A Novel Live Imaging-Based Assay for Visualising Species-Specific Interactions in Gametic Adhesion Molecules

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Abstract

Successful gametic fusion requires species-specific membrane adhesion. However, the interaction of adhesion molecules in gametes is difficult to study in real time through low-throughput microscopic observation. Therefore, we developed a novel live imaging-based adhesion molecule (LIAM) assay to study gametic adhesion molecule interactions in cultured cells. First, we modified a fusion assay previously established for fusogens introduced into cultured cells, and confirmed that our live imaging technique could visualise cell-to-cell fusion in the modified fusion assay. Next, instead of fusogen, we introduced adhesion molecules including a mammalian gametic adhesion molecule pair, IZUMO and JUNO, and detected their temporal accumulation at the contact interfaces of adjacent cells. Accumulated IZUMO or JUNO was translocated to the opposite cells; the mutation in amino acids required for their interaction impaired accumulation and translocation. By using the novel LIAM assay, we investigated the species specificity of IZUMO and JUNO of mouse, human, hamster, and pig in all combinations. IZUMO and JUNO accumulation and translocation were observed in conspecific, and some interspecific, combinations, suggesting potentially interchangeable combinations of IZUMO and JUNO from different species.

Introduction

Sexually reproducing organisms commonly reproduce via the union of different sex cells. Fertilisation is the final process of sexual reproduction; it is achieved through the recognition, adhesion, and fusion of gametes in processes regulated by fertilisation molecules on gametes. Fertilisation molecules have been identified in several species. For example, in mammals, sperm that have passed through the zona pellucida approach the egg and ultimately fertilise it through interactions of fertilisation molecules, such as IZUMO on the sperm and CD9 and JUNO on the egg¹⁻³. Mutants of IZUMO, CD9, and JUNO are completely infertile. Co-crystal structure analysis has demonstrated that IZUMO and JUNO interact as adhesion molecules, and that species-specific binding depends on the conserved amino acid sequence, e.g., W148A of IZUMO and W62A of JUNO, on the interaction surface^{4,5}, although the machinery required for membrane fusion during fertilisation remains unclear. To date, only IZUMO and JUNO have been identified as a gametic adhesion molecule pair. A recent study also reported that the sperm proteins SPACA6, TMEM95, SOF1, FIMP are required for mammalian fertilisation⁶⁻⁹.

In flowering plants, two immotile sperm cells are conveyed by the pollen tube to the female gametes, and the adhesion molecule GAMETE EXPRESSED 2 (GEX2) and fusogen GENERATIVE CELL-SPECIFIC 1/HAPLESS 2 (GCS1/HAP2) on the sperm cell membrane promote fertilisation with the two female gametes, i.e. the egg cell and central cell¹⁰⁻¹², in a unique fertilisation process referred to as double fertilisation. GEX2 has a filamin-like domain similar to the Ig-like domain contained in IZUMO. The green alga *Chlamydomonas reinhardtii* has no GEX2 orthologue; however, FUS1, which contains a filamin-like domain, is expressed on plus-type cells¹³. Recently, FUS1 partner molecule, Minus Adhesion Receptor 1 (MAR1), was identified, which was required not only for gamete adhesion but for focused localization of

GCS1/HAP2 at the mating structure¹⁴. GCS1/HAP2 is an ancestral eukaryotic fusogen widely conserved in plants and protists¹⁵. Crystal structure studies have revealed that GCS1/HAP2 shows homology with the *Caenorhabditis elegans* somatic cell fusogen Epithelial Fusion Failure 1 (EFF-1)¹⁶ and class II viral fusion proteins (e.g. Dengue, Zika, and West Nile viruses)¹⁷. Whether the GCS1/HAP2 fusion mechanism is unilateral or bilateral remains controversial^{18,19}. GCS1/HAP2 does not exist in mammals, and fusogens have not been identified in mammalian gametes. Current studies are seeking to identify the complete set of fertilisation molecules in several species. Missing partner molecules and the function and action mechanisms of known fertilisation molecules require investigation to understand the precise molecular mechanisms of fertilisation.

Genetic loss-of-function experiments with phenotypic analysis have revealed that fertilisation-related molecules are required for gamete adhesion or fusion. However, the precise molecular interactions driving cell membrane adhesion and fusion remain to be elucidated. Recent gain-of-function experiments have investigated the molecular functions of membrane proteins. For example, nematode specific proteins EFF-1 and AFF-1 have been shown to fuse nematode, insect, mammalian and viral membranes^{16,20–23}. More recently, the muscle-specific protein Myomaker was transfected with Myomerger (also called Myomixer and Minion) into fibroblasts to investigate their biochemical function in cell-membrane fusion²⁴. Molecular adhesiveness of a gametic adhesion molecule pair IZUMO-JUNO has been shown by ectopic expression of IZUMO or JUNO in cultured somatic cells^{4,25,26}. Gain-of-function experiments in heterologous cell systems are powerful tools for the elucidation of molecular functions with characteristic activities. Combining this technique with live-cell imaging would allow us to visualise molecular dynamics and functions in real time.

Species-specific and -preferential interactions among fertilisation molecules also require further investigation. Species specificity has been studied in mammals for several decades. Ovulated eggs are surrounded by the zona pellucida; Yanagimachi *et al.* found that golden hamster zona-free oocytes could be fertilised with sperm of various species²⁷. Some proteins involved in male–female interactions have been suggested to be involved in species-specific interactions. The zona pellucida glycoprotein ZP2 is required for species-specific sperm–egg binding through the zona pellucida in mice and humans²⁸. The glycosylphosphatidylinositol (GPI)-anchored protein Bouncer is required for species-specific sperm entry into the egg, and enables cross-species fertilisation between medaka and zebrafish through gene swapping²⁹. Extracellular protein interactions were exploited in the AVIDity-based Extracellular Interaction Screening (AVEXIS) assay, demonstrating conspecific interaction between IZUMO and JUNO in mouse cells; the results suggested that hamster JUNO can interact with human, mouse, and pig IZUMO³⁰, consistent with the crossing result obtained by Yanagimachi *et al.*³¹. However, in flowering plants, gametic interaction appears to be less critical for species-specific fertilisation than the progamic phase of pollen–pistil interactions^{32–34}.

