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JUNO, the receptor of sperm IZUMO1, is expressed by the human oocyte and is essential for human fertilisation

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STUDY QUESTION: Is JUNO protein present at the surface membrane of human oocytes and involved in the fertilisation process?

SUMMARY ANSWER: JUNO protein is expressed on the plasma membrane of human oocytes and its inhibition by a monoclonal antibody completely blocks gamete fusion.

WHAT IS KNOWN ALREADY: Fusion of gamete membranes is the culminating event of the fertilisation process, but its molecular mechanisms are poorly understood. Until now, three molecules have been shown to be essential: CD9 tetraspanin in the oocyte, Izumo1 protein on the sperm and Juno, its corresponding receptor on the oocyte. Oocyte CD9 and sperm IZUMO1 have been identified in human gametes and their interaction is also well-conserved among several mammalian species. The presence of JUNO on human oocytes, however, has not yet been reported, nor has its role in fertilisation been investigated.

STUDY DESIGN, SIZE, DURATION: We selected an anti-human JUNO antibody in order to investigate the presence of JUNO on the oocyte membrane surface and studied its potential involvement in gamete membrane interaction during fertilisation.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Monoclonal antibodies against human JUNO (anti-hJUNO mAb) were produced by immunisation of mice with HEK cells transfected with the putative human JUNO sequence (HEK-hJUNO). These antibodies were used for immunostaining experiments and *in vitro* fertilisation assays with human gametes (GERMETHEQUE Biobank).

MAIN RESULTS AND THE ROLE OF CHANCE: Three hybridoma supernatants, verified by immunostaining, revealed specifically HEK-hJUNO cells. The three purified monoclonal antibodies, FJ2E4 (IgG1), FJ8E8 (IgG1) and FJ4F5 (IgG2a), recognised the soluble recombinant human JUNO protein and, in a western blot of HEK-hJUNO extracts, a protein with an expected MW of 25 kDa. In addition, soluble recombinant human IZUMO protein inhibited the binding of anti-hJUNO mAbs to cells expressing hJUNO. Using these anti-hJUNO mAbs in immunostaining, we identified the presence of JUNO protein at the plasma membrane of human oocytes. Furthermore, we revealed a progressive expression of JUNO according to oocyte maturity. Finally, we showed that human zona-free oocytes, inseminated in the presence of anti-hJUNO mAb, were not fertilised by human sperm. These results suggest that, as seen in the mouse, JUNO is indeed involved in human gamete membrane fusion during fertilisation.

LARGE-SCALE DATA: N/A.

[†]These authors contributed equally to the study.

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LIMITATIONS, REASONS FOR CAUTION: In accordance with French bioethics laws, functional tests were performed using zona-free oocytes, which of course does not fully encompass all normal *in vivo* physiological conditions. However, these *in vitro* tests do provide direct information regarding sperm–oocyte membrane interactions.

WIDER IMPLICATIONS OF THE FINDINGS: Mechanisms of gamete fusion appear to be homologous between mice and humans. However, some differences do exist and analysing the human mechanisms is essential. In fact, this is the first report describing the presence of JUNO on human oocytes and its involvement in human fertilisation. This discovery allows further examination of the understanding of molecular mechanisms that drive gamete fusion: a crucial challenge at a time when infertility affects 16% of reproductively active couples.

STUDY FUNDING/COMPETING INTERESTS: This work was supported by the Agence Nationale pour la Recherche, Grant no. ANR-13-BVS5-0004, and by Association Institut du Cancer et d'Immunogénétique (ICIG). There are no competing interests.

Key words: JUNO protein / IZUMO1 protein / fertilisation / oocyte / human gametes

Introduction

Fertilisation is a fundamental biological process in sexual reproduction, consisting of a series of interactions between sperm and oocyte. Gamete membrane adhesion and fusion is the culminating event of this process, driven by molecular mechanisms that have been widely studied. CD9 was the first essential protein identified on the oocyte's surface. More recently, Izumo1 and Juno proteins were identified as an essential sperm–oocyte binding pair in the mouse (Bianchi *et al.*, 2014). These three proteins are involved in gamete recognition and membrane adhesion, but do not induce membrane fusion (Bianchi *et al.*, 2014).

