Chapter 31

Role of CD9 in Sperm–Egg Fusion and Virus-Induced Cell Fusion in Mammals

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Abstract In mammals, two integral membrane proteins, sperm IZUMO1 and egg CD9, regulate sperm–egg fusion, and their roles are critical but as yet unknown. In such situation, a recent study has shown that CD9-containing exosome-like vesicles, which are released from wild-type eggs, can induce the fusion between sperm and *Cd9*-deficient egg, even though *Cd9*-deficient eggs are highly refractory to the fusion with sperm. This result provides compelling evidence for the crucial involvement of CD9-containing, fusion-facilitating vesicles in sperm–egg fusion. On the other hand, similarities have been observed between the generation of retroviruses in host cells and the formation of small cellular vesicles, termed exosomes, in mammalian cells. The exosomes are thought to regulate intercellular communication through transfer of proteins and RNAs. These collective studies provide an insight into the molecular mechanisms of gamete fusion and other membrane fusion events.

Keywords CD9 • Exosome • Membrane fusion • Tetraspanin

31.1 Introduction

Fertilization is an event that consists of cell–cell adhesion, cell–cell fusion, and activation of cell signaling to allow the resumption of the egg cell cycle (Fig. 31.1). In mammals, two membrane protein families, a cell adhesion molecule “integrin” (Almeida et al. 1995) and a membrane-anchored protease “ADAM (a disintegrin

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Fig. 31.1 Series of steps from sperm–egg interaction to fusion during mammalian fertilization: an overview of mammalian fertilization. Fertilization is divided into multiple steps: interaction of sperm–somatic cells (termed cumulus cells), binding of sperm to the extracellular matrix (termed zona pellucida), and penetration of the egg. After the sperm penetrates the zona pellucida, it can bind and fuse to the egg cell membrane. Successful fertilization requires not only that a sperm and egg fuse, but also that polyspermy block occurs

and metalloprotease)” (Blobel et al. 1992), were biochemically identified and immunocytochemically confirmed to localize on the outer cell membranes of egg and sperm, respectively; furthermore, antibodies against these protein families were shown to significantly reduce the rate of sperm–egg binding and fusion in mice (Almeida et al. 1995). Integrins, which are expressed in many types of cells in animals, mediate cell–cell and cell–matrix interaction and intercellular communication, including cell adhesion and cell–cell fusion (Almeida et al. 1995). On the other hand, the ADAM family has a characteristic domain that is homologous to an extracellular region of integrin family (Evans 2001). Thus, the presence of the domain conserved between integrins and ADAMs indicates that these two protein families play a role in sperm–egg adhesion and/or fusion (Evans 2001). Unexpectedly, when their genetically manipulated mice were produced, both male and female mice displayed no overt anomalies in either sperm–egg fusion or adhesion (Miller et al. 2000; He et al. 2003). In the past, many factors predicted to participate in sperm–egg fusion have emerged in mice, but despite expectations, most were found to be unnecessary (Okabe and Cummins 2007). From these studies, to ensure the continuous success of the reproduction cycle in mice, compatible pathways tuned by overlapping functions of multiple proteins seem to regulate the mechanism of fertilization. In other words, there will be more than one way for a sperm and egg to fuse and the network of multiple pathways may minimize the severity of the malfunction that may occur in sperm or eggs lacking a single gene. Concerning sperm–egg fusion, CD9 on the egg membrane (Miyado et al. 2000; Le Naour et al. 2000; Kaji et al. 2000) and IZUMO1 on the sperm membrane (Inoue et al. 2005) are exceptionally essential factors in gene disruption experiments (Fig. 31.2).