To elucidate the molecular dynamics and function of molecules involved in gametic interactions such as gamete adhesion and fusion, we developed a system to analyse the functions of fertilisation molecules

through live-cell imaging in cultured animal cells. For this gain-of-function approach, we modified a fusion assay developed by Valansi *et al.*¹⁸ and transfected cultured baby hamster kidney (BHK) cells to express a gametic fusogen GCS1/HAP2, which promoted their cell-to-cell fusion. We confirmed that cell-to-cell fusion of BHK cells expressing fusogens could be captured by our live-cell imaging technique using a disc scanning confocal system embedded within the incubator. Then, we introduced adhesion molecules to explore their effects in cultured cells, which lead us to develop a live imaging-based adhesion molecule (LIAM) assay. LIAM assay was shown to be useful for the screen of species-specificity in gametic adhesion molecules IZUMO and JUNO.

Results

Fusogen promotes cell-to-cell fusion in our live-cell imaging system

We aimed to develop a system to study fertilisation molecules in animal cultured cells by live imaging. Various cultured animal cell lines, such as COS-7 and HEK293T, have been used for transfection and expression in previous fertilisation molecule studies; for example, molecular adhesiveness between IZUMO and JUNO was shown by mixing gametes and transfected cultured-cells^{3,26,35}. In this study, because we focused on cell migration and morphology for frequent contact of cells under microscopy, we selected a line of motile BHK cells that did not spontaneously adhere or fuse with each other (Fig. S1). BHK cells were also used in the original fusion assay¹⁸. We investigated whether cell fusion activity could be detected in known fertilisation molecules using our live-cell imaging technique by transfecting BHK cells with RFPcyto as a negative control, and *GCS1/HAP2* to evaluate multinucleation rates. Ectopic expression of fusogens sometimes indicates toxicity to cells or the whole organism^{16,18,21,36}; therefore, tight regulation was required to ensure expression at the preferred times. For this purpose, we used a mifepristone-inducible system as used in the fusion assay¹⁸.

To capture cell-to-cell fusion by live imaging, we used a Nipkow disc scanning confocal system embedded within the incubator to monitor cells in 1 mm × 1 mm areas every 6 min under stable low-phototoxicity conditions (Fig. 1). In each captured video, 130-476 cells expressing RFPcyto were monitored. When the gametic fusogen GCS1 (*Arabidopsis thaliana* GCS1; AtGCS1) was expressed, cell-to-cell fusion was observed (Fig. 1A, arrows; Video 1). At 12 h after induction, the multinucleation rate increased significantly, to $2.2 \pm 0.4\%$ ($n = 4$; $P < 0.05$; Dunnett's test; Fig. 1B). To confirm that cell-to-cell fusion depended on the fusogen activity of the expressed proteins, we transfected the loop-deletion *GCS1* (*GCS1Δloop*). Amino acids 166–178 in AtGCS1 represent a hydrophobic loop structure, which is necessary for membrane fusion¹⁷. The multinucleation rate was $1.2 \pm 0.2\%$ ($n = 3$), which was not significantly different from the negative control (Fig. 1B). This result suggested that fusogen-dependent cell-to-cell fusion was visualised by live imaging.

Our live imaging system is useful to monitor expression of a fluorescent marker upon cell-to-cell fusion. The dependency of individual fusion on the expression of transfected genes from 4 to 12 h after expression induction is shown in Fig. 1C. In experiments for wild-type GCS1, a combination of two cells labelled with red fluorescent protein (RFP) was more likely to fuse than a combination of labelled and non-labelled cells. This is partly due to the frequent fusion of daughter cells after cell division. Our result is consistent with the previous report that GCS1, as well as EFF-1, bilaterally promoted cell-to-cell fusion in the fusion assay¹⁸. Together, these results suggest that our live imaging technique is compatible with a modified fusion assay for the study of fusogen functions in cultured cells.

Adhesion molecules accumulate at the contact interfaces of adjacent cells

The fusion assay has never previously been used to evaluate gametic adhesion molecule function; conventional methods include adhesion and aggregation assays^{37,38}. To develop a simple method suitable for live imaging-based studies, we introduced adhesion molecule pairs into the modified fusion assay.

First, we transfected the somatic adhesion molecule E-cadherin into BHK cells. Cadherins have been shown to play a role in Ca^{2+} -dependent cell–cell adhesion³⁹. The transfection of a single cadherin gene can potentially induce interaction of cadherin expressed in different cells (homophilic adhesion), eliminating the necessity of introducing an adhesion molecule pair such as IZUMO and JUNO (heterophilic adhesion). Cadherin was visualised by translational fusion with GFP, and no toxicity was observed despite the lack of an inducible gene expression system for cadherin in this experiment. E-cadherin-expressing BHK cells in our system did not adhere to neighbouring cells to show aggregation. E-cadherin appeared to be localised at the cell membrane and secretory pathways of the cell, showing no polarisation (Fig. 2A). However, upon temporary contact between E-cadherin-expressing cells, E-cadherin tended to accumulate at the contact interface (Fig. 2A, 7:30; Video 2); fluorescent intensity of E-cadherin-GFP increased specifically at the interface of cell contact (Fig. 2B, 7:30). This accumulation disappeared as cells detached (Fig. 2B, 8:00).