CD9, an integral membrane protein belonging to tetraspanin superfamily (TM4), is expressed by a wide variety of cells (Hemler, 2005; Levy and Shoham, 2005; Charrin *et al.*, 2014). *Cd9* gene knock-out causes a drastic decrease of murine female fertility due to the loss of oocyte membrane fusogenic ability (Kaji *et al.*, 2000; Le Naour *et al.*, 2000; Miyado *et al.*, 2000). CD9, as facilitator, regulates the distribution of oocyte membrane proteins and creates solid focal adhesion points, most likely forming a cluster of sperm receptors and anchoring them to the cytoskeleton (Jegou *et al.*, 2011). Izumo1 is a member of the immunoglobulin superfamily. It is present and detected on the membrane of the head of acrosome-reacted sperm (Satouh *et al.*, 2012). Male mice that lack *Izumo1* are infertile because their sperm, which otherwise appear to have normal features, are unable to fuse with oocytes (Inoue *et al.*, 2005).

A soluble form of Izumo1 was used to identify Juno, its receptor on the oocyte membrane. Juno is a GPI-anchored protein that was formerly identified as a putative folate receptor (Folr4), expressed by CD4+ CD25+ regulatory T lymphocytes in mice (Yamaguchi *et al.*, 2007). In a *Juno* knock-out mouse model, females are healthy but infertile; producing normal mature oocytes that are unable to fuse with wild-type acrosome-reacted sperm. Interestingly, CD9 has been shown to regulate the interaction between Juno and Izumo1 (Chalbi *et al.*, 2014) in wild-type mice.

IZUMO1 and CD9 proteins are present at the surface of human gametes and *in vitro* fertilisation assays have demonstrated their roles in human fertilisation (Inoue *et al.*, 2005; Ziyyat *et al.*, 2006). Furthermore, the Izumo1–Juno interaction has been shown to be conserved within several mammalian species, including human (Bianchi *et al.*, 2014; Chalbi *et al.*, 2014). Its crystal structure revealed that IZUMO1 forms a complex with JUNO and undergoes a conformational change at the N-terminal domain when this binding occurs (Aydin *et al.*, 2016; Ohto *et al.*, 2016). However, whether JUNO is expressed by human oocytes and plays a crucial role in human fertilisation has yet to be determined. For this reason, we investigated the presence of JUNO on the surface of human oocytes and its potential involvement in gamete membrane interaction during fertilisation.

Materials and Methods

Antibodies, recombinant proteins and transfected cell lines

Western blotting on immunoprecipitates of biotinylated cell extracts was performed using Alexa Fluor 680-labelled streptavidin (Invitrogen, Thermo Fisher Scientific, Massachusetts, USA), which was read with the Odyssey Infrared Imaging System (LI-COR Biosciences, Nebraska, USA). Alternatively, after primary antibodies were applied to unlabelled cells, secondary reagents labelled with Alexa Fluor 680 or Dylight-800 were used (Thermo Fisher Scientific). Rat anti-mouse Izumo1 Mab125 was provided by M. Okabe (Research Institute for Microbial Diseases, Osaka University, Japan; (Inoue *et al.*, 2013)). Anti-HSP70 mouse mAb was purchased from BD Biosciences Pharmingen (California, USA).

K562-Iz-EGFP cells are K562 cells that have been transfected with a mouse Izumo1–EGFP fusion protein, as previously described, and thus are able to bind to zona-free human oocytes (Chalbi *et al.*, 2014). HEK cells expressing human JUNO (HEK-hJUNO) were produced by transfection with the putative human sequence. An expression vector containing the coding region of the human orthologue of mouse Juno/Folr4 cDNA (a PCR amplified sequence of the cDNA NM_001199206 inserted at EcoR1 and Xho1 sites of pcDNA3.1(+)) was purchased from Life Technologies (Thermo Fisher Scientific). The plasmid was amplified and subsequently used for HEK cell transfection in conjunction with FuGENE HD transfection reagent (Promega Corp., Wisconsin, USA). A plasmid encoding Red Fluorescent Protein (RFP) was also co-transfected. After 4 days, high-expressing RFP HEK cells were selected by cell sorting (FACSAria II flow cytometer, Becton-Dickinson, New Jersey, USA) and further selection of human JUNO expressing cells was ensured by the addition of G418 to the culture medium.