Fig. 31.2 Players identified in sperm–egg fusion. IZUMO1 is expressed on the sperm membrane, and *Izumo1*-deficient sperm show a defect in fusion with the egg cell membrane. CD9 is expressed on the egg cell membrane and functions in fusion with the sperm. Two membrane proteins, IZUMO1 and CD9, are essential for sperm–egg fusion in mice. Direct interaction between CD9 and IZUMO1 has not been identified, and the unidentified sperm and egg factors may be involved in sperm–egg fusion. After sperm–egg membrane fusion occurs, a sperm factor triggers Ca^{2+} oscillations and initiates egg activation in mammals

31.2 CD9 and Its Role in Cellular Function

The CD9 gene encoding a 24-kDa protein is transcribed in all types of mammalian cells (Hemler 2008). This protein is localized on the cell membranes and partially on endosomes, and it is expected to be involved in cell–cell adhesion, because CD9 associates with the integrin family (Hemler 2008). CD9 is known as a motility-related protein 1 (MRP-1), which plays a role in suppressing tumor metastasis (Miyake et al. 1991). CD9 has two extracellular loops, four transmembrane domains, and two short cytoplasmic domains. Its functional domain is expected to be included in a large extracellular loop (LEL) (Fig. 31.3), because CD9 associates with other membrane proteins via LEL *in vitro* (Hemler 2008). In addition, because the amount of CD9 in mesenchymal and embryonic stem cells is significantly higher than in fibroblastic cells, CD9 is useful as a cell-surface marker for isolating undifferentiated cells from cell pools in mice and humans (Akutsu et al. 2009).

Despite two decades of effort, however, the *in vivo* role of CD9 was unclear, and therefore three laboratories have independently generated *Cd9*^{−/−} mice (Miyado et al. 2000; Le Naour et al. 2000; Kaji et al. 2000). Consequently, all strains of the *Cd9*^{−/−} mice have shown severe female subfertility whereas the *Cd9*^{−/−} male mice were fertile. Moreover, the *Cd9*^{−/−} female mice exhibited severely reduced sperm

Fig. 31.3 Structural features of tetraspanin CD9, a member of the tetraspanin family, termed tetraspanin, and its molecular mass is 24 kDa. The structural features of CD9 include four transmembrane domains, two extracellular loops, short and large extracellular loops (SEL and LEL), and two short cytoplasmic tails. CD9 has cysteine-cysteine-glycine (CCG) residues (amino acids 152–154) as a tetraspanin-specific motif and two other cysteines within LEL

fusion ability. Since these findings, CD9 has been studied as one of the crucial factors in sperm–egg fusion in mammals. A functionally essential domain of CD9 is predicted to be located within the LEL (Zhu et al. 2002; Kaji et al. 2002); however, even though CD9-binding proteins have been identified in non-gamete cells, LEL-binding, potentially fusion-related proteins have not been found yet.

31.3 Tetraspanin

CD9 belongs to a membrane protein superfamily collectively termed “tetraspanin” that encompasses 35 members in mammals (including CD9, CD37, CD53, CD63, CD81, CD82, and CD151) (Hemler 2008), 30 in nematodes (Moribe et al. 2004), and 30 in flies (Todres et al. 2000). In mammals, tetraspanin is related to infectious diseases; in particular, these proteins are involved in cell–cell transmission of human immunodeficiency virus (HIV-1) (Garcia et al. 2005; Wiley and Gummuluru 2006). After its primary infection into host cells, CD9, CD63, CD81, and CD82 are enriched at HIV-1 budding sites of HIV-1 virions. When tetraspanin-containing HIV-1 particles are secondarily formed and released from host cells, they become tenfold more infectious than the cell-free virus particles. In mice, CD81 is linked to infection of hepatocytes with the malaria parasite (Silvie et al. 2003).

Malaria sporozoites, a cell form that infects new hosts, are transmitted into the liver of the mammalian host through bites from infected mosquitoes, but the sporozoites fail to infect *Cd81*^{-/-} mouse hepatocytes, suggesting that CD81 is involved in sporozoite entry into hepatocytes as a host factor. Otherwise, *Cd81*^{-/-} female mice are subfertile, because *Cd81*-deficient eggs exhibit impaired sperm fusion ability (Rubinstein et al. 2006; Tanigawa et al. 2008). CD81 is expressed on *Cd9*-deficient eggs, and CD9 is also expressed on *Cd81*-deficient eggs at an expression rate comparable with that of wild-type eggs, indicating that CD9 and CD81 work independently in sperm–egg fusion (Ohnami et al. 2012).