Next, we introduced mouse IZUMO and JUNO to determine whether a similar temporal accumulation at the contact interface of adjacent cells might occur even in heterophilic adhesion molecules. We developed a mixing assay, in which cells expressing IZUMO and JUNO were mixed at 4 hours after independent transfection and were incubated for 20 hours. IZUMO and JUNO were designed as translational fusion with fluorescent proteins. At 4 to 12 hours after the induction of IZUMO and JUNO expression by mifepristone, we found that IZUMO temporally accumulated at the contact interfaces of JUNO-expressing cells (47% of contacting cells, $n = 53$; Fig. 2C-E; Video 3).

We examined whether IZUMO accumulation is dependent on the interaction of IZUMO and JUNO expressed in adjacent cells. When IZUMO and JUNO were expressed simultaneously in the same cell by

co-transfection, neither molecule accumulated on the cell surface (0%, n = 63; Fig. 2E). Next, we constructed a mutant IZUMO lacking the β -hairpin region (IZUMO Δ) and examined the interaction between IZUMO Δ and JUNO. The central β -hairpin region of IZUMO is required for JUNO binding, as has been shown by co-crystal structure analysis^{4,5}. When IZUMO Δ - and JUNO-expressing cells were mixed, accumulation of IZUMO was rarely observed (7% of cases, n = 59; Fig. 2E). We also co-transfected JUNO with CD9, an egg tetraspanin required for gamete fusion², to determine whether CD9 modulates IZUMO accumulation. When IZUMO- and JUNO/CD9-expressing cells were mixed, IZUMO accumulation and frequency were unchanged, consistent with the finding that CD9 is independent of gamete adhesion^{2,40} (50%, n = 42; Fig. 2E). These results demonstrate the usefulness of this assay for visualising the dependence of adhesion molecule interactions on the affinity of introduced adhesion molecule pairs. We named this assay system the live imaging-based adhesion molecule (LIAM) assay, which we propose for the examination of transfected adhesion molecules at cell contact sites.

Accumulation and translocation of adhesion molecules in the LIAM assay depended on critical amino acids

Subsequent experiments were conducted using high-throughput analysis in a non-inducible gene expression system (cytomegalovirus promoter), because we found that the inducible gene expression system was unnecessary for cadherin, IZUMO, and JUNO (translational fusion with fluorescent proteins). Prior to contact between mouse IZUMO- and JUNO-expressing cells, these adhesion molecules were distributed evenly throughout the cell, as also occurs in the inducible gene expression system. Accumulation was observed upon cell contact; however, both IZUMO and JUNO showed accumulation (Fig. 3A; Video 4). IZUMO accumulation was observed in 72% of contacting cells (39/54; Fig. 3B), whereas JUNO accumulation was observed in only 37% (20/54; Fig. 3B). IZUMO and JUNO accumulation were always simultaneously observed in the same cell pairs (opposite sides of a single contact site) when JUNO accumulation was observed.

To determine whether accumulation was dependent on the amino acid residues of IZUMO and JUNO for molecular interaction, we performed a LIAM assay using mutant IZUMO and JUNO, according to previous crystal structure analysis results^{4,5} (Fig. 3A). When mouse mutant IZUMO W148A-expressing cells were mixed with mouse JUNO-expressing cells, accumulation was not observed (0% of 45 contacting cells; Fig. 3B). When mouse IZUMO-expressing cells and mouse mutant JUNO W62A-expressing cells were mixed, accumulation was again not observed (0% of 67 contacting cells; Fig. 3B). Prior to cell contact, IZUMO and JUNO expression and localisation were unchanged by these point mutations (Fig. 3A). Next, we tested the sperm transmembrane protein SPACA6, which is required for fertilisation^{6,7}, instead of IZUMO, which shows structural similarity with SPACA6⁶ (Fig. S2), and found that neither SPACA6 nor JUNO accumulated (Fig. 3A). These results indicate that IZUMO and JUNO accumulation depends on amino acids critical for their interaction, according to the LIAM assay.

In 38% (15/39) cells showing IZUMO accumulation, IZUMO was found to translocate to the plasma membrane of the opposite cell (Fig. 3C). Several fluorescent foci of IZUMO or JUNO on the opposite cell were observed upon the detachment of contacting cells (Video 5). IZUMO was translocated to JUNO-expressing cells in 27% of contacting cells (15/54; Fig. 3D) and JUNO was translocated to IZUMO-expressing cells in 7.4% of contacting cells (4/54; Fig. 3D). This translocation of IZUMO and JUNO was unilateral, not bilateral, in each cell pair, and translocation was selectively observed for either IZUMO or JUNO in each cell pair. In addition, IZUMO or JUNO translocation was always observed after IZUMO accumulation, but not necessarily after JUNO accumulation, perhaps due to the difficulty associated with visualising JUNO accumulation on the cell membrane.

This translocation was dependent on the amino acid sequence of IZUMO and JUNO required for the interaction (Fig. 3D). When mutant IZUMO W148A or mutant JUNO W62A was used, neither translocation nor accumulation was observed (Fig. 3D). SPACA6 and JUNO also did not induce translocation (Fig. 3D). In subsequent experiments, this translocation of adhesion molecules was also monitored in the LIAM assay.