Production of fully glycosylated human IZUMO122–254 and JUNO20–228 recombinant proteins (called r-hIZUMO and r-hJUNO in the following sections) in the laboratory of JE Lee were detailed previously (Aydin *et al.*, 2016). Briefly, the DNA sequences of IZUMO1 (residues 22–254) and JUNO (residues 20–228) with a BiP signal peptide, and C-terminal thrombin cleavage site and 10x His-tag were codon optimised

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and subcloned into a pMT-puromycin vector for stable expression in *Drosophila melanogaster* S2 cells (Invitrogen). All cells were confirmed to be mycoplasma free. Cultures of S2 cells were grown in Insect-XPRESS serum-free medium (Lonza) supplemented with 1X antibiotic-antimycotic (Gibco) and 6 µg/ml puromycin (Bioshop Canada) in 2-l vented Erlenmeyer shaker flasks at 27°C and 110 rpm. Expression was induced with 500 µM Cu_2SO_4 final concentration and purification from cultured media 6-days post-induction was by standard Ni-NTA affinity chromatography (Qiagen). Ni-purified r-hIZUMO and r-hJUNO were thrombin cleaved at a 1:1000 (w/w) enzyme to protein ratio (EMD Millipore) to remove the C-terminal 10x His-tag and subsequently further purified by size exclusion chromatography on a custom Superdex 200 prep grade 10/600 column equilibrated in 1x PBS and 5% (v/v) glycerol. Peak fractions were pooled, quantitated by A280 and frozen at −80°C prior to use.

Intercellular adhesion of HEK-hJUNO to K562-Iz-EGFP cells

HEK-hJUNO cells were first labelled with the mAb anti-HLA Class I W6/32 and goat anti-mouse Alexa 647 as the secondary labelled antibody (Thermo Fisher Scientific). Labelled HEK-hJUNO cells were washed three times to remove secondary antibodies and 10^5 cells were mixed with 10^6 K562-Iz-EGFP cells in 500 µl DMEM-10% FCS and gently agitated for 3 h. The cell suspension was then observed under fluorescence microscopy and quantified in an Accuri flow cytometer. This combination was used to enhance the population of highly-expressed hJUNO HEK cells by using flow cytometry to sort for cell doublets of HEK-hJUNO/K562-Iz-EGFP (data not shown). To evaluate the effect of antibodies against human JUNO on intercellular adhesion, the antibodies were added to the cell mixture at a concentration of 20 µg/ml.

Monoclonal antibody production against human JUNO

Before immunising the mice, we verified biochemically that the complex formed between mouse Izumo1–EGFP and human JUNO could be pulled down by GFP trap (Chromotek). To facilitate this process, we labelled the surface of 2×10^7 HEK and HEK-hJUNO cells expressing high levels of JUNO with biotin. Each sample was incubated with 10^8 K562-Iz-EGFP cells. Cells were then co-incubated and subsequently extracted in Triton X100 lysate buffer (10 mM Tris (pH 7.4), 150 mM NaCl, 0.02% NaN3, 1 mM phenylmethylsulfonyl fluoride, 0.5 mg/ml leupeptin, 1 mg/ml pepstatin A and 10 kallikrein-inactivating units/ml aprotinin) containing 1% Triton X100, as shown in Supplemental Figure S1A. After centrifugation at 10 000 x*g*, the supernatant was collected and a pull-down with 60 µl GFP trap beads was performed. The bead suspension was then washed four times with the lysis buffer and resuspended in SDS-Laemmli. The precipitates were then separated by 5–15% SDS-polyacrylamide gel electrophoresis under non-reducing conditions and transferred to a PVDF membrane (Amersham Pharmacia Biotech, UK). Western blotting on immunoprecipitates was performed with Alexa Fluor 680-labelled streptavidin (Invitrogen) and viewed using the Odyssey Infrared Imaging System (LI-COR Biosciences) (Supplementary Figure S1).