More than 60 members of tetraspanin are known in plants (Huang et al. 2005; Chiu et al. 2007). Tetraspanin-like proteins have also been identified in fungi, and their molecular mass (more than 200 kDa) is greater than those of tetraspanin identified in animals and plants (20–30 kDa) (Lambou et al. 2008). An appressorium is a specialized cell typical of many fungal plant pathogens that is used to infect host plants. By analyzing a nonpathogenic mutant, *punchless*, isolated from the rice blast fungus *Magnaporthe grisea*, tetraspanin-like PLS1 (MgPLS1) has been shown to control the appressorial function, which is essential for fungal penetration into host leaves (Clergeot et al. 2001). Similarly, *Colletotrichum lindemuthianum* PLS1 (CIPLS1) is a functional homologue of MgPLS1. The nonpathogenic *CIPLS1*-deficient mutant to bean leaves exhibits a defect in the formation and positioning of the penetration pore (Veneault-Fourrey et al. 2005). As the invasion of pathogenic fungi into leaves is an event closely related to membrane fusion events, these studies indicate that tetraspanin-like PLS1s are involved in the membrane fusion-related event between fungus and plant.

Taken together, these results suggest that tetraspanin is related to membrane fusion-related events in multicellular organisms. On the other hand, the physiological activities of tetraspanin are still unknown, and its fusogenic activity corresponding to fusogenic transmembrane proteins, such as syncytin identified in human placenta (Mi et al. 2000), and virus envelope proteins (Hernandez et al. 1996), has not been identified.

31.4 Tetraspanin as an Exosome Component

In mammals, cell-cultured media contain nanosized membrane vesicles, but they were not attractive to researchers because they could not be structurally distinguished from the debris of dead cells (Couzin 2005). Recent studies have shown that the vesicles, termed exosomes, are derived from living cells, but not dead cells; furthermore, they have been proven to play a significant role in the mediation of adaptive immune reactions to pathogens and tumors through the enhancement of antigen-specific T-cell responses (Couzin 2005; Simons and Raposo 2009). Besides immune cells, the exosomes are released from a wide range of normal and malignant mammalian cell types, and their diameter is estimated to range from 50 to

90 nm (Simons and Raposo 2009). The protein composition of exosomes varies with the origin of cells, yet the exosomes commonly contain a ganglioside GM3, two kinds of heat shock proteins (HSP70 and HSP90), and tetraspanin (Simons and Raposo 2009). The exosomes also contain transcripts, mRNA and microRNA, which are thought to be shuttled from one cell to another, thereby influencing protein synthesis in recipient cells (Valadi et al. 2007).

31.5 Exosome-Like Vesicles Released from Eggs

In eggs, two reports have demonstrated the contribution of CD9 in the organization of the egg cell membrane. First, CD9 is transferred from the egg to the fertilizing sperm present in the perivitelline space, implying the involvement of a process similar to trogocytosis, which is a mechanism for the cell-to-cell contact-dependent transfer of membrane fragments from antigen-presenting cells to lymphocytes in immune responses for pathogens (Barraud-Lange et al. 2007). Second, CD9 deficiency alters the length and density of microvilli on the egg cell membrane (Runge et al. 2007).