Species specificity in JUNO and IZUMO interactions suggested by the LIAM assay

Using the LIAM assay, we investigated the species specificity of IZUMO and JUNO from mouse, human, hamster, and pig (Fig. 4; Tables 1–4). Among these four mammalian species, mice are most closely related to hamsters, and humans and pigs are phylogenetically close to each other⁴¹. Molecular phylogenetic analysis results for IZUMO and JUNO proteins were consistent with the phylogenetic relationships among these four species (Figs. S3–5). The mouse IZUMO protein shares 61%, 52%, and 50% homology (identity) with hamster, human, and pig IZUMO proteins, respectively (Figs. S3, S5). In contrast, the mouse JUNO protein shares 74%, 68%, and 65% homology with hamster, human, and pig JUNO proteins, respectively (Figs. S4, S5). These four species were selected because species specificity has been observed biochemically in their IZUMO and JUNO interactions, in some but not all combinations, using the AVEXIS assay³. In the AVEXIS assay, IZUMO and JUNO interaction was shown in all conspecific combinations, hamster JUNO and all other three species, and mouse JUNO and human IZUMO but not in human JUNO and mouse IZUMO.

Table 1
IZUMO accumulation in conspecific and heterospecific cell combinations. Numbers in brackets are the numbers of contacted cells for each combination.

IZUMO				
JUNO	Mouse	Human	Hamster	Pig
Mouse	72% (54)	0.0% (50)	31% (36)	0.0% (64)
Human	50% (38)	28% (51)	0.0% (58)	48% (63)
Hamster	78% (45)	0.0% (46)	77% (57)	13% (84)
Pig	0.0% (25)	0.0% (38)	0.0% (37)	24% (41)

We examined all combinations of IZUMO and JUNO from mouse, human, hamster, and pig cells using the LIAM assay. We began with conspecific combinations. Mouse IZUMO and JUNO showed accumulation in 72% (39/54; Fig. 3; Table 1) and 37% (20/54; Fig. 3; Table 2) of contacting cells, respectively.

Translocation of mouse IZUMO and JUNO was also observed, in 27% (15/54; Table 3; Fig. 3C) and 7.4% (4/54; Table 4) of contacting cells, respectively. This finding is consistent with previous AVEXIS assay results³, which showed that mouse IZUMO and JUNO show relatively strong binding. In this study, we observed IZUMO accumulation as often as JUNO accumulation in human IZUMO × human JUNO interactions (IZUMO accumulation: 28%, 14/51, Table 1; JUNO accumulation: 29%, 15/51, Table 2). In this combination, IZUMO translocation was not observed (Table 3), whereas JUNO translocation was frequently observed (64%, 33/51, Table 4, Fig. 4A). In hamster IZUMO × hamster JUNO, IZUMO and JUNO accumulation (IZUMO: 77%, 41/57, Table 1; JUNO: 28%, 16/57, Table 2) and translocation (IZUMO: 31%, 18/57, Table 3; JUNO: 28%, 16/57, Table 4, Fig. 4B) were observed, consistent with mouse IZUMO and JUNO. In pig IZUMO × pig JUNO interactions, we observed IZUMO accumulation at a low rate (24%, 10/41, Table 1, Fig. 4C). Conspecific interaction of IZUMO and JUNO was confirmed for all combinations, and the frequencies of IZUMO and JUNO accumulation and translocation differed among species.

Table 2
JUNO accumulation in conspecific and heterospecific cell combinations. Numbers in brackets are the numbers of contacted cells for each combination.

IZUMO				
JUNO	Mouse	Human	Hamster	Pig
Mouse	37% (54)	2.0% (50)	25% (36)	0.0% (64)
Human	0.0% (38)	29% (51)	4.1% (58)	49% (63)
Hamster	0.0% (45)	0.0% (46)	28% (57)	7.1% (84)
Pig	0.0% (25)	0.0% (38)	0.0% (37)	0.0% (41)

Table 3
IZUMO translocation in conspecific and heterospecific cell combinations. Numbers in brackets are the numbers of contacted cells for each combination.

IZUMO				
JUNO	Mouse	Human	Hamster	Pig
Mouse	28% (54)	0.0% (50)	0.0% (36)	0.0% (64)
Human	0.0% (38)	0.0% (51)	0.0% (58)	3.2% (63)
Hamster	0.0% (45)	0.0% (46)	32% (57)	0.0% (84)
Pig	0.0% (25)	0.0% (38)	0.0% (37)	0.0% (41)

Next, we examined heterospecific interaction between mouse IZUMO and human, hamster, and pig JUNO. When mouse IZUMO-expressing cells and human JUNO-expressing cells were mixed, mouse IZUMO accumulated at the cell interface (50%, 19/38; Table 1), but neither JUNO accumulation nor IZUMO/JUNO translocation was observed (Tables 2–4). When mouse IZUMO-expressing cells and hamster JUNO-expressing cells were mixed, only mouse IZUMO accumulated (78%, 35/45, Table 1), consistent with mouse IZUMO and human JUNO. When mouse IZUMO-expressing cells and pig JUNO-expressing cells were mixed, we observed neither accumulation nor transference. These results indicate that the translocation of adhesion molecules to opposite cells correlated well with conspecific interactions.

Table 4
JUNO translocation in conspecific and heterospecific cell combinations. Numbers in brackets are the numbers of contacted cells for each combination.