We induced immunisation of Balb/c mice with HEK-hJUNO cells (three intraperitoneal injections of 10^7 cells in 500 µl PBS, every 2 weeks). One month after the last injection, two immunisation boosts were given at 8-days' interval with purified hJUNO. The protocol for this purification of hJUNO is as explained above, except the biotinylation step that was skipped. To have sufficient material for immunisation, we incubated 2×10^8 HEK-hJUNO with 4×10^8 K562-Iz-EGFP cells. The mixed cell suspension was centrifuged and the pellet was resuspended in the lysis buffer. Then 400 µl GFP trap beads were used to pull-down the molecular complexes. The bead suspension was then washed four times with the lysis buffer; the beads were resuspended in 400 µl PBS, and then injected intraperitoneally in the mouse. After 4 days, the mouse was sacrificed and spleen cells were recovered for fusion with X63 myeloma cells. After a PEG-induced fusion, cells were distributed in 96-well microplates for aminopterin selection of fused cells. After 10 days, supernatants were then screened with a mixture of HEK-hJUNO cells and HEK-EGFP cells. Mouse monoclonal antibodies against hJUNO were detected by flow cytometry using an Alexa 647-labelled goat anti-mouse antibody (Thermo Fisher Scientific). Thus, HEK cells expressing hJUNO were labelled by the secondary reagent only when the supernatant contained anti-hJUNO specific mAbs (Supplementary Figure S2).

Inhibition of the binding of anti-hJUNO mAbs to HEK-hJUNO cells by rHu-Izumo soluble protein

HEK-hJUNO cells (5×10^5) were incubated with increasing concentrations of rHu-Izumo soluble protein (doubling concentrations from 0.03 to 4.5 µg/ml) for 15 min in a 100-µl volume. Anti-hJUNO and Ts9 (CD9) control mAbs were then added for 15 min at saturating concentrations. The cell suspension were washed three times and incubated in 50 µl with Alexa 647-labelled goat anti-mouse antibody. After washing, the labelling was measured by flow cytometry. All incubations and washings were performed at 4°C in DMEM medium containing 10% foetal calf serum.

Human gametes

Human gametes that were not used for patients were donated to research in accordance with French bioethics laws. GERMETHEQUE Biobank (BB-0033–00081), partner site of PARIS-COCHIN, provided 110 oocytes and 11 sperm samples for the purposes of this project, in addition to informed consent from each patient for the use of their materials (CPP 2.15.27). The gametes were systematically de-identified. The GERMETHEQUE pilot study committee approved the design of this study on 30 March 2016 under the number 20160304. The Biobank registered declaration DC-2014–2202 and authorisation AC-2015–2350.

In accordance with the Assisted Reproductive Technologies guidelines of Cochin Hospital Reproductive Medical Centre, either conventional IVF or ICSI was as used as the fertilisation method based on semen sample characteristics and the couple's medical history. Oocytes and/or embryos were incubated in a 40-µl drop of single-step medium (Global®, LifeGlobal®, USA) under mineral oil at 37°C in a 5% CO_2, 5% O_2 humidified gas atmosphere. Fertilisation was assessed 18 h after either insemination or injection; normal fertilisation was defined as the presence of two distinct pronuclei. At Day 2 after insemination, either immature oocytes (germinative vesicle (GV) and metaphase I (MI)) or mature oocytes (unfertilised metaphase 2 (M2) and *in vitro* matured oocytes) were included in the GERMETHEQUE Biobank and dedicated to this study. Spermatozoa were collected from excess fresh sperm derived from IVF attempts and were used on the day of collection.

Immunostaining of human oocytes

The staining procedure of human oocytes included an initial incubation with anti-hJUNO mAb (see section '*monoclonal antibodies production*') at a concentration of 20 µg/ml for 20 min, followed by several washes in M16® microdroplets, then a second incubation with an anti-mouse Alexa 488 antibody at 10 µg/ml for 20 min (Thermo Fisher Scientific, Massachusetts, USA). DNA content was detected by incubating with Hoechst 33342 for 10 min (Thermo Fisher Scientific Massachusetts, USA) at 5 µg/ml followed by serial washes in M16® culture medium. Stained oocytes were mounted on Nunc® Lab-Tek® II chambered cover glass (Sigma-Aldrich, Missouri,

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USA) for confocal analysis. The primary antibody was omitted in the control group.

When fresh oocytes were used, incubation was performed in microdroplets of M16® culture medium (Sigma-Aldrich, Missouri, USA), under mineral oil, for 20 min at 37°C or 4°C, in a 5% CO_2 atmosphere.

