning of sperm-egg contact as CD9 increases adhesion. Possibly, via interaction of CD9 with adhesion molecules, CD9 can participate in egg activation process. Jégou *et al.* (2011) studied CD9 function in fertilization process using force measurement technique and different types of adhesion interaction between gametes were distinguished. Although, CD9-deficient eggs showed reduced fusion rate, number of bound sperm was increased compared to wild type mice probably due to increase of accessible sperm adhesion sites. It is important to note that disappearance of strong type adhesion interaction has been revealed and the authors suggested CD9 function directly in the adhesion site on sperm and egg membrane before the fusion occurs and consequently successful fusion is a direct consequence of CD9 controlled adhesion properties. According to Higginbottom *et al.* (2003), it is likely that CD9 interacts with different egg proteins, involved in sperm binding or fusion, respectively, and brings them together into close proximity. Similar mechanism for the other tetraspanins has been reported in virus-induced cell-cell fusion by Loffler *et al.* (1997).

The ability of tetraspanins to create molecular complexes between each other and other membrane proteins is crucial for their functionality. Boucheix and Rubinstein (2001) and Hemler (2003) differentiated particular primary complexes (tetraspanin and one partner molecule) according to the solubility in various detergents and using cross-linking experiments. Therefore, for better elucidation of CD9 function, other web-associating molecules should be also mentioned. In CD9 positive vesicle population isolated from bovine epididymis, three proteins were represented mostly, P25b, GliPr1L1 and MIF whereas all of them are involved in sperm function as described Parent *et al.* (1999), Frenette *et al.* (2003),

Eikhoff *et al.* (2004), Caballero *et al.* (2012). When tetraspanin-enriched microdomains involved in mechanism of molecular transfer regarding CD9 molecule have been analyzed, CD26 and CD224 were found to be associated with CD9-positive microvesicles isolated from bovine epididymis in the tetraspanin web. Moreover, CD26 synergic effect with CD9 was observed in the experiments where transfer of molecules from microvesicles to epididymal sperm has been examined. The study of Glazar and Evans (2009) demonstrated from co-immunoprecipitation experiments that CD9 and IgSF8 are in the first-level interaction in mouse egg and described interaction of CD9 with IgSF8 in a primary cell type for the first time. CD9 could have a major role in mouse fertilization through the association with other membrane proteins on egg membrane within the tetraspanin web, potentially also as IgFS8. CD81 is a tetraspanin that associates with several β1 integrins and also with CD9 molecule (Hemler 1998). Although CD81 shares homologous transmembrane domains with CD9 and similar biological function, different subcellular oocyte localisation has been observed (Horváth *et al.* 1998). CD81 was predominantly localized in the zona-pellucida whereas CD9 in perivitelline space. Both proteins were found to be a part of oocyte exosomes (Ohnami *et al.* 2012). In IVF assay, anti-CD81 antibody caused moderate inhibition of sperm-egg binding and weak influence on fusion as referred Takahashi *et al.* (2001). On the other hand, microinjection experiments in study of Ohnami *et al.* (2012) showed that force expression of CD9 can improve the fusion rate decreased due to CD81-deficiency in oocytes. Reverse ability of CD81 has not been achieved. Therefore the conductive role of CD81 to the oocyte CD9 in the sperm-egg fusion has been suggested. Eventually, CD81can be involved in CD9 transfer to sperm membrane. Another differentiation antigen CD151 links α3β1 and α6β1 integrin to other tetraspanins, including CD9 (Ito *et al.* 2003, Charrin *et al.* 2003b). Rubinstein *et al.* (1996) found out that CD9 as well as CD63, CD81 and CD82 organize a complex with other associated molecules as the VLA integrins and HLA-DR antigens. Recently,

interactions between CD9 and CD49B, CD63 and IZUMO1 during human fertilization were predicted using several computational approaches (Sabetian *et al.* 2014). Some authors (Ellerman *et al.* 2003) concluded CD9 as a receptor for PSG17 (a member of pregnancy-specific glycoprotein family), which, similar to IZUMO, is a member of the immunoglobulin superfamily. CD9-deficient eggs were unable to bind PSG17 molecule and moreover, sperm-egg fusion was blocked when the egg PSG17 binding site was mutated or not available. They suggested possible CD9 function in sperm-egg fusion through the binding the sperm PSG17.

Conclusion

The involvement of CD9 in fertilization process is likely to be related to tetraspanin character of this molecule. Belonging to this protein superfamily predicts this protein to bind other molecules in tetraspanin web complex, comprising large spectrum of potential associating partners. In all presented studies, some discrepancies in achieved results were obvious, mainly due to various experimental designs and the role of CD9 has been discussed in relation to all events of fertilization process: acrosome reaction, sperm-binding, sperm-penetration, sperm-egg fusion and eventually, in egg activation. Summarizing, although these findings did not prove any hypothesis, the indispensable role of CD9 in fertilization process was not excluded and the precise role of CD9 remains unexplained.

Conflict of Interest

There is no conflict of interest.

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	Molecule	References
Male system	CD46	Anderson *et al*. 1989, Inoue *et al*. 2003, Mizuno *et al*. 2004, Antalíková *et al*. 2007
	CD35 (CR1; complement receptor 1)	Anderson *et al*. 1993
	CD11b/CD18 (R3; complement receptor 3)	Anderson *et al*. 1993
	CRISP1 (epididymal protein DE)	Da Ros *et al.* 2008
	CRISP2	Busso *et al*. 2007
	calmodulin	Courtot *et al.* 1994
	IZUMO	Okabe *et al*. 1987
	CD52	Hale *et al*. 1993, Michalková *et al*. 2010
	CD55	He *et al*. 2000
	fertilinα, fertilin β, cyritestin	Bigler *et al*. 2000, Blobel 2000, Evans *et al.* 1997, Evans *et al.* 2002, Yuan *et al.* 1997
	Crry	Xu *et al*. 2000
	CD59	Rooney *et al*. 1992
Female system	CD35	Anderson *et al*. 1993
	CD46	Taylor and Johnson 1996
	Integrins (α6β1, α3β1, α5β1, αvβ3)	Reviewed in Rubinstein *et al.* 2006
	CD81	Ziyyat *et al*. 2006
	CD151	Ziyyat *et al*. 2006
	CD98	Takahashi *et al*. 2001
	CD52	Hasegawa *et al*. 2008
	CD55	He *et al*. 2000
	IgSF8	Glazar and Evans 2009
	CD59	Rooney and Morgan 1992
	JUNO	Bianchi *et al.* 2014

Table 1

Some of the molecules detected on gamete surface or in male and female genital tract with potential involvement in fertilization process.