IZUMO				
JUNO	Mouse	Human	Hamster	Pig
Mouse	7.4% (54)	0.0% (50)	0.0% (36)	0.0% (64)
Human	0.0% (38)	65% (51)	0.0% (58)	32% (63)
Hamster	0.0% (45)	0.0% (46)	28% (57)	0.0% (84)
Pig	0.0% (25)	0.0% (38)	0.0% (37)	0.0% (41)

Next, we examined heterospecific interaction between human IZUMO and mouse, hamster, and pig JUNO. Neither accumulation nor translocation was detected in any combination (Tables 1–4). When hamster IZUMO-expressing cells and mouse JUNO-expressing cells were mixed, IZUMO and JUNO accumulation were detected, although at lower rates than for conspecific combinations (mouse IZUMO × mouse JUNO,

hamster IZUMO × hamster JUNO) (Tables 1 and 2). This result is consistent with the crossing results reported by Yanagimachi et al.²⁷ and those of a previous AVEXIS assay³. No translocation was observed (Tables 3 and 4). Finally, we combined pig IZUMO-expressing cells with mouse, human, and hamster JUNO-expressing cells. When pig IZUMO-expressing cells and mouse JUNO-expressing cells were mixed, neither accumulation nor translocation was observed (Tables 1–4). However, when pig IZUMO-expressing cells and human JUNO-expressing cells were mixed, IZUMO and JUNO accumulation was detected. Unexpectedly, translocation of both IZUMO and JUNO was detected in this heterospecific combination of pig IZUMO and human JUNO (Tables 1–4, Fig. 4D). This strongly positive result was obtained using our LIAM assay, but has not been tested previously using other assays.

Discussion

In this study, we developed the LIAM assay to study gametic adhesion molecules in cultured animal cells. The LIAM assay represents a powerful complement to the fusion assay¹⁸ for microscopic examination of the functions of fertilisation molecules in various organisms under highly controllable conditions. As previously observed in fusogens including plant GCS1/HAP2 and nematode EFF-1, the functions of the mammalian adhesion molecule pair IZUMO and JUNO were assessed at the contact interfaces of adjacent cells. Two phenotypes of adhesion molecule dynamics, i.e., accumulation and translocation, were detected by the LIAM assay (Fig. 3). The accumulation of homophilic and heterophilic somatic adhesion molecules^{39,42,43}, as well as gametic IZUMO and JUNO³, has been reported at the interface of adhered cultured cells; however, our live imaging-based assay suggested that temporal contact was sufficient to induce the intracellular accumulation of adhesion molecules in non-aggregating cells. Such translocation is an interesting phenomenon that may be caused by protein extraction from the membrane of the adjacent cell during contact. Monodirectional translocation (i.e. selective translocation of IZUMO or JUNO in a single cell pair) and stable foci of translocated proteins suggest that some cell activity, such as endocytosis, trogocytosis⁴⁴, or exosome reception⁴⁵ within a cell pair, is involved in the translocation and compartmentalisation of adhesion molecules, depending on the molecule combination.

Accumulation and translocation were strictly dependent on the structures of the tested molecules (Fig. 3). Single amino acid mutations in IZUMO or JUNO, which are critical for their interaction, abolished these signals completely. Other loop and domain deletions, as well as the swapping of IZUMO and SPACA6, also resulted in drastic decreases in these signals (Figs. 2 and 3). These results suggest that the LIAM assay successfully detected interactions within the adhesion molecule pairs. Consistently, IZUMO and JUNO that had simultaneously accumulated within a cell pair were localised together across the contact site (Fig. 3). The frequencies of accumulation and translocation detected by the LIAM assay were strongly correlated with conspecific combinations in which direct interaction of IZUMO and JUNO have been shown biochemically using an AVEXIS assay³ (Tables 1–4). This was also consistent with the detection of some heterospecific but compatible combinations, such as mouse sperm IZUMO and human egg JUNO^{25,26} or hamster egg JUNO³ (Table 1). The application of other analysis techniques, such as fluorescence resonance energy transfer (FRET), would provide insights into the interaction of adhesion

molecules observed in our LIAM assay; combining the LIAM assay with FRET would be a very powerful method for visualising molecular interactions in real time, which has not been attempted for gametic interactions in any organisms to date.

The advantages of the LIAM assay include higher throughput to identify potential molecule pairs showing heterophilic and homophilic adhesion activity among many combinations. After confirming conspecific interaction of IZUMO and JUNO from mouse, human, hamster, and pig, we identified a candidate combination for strong heterospecific binding: pig IZUMO and human JUNO. Among the tested four species, human and pig possess relatively close IZUMO and JUNO (Fig. S5). Evaluation of the adhesiveness of pig sperm and a human egg would be of interest, and to our knowledge has not been reported. The LIAM assay showed positive results for hamster JUNO with a wide range of IZUMO from mouse, pig, and hamster, but not human, which is largely consistent with previous reports that hamster zona-free eggs can be fertilised by sperm of various mammals³¹, and that hamster JUNO can interact with IZUMO from various species³⁰. It may be possible to determine why hamster JUNO is compatible with IZUMO from various mammals in future studies, via detailed amino acid swapping analyses performed using the LIAM assay.

An anticipated direction of *in vitro* studies using LIAM and fusion assays is reconstitution of the gametic fertilisation machinery in cultured cells, which would be a powerful tool for the study of fertilisation molecules as a machinery complex. In LIAM assay, we did not observe cell-to-cell fusion, consistent with the previous report that ectopic expression of adhesion molecules is not sufficient for cell-to-cell fusion of cultured somatic cells⁴⁶. Reconstitution of the fertilisation machinery complex under microscopy is a promising direction for future research to clarify these molecular dynamics and functions. In mammals, partner molecules for the sperm proteins SPACA6, TMEM95, SOF1, FIMP, as well as a fusogen, remain elusive^{6–9}. The identification of a complete set of fertilisation molecules, which is in progress in some organisms including *Chlamydomonas*¹⁴, would contribute significantly to reconstitution in cultured cells or liposomes.

Methods

Cell culture and DNA transfection

BHK-21 cells were used in this study (RCB1423; RIKEN Cell Bank, Tsukuba, Japan). BHK cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM; Wako, Osaka, Japan) containing 10% foetal bovine serum (FBS). Cells were cultured at 37°C in 5% CO₂. Plasmids were transfected into cells using 20 µL jetPRIME (PolyPlus-transfection, Illkirch-Graffenstaden, France) in 200 µL for every 8-well chambered coverglass (IWAKI, Tokyo, Japan).