When oocytes were fixed in 4% paraformaldehyde solution for 30 min prior to immunostaining, each step was performed in PBS-3% BSA (Sigma-Aldrich, Missouri, USA) at room temperature. Confocal detection of JUNO protein was performed using a Spinning Disk L1 515 settled on a DMI6000 inverted microscope (Leica, Wetzlar, Germany), using a 40x objective. ImageJ software was used for image analysis (Schneider *et al.*, 2012). The acquisition was carried out under consistent conditions: Spinning Disk microscope, Bin 1, 20 MHz, exposure time 200 ms. The fluorescence intensity of fresh anti-hJUNO stained oocytes was calculated using the 'integrated density' of the set measurement, which corresponds to the sum of pixel values in the selected image. The value of the negative control was systematically subtracted.

In vitro fertilisation assays

Metaphase II stage oocytes were used for fertilisation assays: either M2 unfertilised oocytes or *in vitro* matured M2 oocytes. Because French bioethical laws forbid the production of human embryos for research purposes, fertilisation assays were performed in zona-free conditions that facilitate polyspermic fertilisation and thus prevent normal embryo development. The Zona pellucida (ZP) was removed chemically using acidic Tyrode's solution (pH 2.5; Sigma-Aldrich), at room temperature, for up to 20 s, with gentle pipetting, under observation until dissolution of the ZP was complete. Oocytes were then immediately washed five times in culture medium and kept at 37°C and 5% CO_2 atmosphere to recover for 2 h (Kellom *et al.*, 1992). Before insemination, zona-free oocytes were incubated in a Hoechst solution then gently washed. If membrane fusion occurs, the dye present in the oocyte cytoplasm freely diffuses into the sperm head and generates an increased blue fluorescent signal. Zona-free oocytes were co-incubated overnight with 4000 capacitated motile human spermatozoa in 20-µl microdroplets of M16® culture medium, under mineral oil, at 37°C and 5% CO_2 atmosphere. Then, they were gently washed in M16® and mounted directly between a glass slide and cover slip in Vectashield® Mounting Medium (Vector Labs, California, USA) for observation under UV light (Nikon Eclipse E600) to assess fertilisation.

In order to test the involvement of JUNO protein, fertilisation was performed in the presence of anti-hJUNO mAb at a final concentration of 20 µg/ml (test group). In the control groups, Ts53 (mouse anti-human CD53) and 12A12 (mouse anti-human CD55) (Lozahic *et al.*, 2000) mAb were used in place of anti-hJUNO mAb (CD55 is a GPI-anchored protein, expressed by oocytes and not involved in fertilisation whereas CD53 is a tetraspanin not expressed on oocytes.) Ts53 and 12A12 mAb were produced in the E. Rubinstein and C. Boucheix laboratory. Before insemination, zona-free oocytes were pre-incubated for 15 min with the appropriate antibody. Fertilisation was considered to have occurred when oocytes were observed to contain at least one visibly fluorescent decondensed sperm head within their cytoplasm. The fertilisation rate (number of fertilised oocytes/total number of inseminated oocytes) and fertilisation index (number of decondensed sperm nuclei/total number of inseminated oocytes) were compared between the test and control groups.

Statistical analyses

Statistical analysis was performed using GraphPad Prism (GraphPad® software, Inc). Comparisons were made using the Kruskal–Wallis test and the Mann and Whitney test as indicated below. Differences were considered statistically significant for P values <0.05.

Results

HEK-hJUNO cells adhere to K562-Izumo1-EGFP cells

We have previously shown that mouse Izumo1 interacts with a receptor on human oocytes that was suspected of being human Juno (Chalbi *et al.*, 2014). We verified that K562-Izumo1-EGFP cells indeed adhered to HEK-hJUNO cells by both fluorescent microscopy and flow cytometry. K562-Izumo1-EGFP cells bound to HEK-hJUNO cells with a strong but thin fluorescence enhancement in the adherence zone (Fig. 1A), which was consistent with our previous results showing binding of K562-Izumo1-EGFP cells to human oocytes (Chalbi *et al.*, 2014). In a flow cytometry bi-parametric plot, free HEK-hJUNO cells labelled with anti-HLA class I mAb W6/32 shifted along the FL4 axis and could be seen in the upper left quadrant (Fig. 1B left), whereas free K562-Izumo1-EGFP cells shifted along the FL1 axis (Fig. 1B right). When cells were co-incubated, cellular complexes appeared as double-labelled events in the Q2 upper-right quadrant (Fig. 1C). The percentage of HEK-hJUNO cells adhering to K562-Izumo1-EGFP cells corresponds to the ratio of HEK-hJUNO cells present in the upper-right quadrant (Q2) versus the sum Q1 + Q2:0.9/(0.9 + 1.15) = 43.9%. This property of adherence of HEK-hJUNO to K562-Izumo1-EGFP cells was used to isolate hJUNO for further immunisation assays.