On the other hand, a recent study has reported the potential of enhanced green fluorescent protein-tagged CD9 (CD9-EGFP) as a reporter protein for studying sperm-egg fusion in living mouse eggs (Miyado et al. 2008). Interestingly, in eggs just before fertilization, CD9-EGFP is significantly accumulated within the perivitelline space that completely surrounds the eggs and lies between the egg cell membrane and the zona pellucida. Consistent with the images from CD9-EGFP, immunoelectron microscopic analysis of wild-type eggs has revealed that CD9 is not only present in the perivitelline space but is also incorporated into vesicles of varying size (50–200 nm in diameter) without a sectional profile of a typical lipid bilayer. Furthermore, in opossums (Talbot and DiCarantonio 1984) and humans (Dandekar et al. 1992), as well as in mice, membrane vesicles have been detected by electron microscopy within the perivitelline space of their eggs. Moreover, recent study has demonstrated that the vesicles identified in mouse eggs share CD9, GM3, and HSP90 with exosomes, and these components are absent in eggs lacking CD9 and are reproduced by CD9-EGFP expression restricted to the eggs (Miyado et al. 2008). These results provide two types of evidence for the nature of CD9 in mouse eggs. First, CD9-incorporated exosome-like vesicles are produced in mouse eggs and are released outside the egg cell membrane just before fertilization. Second, CD9 is essential for the formation and release of the exosome-like vesicles (hereafter referred to as egg exosomes) in mouse eggs.

CD9-containing egg exosomes render sperms capable of fusing with *Cd9*-deficient eggs (Miyado et al. 2008) (Fig. 31.4). *Cd9*-deficient eggs cannot fuse with eggs, but the coexistence of wild-type eggs results in 60–70 % of the *Cd9*-deficient eggs fusing with at least one sperm. Thus, sperm can fuse with *Cd9*-deficient eggs with impaired microvilli via the egg exosomes of wild-type eggs, which means that the egg exosomes, but not the microvilli, are essential for sperm-egg fusion.

Fig. 31.4 Overview of studies of *Cd9*-deficient eggs. In wild-type eggs, CD9-containing egg exosomes are released from wild-type eggs before any interaction with the sperm (*upper right*). Shortly after the sperm penetrates the perivitelline space, the egg exosomes are transferred on the acrosome-reacted sperm head. Then, a sperm fuses with the egg cell membrane. Interaction between sperm and exosomes is an essential step for sperm-fusing ability. In contrast, *Cd9*-deficient eggs cannot release egg exosomes, which are correlated with the formation of microvilli on the egg cell membrane (*upper left*). Sperm cannot fuse to the cell membrane of the *Cd9*-deficient egg. However, when the zona pellucida is removed from the eggs, the sperm is able to interact with the egg exosomes released from wild-type eggs and can fuse with the *Cd9*-deficient egg (*lower left*). By coincubation with wild-type eggs, the sperm can fuse with a similar number of *Cd9*-deficient and wild-type eggs. Intracytoplasmic sperm injection (ICSI) is an in vitro fertilization procedure in which a single sperm head is injected directly into an egg (*lower right diagram*). This procedure is most commonly used to overcome male infertility and fusion defects in *Cd9*-deficient eggs

31.6 Membrane Fusion and Exosomes

The close relationship between egg exosomes and sperm–egg fusion raises the question of how egg exosomes facilitate fusion. According to a previous report, exosomes contain both functional mRNA and microRNA, which are shuttled from one cell to another, affecting recipient cell ability to produce protein (Valadi et al. 2007). Moreover, HIV-1 utilizes the exosome biogenesis pathway for the formation of infectious particles, and in macrophages, HIV-1 assembles into an intracellular plasma membrane domain-containing tetraspanin (i.e., CD9, CD81, CD53, or CD63) (Wiley and Gummuluru 2006). Thus, the exosomes may play at least two roles in regulating cell function: first, shuttling proteins and RNAs (mRNAs and microRNAs) from one cell to another, and second, forming infectious particles. In fertilization, these two actions may be required for sperm–egg fusion in mammals. The studies of exosomes underscore the relevance of CD9 for healthy and pathogenic cell–cell fusion processes and may present a useful strategy for regulating the cell-to-cell spread of specific viruses and fertilization ability.

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