Plasmid construction

Arabidopsis thaliana GCS1/HAP2 coding sequence (CDS) fragments were amplified from pSN30 (a gift from Dr. Shiori Nagahara) by polymerase chain reaction (PCR) using the primers listed in Supplemental Table 1. *Arabidopsis thaliana* GEX2, *Caenorhabditis elegans* eff-1, mouse *Izumo*, *Juno*, *Cd9*, or *Spaca6* and *Chlamydomonas reinhardtii* GCS1/HAP2, *FUS1* CDS fragments were amplified from cDNA in each organism. Human, hamster, and pig *Izumo* and *Juno* were synthesised artificially (Integrated DNA technologies, Tokyo, Japan). Mouse E-cadherin CDS fragments were derived from mouse E-cadherin green fluorescent protein (GFP; 67937; Addgene, Watertown, MA, USA). To visualise the proteins, these fragments were fused to fluorescent protein sequences by PCR and then cloned into pGENE B or replaced with an E-cadherin-GFP insert after double digestion with restriction enzymes using the Gibson assembly (NEB, Ipswich, MA, USA). For inducible expression using mifepristone in BHK cells, we used the GeneSwitch System (Invitrogen, Waltham, MA, USA).

Microscopy and image acquisition for live cell imaging

Time-lapse images used to detect fusing cells and their interaction were obtained using a spinning disk confocal system (CellVoyager CV1000; Yokogawa Electric, Tokyo, Japan) equipped with 405-, 488-, and 561-nm diode lasers. The incubator equipped with the microscope was set at 37°C and 5% CO₂. Confocal images were acquired using 10× (numerical aperture [NA], 0.40; working distance [WD], 3.1 mm; 10×UPLSAPO; Olympus, Tokyo, Japan) and 40× (NA, 0.95; WD, 0.188 mm; 40×UPLSAPO; Olympus) dry objective lenses. The exposure time was 100 ms for Venus, mCherry, and mTurquoise2. Fluorescence was acquired through band-pass (BP) filters: BP 447/60 for mTurquoise2, BP 525/50 for Venus, and BP 617/73 for mCherry. Image analyses were performed using CV1000 software (Yokogawa Electric) and the Fiji online tool (<http://fiji.sc/>) was used to adjust the brightness and contrast.

Multinucleation analysis

To evaluate multinucleation rates, we co-transfected BHK cells with pGENE and pSWITCH. One day prior to transfection, BHK cells were cultured at 5.0×10^4 cells/mL. At 4 h after transfection, expression vectors were induced by addition to DMEM containing 10% FBS and 10^{-4} mM mifepristone. At 3–4 h post-induction, images of the cells were acquired every 6 min for 12 h, using the CV1000 system at a magnification of 10× to record cell-to-cell fusion. We counted the transfected living cells and evaluated transfection efficiency as 20% of fluorescence. The multinucleation rate was defined as the ratio between the number of multinucleated living cells (Mc) and expressed living cells (EC), as follows: % Multinucleation = $\text{Mc}/\text{Ec} \times 100$. Each experiment was repeated at least three times.

Mixing assay for adhesion molecule pairs

For interaction experiments, plasmids were transfected into BHK cells. At 4 h post-transfection, the cells were washed with DMEM without FBS and detached using 0.05% trypsin-EDTA. BHK cells were

resuspended in DMEM with 10% FBS, and equal amounts of cells were mixed and incubated for 20 h in an 8-well chambered coverglass (IWAKI). After incubation, cell images were acquired using the CV1000 system at a magnification of 40× to record interactions. Adhesion was defined as accumulation at the interface of the cell membrane. We calculated the accumulation and translocation rates as the proportion of cells showing accumulation or translocation among all contacting cells, as follows: % Accumulation or translocation = cells showing accumulation or translocation/contacting cells × 100. Each experiment was repeated at least three times.

Statistical analyses

For multinucleation analysis, at least three independent technical replicates were performed. We used Dunnett's test to compare the means of transfected genes with those of the negative control (RFP). Significant differences were evaluated at $P < 0.05$.

Declarations

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Author contributions

K.P.N. designed the study, which was supervised by T.H. K.P.N. conducted all experiments, with advice from C.V., D.K., N.S., and B.P. K.P.N., and T.H. wrote the manuscript with input from the other authors.

Competing interests

The authors declare no competing interests.

References

1. Inoue, N., Ikawa, M., Isotani, A. & Okabe, M. The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. *Nature* **434**, 234–238 (2005).
2. Miyado, K. *et al.* Requirement of CD9 on the egg plasma membrane for fertilization. *Science* **287**, 321–324 (2000).