Selection of three monoclonal antibodies produced against human JUNO

The adhesion of HEK-hJUNO to K562-Izumo1-EGFP suggested that it could be possible to use this system to isolate hJUNO in an active form. To test this hypothesis, we labelled the surface proteins of HEK-hJUNO with biotin before incubation of these cells with unlabelled K562-Izumo1-EGFP cells, and we induced the lysis of cell complexes. Izumo1–EGFP was then immunoprecipitated using GFP trap beads and biotin-labelled proteins present in the immunoprecipitate were visualised using Alexa Fluor 680-labelled streptavidin. As shown in Supplementary Figure S1, a major biotin-labelled band of 25 kDa (the size expected for JUNO) had been immunoprecipitated with Izumo1 when K562-Izumo1-EGFP cells were incubated with HEK-hJUNO cells, but not with parental HEK cells. In the same blot, the pull-down of Izumo1–EGFP fusion protein in both samples (apparent MW slightly above 70 kDa) was confirmed by blotting the membrane with an anti-Izumo1 mAb and a Dylight-800 goat anti-mouse antibody. Thus, hJUNO/Izumo1 complexes that were formed during co-incubation of HEK-hJUNO and K562-Izumo1-EGFP cells remained stable during the lysis process. We then used these Izumo1/hJUNO complexes as a booster in mice immunised with HEK-hJUNO cells.

Six hybridoma supernatants were tested and were able to stain HEK-hJUNO cells, but not HEK cells (examples are shown in Supplementary Figure S2). After biochemical control, three hybridomas were retained for further re-cloning and used in subsequent experimental studies. As shown in Fig. 2, the three monoclonal antibodies FJ2E4 (IgG1), FJ8E8 (IgG1) and FJ4F5 (IgG2a) recognised in a western blot of HEK-hJUNO extracts, but not in the lysates of parental HEK cells, a protein with an apparent MW of 25 kDa: the same size as the biotin-labelled protein precipitated by GFP trap beads from the HEK-hJUNO/K562-Izumo1-EGFP cell mixture (Supplementary Figure S1).