3. Bianchi, E., Doe, B., Goulding, D. & Wright, G. J. Juno is the egg Izumo receptor and is essential for mammalian fertilization. *Nature* **508**, 483–487 (2014).
4. Ohto, U. *et al.* Structure of IZUMO1-JUNO reveals sperm-oocyte recognition during mammalian fertilization. *Nature* **534**, 566–9 (2016).
5. Aydin, H., Sultana, A., Li, S., Thavalingam, A. & Lee, J. E. Molecular architecture of the human sperm IZUMO1 and egg JUNO fertilization complex. *Nature* **534**, 562–565 (2016).
6. Noda, T. *et al.* Sperm proteins SOF1, TMEM95, and SPACA6 are required for sperm-oocyte fusion in mice. *Proc. Natl. Acad. Sci. U. S. A.* **117**, (2020).
7. Barbaux, S. *et al.* Sperm SPACA6 protein is required for mammalian Sperm-Egg Adhesion/Fusion. *Sci. Rep.* **10**, 1–15 (2020).
8. Fujihara, Y. *et al.* Spermatozoa lacking Fertilization Influencing Membrane Protein (FIMP) fail to fuse with oocytes in mice. *Proc. Natl. Acad. Sci. U. S. A.* **117**, 9393–9400 (2020).
9. Lamas-Toranzo, I. *et al.* TMEM95 is a sperm membrane protein essential for mammalian fertilization. *Elife* **9**, 1–18 (2020).
10. Mori, T., Kuroiwa, H., Higashiyama, T. & Kuroiwa, T. GENERATIVE CELL SPECIFIC 1 is essential for angiosperm fertilization. *Nat. Cell Biol.* **8**, 64–71 (2006).
11. von Besser, K., Frank, A. C., Johnson, M. a & Preuss, D. Arabidopsis HAP2 (GCS1) is a sperm-specific gene required for pollen tube guidance and fertilization. *Development* **133**, 4761–4769 (2006).
12. Mori, T., Igawa, T., Tamiya, G., Miyagishima, S. & Berger, F. Gamete Attachment Requires GEX2 for Successful Fertilization in Arabidopsis. *Curr. Biol.* **24**, 170–175 (2014).
13. Misamore, M. J., Gupta, S. & Snell, W. J. The Chlamydomonas Fus1 protein is present on the mating type plus fusion organelle and required for a critical membrane adhesion event during fusion with minus gametes. *Mol. Biol. Cell* **14**, 2530–2542 (2003).
14. Pinello, J. F., Liu, Y. & Snell, W. J. MAR1 links membrane adhesion to membrane merger during cell-cell fusion in Chlamydomonas. *Dev. Cell* **56**, 2021.09.03.458930 (2021).
15. Steele, R. E. & Dana, C. E. Evolutionary history of the HAP2/GCS1 gene and sexual reproduction in metazoans. *PLoS One* **4**, 1–5 (2009).
16. Shemer, G. *et al.* EFF-1 is sufficient to initiate and execute tissue-specific cell fusion in *C. elegans*. *Curr. Biol.* **14**, 1587–1591 (2004).
17. Fédry, J. *et al.* The Ancient Gamete Fusogen HAP2 Is a Eukaryotic Class II Fusion Protein. *Cell* **168**, 904–915 (2017).
18. Valansi, C. *et al.* Arabidopsis HAP2/GCS1 is a gamete fusion protein homologous to somatic and viral fusogens. *J. Cell Biol.* **216**, 571–581 (2017).
19. Zhang, J. *et al.* Species-specific gamete recognition initiates fusion-driving trimer formation by conserved fusogen HAP2. *Nat. Commun.* **12**, 1–12 (2021).
20. Mohler, W. A. *et al.* The type 1 membrane protein EFF-1 is essential for development cell fusion. *Dev. Cell* **2**, 355–362 (2002).

21. Podbilewicz, B. *et al.* The C. elegans Developmental Fusogen EFF-1 Mediates Homotypic Fusion in Heterologous Cells and In Vivo. *Dev. Cell* **11**, 471–481 (2006).
22. Sapir, A. *et al.* AFF-1, a FOS-1-Regulated Fusogen, Mediates Fusion of the Anchor Cell in C. elegans. *Dev. Cell* **12**, 683–698 (2007).
23. Avinoam, O. *et al.* Conserved eukaryotic fusogens can fuse viral envelopes to cells. *Science* **332**, 589–592 (2011).
24. Quinn, M. E. *et al.* Myomerger induces fusion of non-fusogenic cells and is required for skeletal muscle development. *Nat. Commun.* **8**, 1–9 (2017).
25. Chalbi, M. *et al.* Binding of sperm protein Izumo1 and its egg receptor Juno drives Cd9 accumulation in the intercellular contact area prior to fusion during mammalian fertilization. *Development* **141**, 3732–9 (2014).
26. Kato, K. *et al.* Structural and functional insights into IZUMO1 recognition by JUNO in mammalian fertilization. *Nat. Commun.* **7**, 1–9 (2016).
27. Yanagimachi, R., Yanagimachi, H. & Rogers, J. The Use of Zona-Free Assessment Ova as a Test-System Spermatozoa. *Biol. Reprod.* **15**, 471–476 (1976).
28. Avella, M. A., Baibakov, B. & Dean, J. A single domain of the ZP2 zona pellucida protein mediates gamete recognition in mice and humans. *J. Cell Biol.* **205**, 801–809 (2014).
29. Herberg, S., Gert, K. R., Schleiffer, A. & Pauli, A. The Ly6/uPAR protein Bouncer is necessary and sufficient for species-specific fertilization. *Science* **361**, 1029–1033 (2018).
30. Bianchi, E. & Wright, G. J. Cross-species fertilization: The hamster egg receptor, Juno, binds the human sperm ligand, Izumo1. *Philos. Trans. R. Soc. B Biol. Sci.* **370**, 1–4 (2015).
31. Yanagimachi, R. Zona-free hamster eggs: Their use in assessing fertilizing capacity and examining chromosomes of human spermatozoa. *Gamete Res.* **10**, 187–232 (1984).
32. Fujii, S. *et al.* A stigmatic gene confers interspecies incompatibility in the Brassicaceae. *Nat. Plants* **5**, 731–741 (2019).
33. Takeuchi, H. & Higashiyama, T. Tip-localized receptors control pollen tube growth and LURE sensing in Arabidopsis. *Nature* **531**, 245–248 (2016).
34. Fedry, J. *et al.* Evolutionary diversification of the HAP2 membrane insertion motifs to drive gamete fusion across eukaryotes. *PLoS Biol.* **16**, 1–26 (2018).
35. Inoue, N., Hagihara, Y., Wright, D., Suzuki, T. & Wada, I. Oocyte-triggered dimerization of sperm IZUMO1 promotes sperm-egg fusion in mice. *Nat. Commun.* **6**, 1–12 (2015).
36. Jin, H., Carlile, C., Nolan, S. & Grote, E. Prm1 prevents contact-dependent lysis of yeast mating pairs. *Eukaryot. Cell* **3**, 1664–1673 (2004).
37. Liu, P. *et al.* Inflammatory Smooth Muscle Cells Induce Endothelial Cell Alterations to Influence Cerebral Aneurysm Progression via Regulation of Integrin and VEGF Expression. *Cell Transplant.* **28**, 713–722 (2019).

38. Tamura, K., Shan, W. S., Hendrickson, W. A., Colman, D. R. & Shapiro, L. Structure-function analysis of cell adhesion by neural (N-) cadherin. *Neuron* **20**, 1153–1163 (1998).
39. Nagafuchi, A., Shirayoshi, Y., Okazaki, K., Yasuda, K. & Takeichi, M. Transformation of cell adhesion properties by exogenously introduced E-cadherin cDNA. *Nature* **329**, 341–343 (1987).
40. Umeda, R. *et al.* Structural insights into tetraspanin CD9 function. *Nat. Commun.* **11**, 1–7 (2020).
41. Nishihara, H., Hasegawa, M. & Okada, N. Pegasoferae, an unexpected mammalian clade revealed by tracking ancient retroposon insertions. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 9929–9934 (2006).
42. Fogel, A. I. *et al.* SynCAMs organize synapses through heterophilic adhesion. *J. Neurosci.* **27**, 12516–12530 (2007).
43. Tang, H. *et al.* Architecture of cell–cell adhesion mediated by sidekicks. *Proc. Natl. Acad. Sci. U. S. A.* **115**, 9246–9251 (2018).
44. Bettadapur, A., Miller, H. W. & Ralston, K. S. Biting Off What Can Be Chewed: Trogocytosis in Health, Infection, and Disease. *Infect. Immun.* **88**, e00930-19 (2020).
45. Miyado, K. *et al.* The fusing ability of sperm is bestowed by CD9-containing vesicles released from eggs in mice. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 12921–12926 (2008).
46. Shilagardi, K. *et al.* Actin-propelled invasive membrane protrusions promote fusogenic protein engagement during cell-cell fusion. *Science* **340**, 359–363 (2013).

Figures

Figure 1

Quantification of multinucleation using live imaging. (A) Time-lapse images from a fusion assay. Baby hamster kidney (BHK) cells were transfected by plasmids for expression of red fluorescent protein (RFP) (magenta) and GCS1 (co-transfection). Arrows and arrow heads indicate contacting and fused cells, respectively. Time (h:min) after the start of observation is shown (see Video 1). Bars: 20 μ m. (B) Multinucleation frequency of BHK cells determined by the expression of GCS1 and GCS1 Δ Loop. The use of mutant proteins GCS1 Δ Loop is explained in detail in the text. Dots indicate multinucleated cells among cultured cells, determined using a fusion assay; bars indicate the average values. Dunnett's test was used to compare each gene to the negative control. * $P < 0.05$. (C) Dependency of cell-to-cell fusion on co-transfected RFP expression. Bars represent the numbers of multinucleated cells, determined by fusion of RFP-labelled and non-labelled cells (unilateral fusion, light grey bars), fusion of RFP-labelled cells (bilateral fusion, dark grey bars), and fusion of non-labelled cells (not determined, black bars; all cells in this category began to express RFP after fusion).

Figure 2

Adhesion molecules accumulated at the contact interface of adjacent cells. (A) Time-lapse images of E-cadherin-expressing cells. Box indicates the region where cell membrane fluorescent intensity was measured in (B). Time (h:min) since the start of observation is shown (see Video 2). Bar: 20 μ m. (B) Fluorescence intensity profile of cells is shown at the top. (Left panel) Cells prior to contact; (middle) cells immediately after contact; (right) cells immediately after detachment. (C) Time-lapse images of IZUMO-expressing cells (green) and JUNO expressing-cells (magenta). Time (h:min) since the start of observation is shown (see Video 3). Bar: 20 μ m. Arrow heads indicate IZUMO accumulation at the contact site. (D) Fluorescence intensity profile of IZUMO in is cells shown at the top. (E) IZUMO accumulation rate for various treatment combinations. Numbers indicate contacted cell pairs observed by live imaging.

Figure 3

The accumulation and translocation of IZUMO and JUNO depended on their amino acid sequences. (A) LIAM assay with mouse IZUMO/SPACA6 and JUNO. Mixing of IZUMO-expressing cells (green) and JUNO-expressing cells (magenta) induced IZUMO accumulation (top panels) or simultaneous accumulation of IZUMO and JUNO (middle panels) (see Video 4). Arrow heads indicate accumulation of IZUMO or JUNO. Point mutations of amino acids critical for binding, i.e., W148A IZUMO (bottom left) and W62A JUNO (bottom middle), impaired accumulation. The sperm membrane protein SPACA6 (bottom right) did not induce accumulation of JUNO. Bars: 10 μ m. (B) IZUMO/SPACA6 and JUNO accumulation rates. (C) Translocation of IZUMO to an adjacent cell. Note that translocated signals (enclosed by dashed lines) continued to act as fluorescence foci. Bars: 10 μ m. (D) IZUMO/SPACA6 and JUNO translocation rates.

Figure 4

(A–D) Translocation and accumulation of conspecific and heterospecific combinations. Dashed lines surround molecular translocation sites (see Video 5). Arrow head in (C) indicates IZUMO accumulation; no translocation was observed in this conspecific combination (pig). Bars: 10 μ m.

Supplementary Files

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- [Video5translocation.avi](#)