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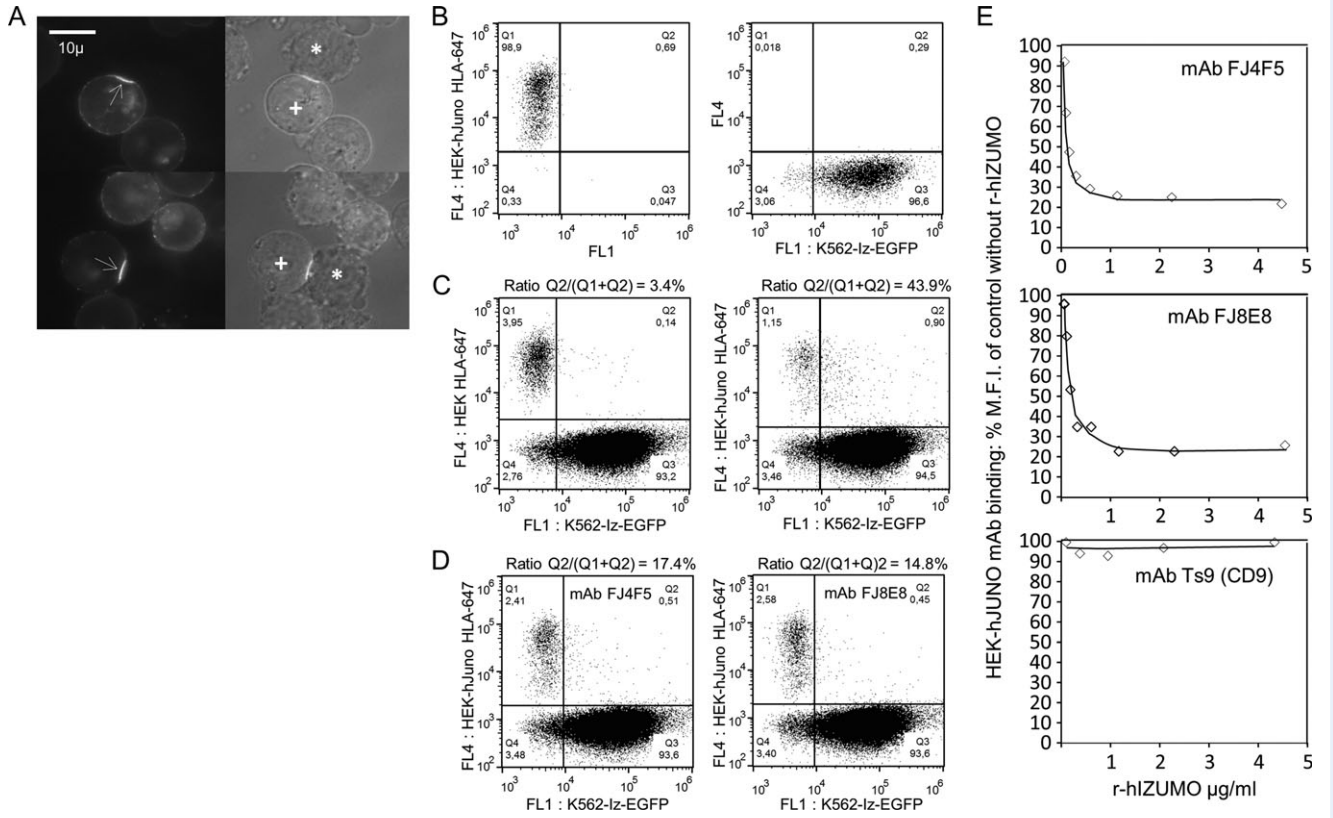

Figure 1 Analysis of the interaction between HEK-hJUNO and K562-Iz-EGFP cells. (**A**) The adherence of K562-Iz-EGFP cells (+) to HEK-hJUNO cells (*) results in an enhancement of the fluorescence in the contact area (white arrow). Left: fluorescence, Right: superimposed fluorescence and transmission lights. (**B**) HLA labelled HEK-hJUNO and K562-Iz-EGFP cells were analysed separately to show the respective positions of the cells in bi-parametric fluorescence plots. (**C**) In the HEK/K562-Iz-EGFP mixture (left plot), only a few events appear in the Q2 upper-right quadrant indicating that there are equally few cell complexes combining at least one HEK cell (not transfected with Juno) with one K562-Iz-EGFP cell. Conversely, the right plot shows 43.9% of HEK-hJUNO cells that form complexes with K562-Iz-EGFP cells visible in the Q2 quadrant (Q2/Q1 + Q2 = 0.9/0.9 + 1.15). Remaining uncomplexed HEK cells are visible in the Q1 quadrant. (**D**) Monoclonal antibodies produced against hJUNO partially inhibit the intercellular adherence. 0.51/2.41 + 0.51 = 17.4% of HEK-hJUNO adhering cells in presence of mAb FJ4F5. 0.45/2.58 + 0.45 = 14.8% of HEK-hJUNO adhering cells in presence of mAb FJ8E8. This reflects an inhibitory effect of 60% and 66% for anti-hJUNO mAb FJ4F5 and FJ8E8, respectively. (**E**) The mAbs binding curves show the % mean fluorescence intensity (M.F.I.) of the mAbs binding to HEK-hJUNO cells according to increasing concentrations of r-hIZUMO protein (doubling concentrations from 0.03 µg to 4.5 µg/ml). A partial mAb binding inhibition was observed with a plateau starting at 1.12 µg/ml. For the mAbs FJ4F5 and FJ8E8, an inhibition of 80% was obtained whereas the inhibition of binding was lower with FJ2E4 (45%). The binding of Ts9 mAb was not inhibited by r-hIZUMO (only the highest concentrations were tested). The experiments were repeated twice. Representative curves are shown.

These antibodies also recognise the soluble r-hJUNO protein. Interestingly whereas the FJ8E8 and FJ4F5 WB signals of the HEK-HJuno cellular extracts are stronger than the soluble r-hJUNO protein, the reverse is observed with FJ2E4, indicating that these antibodies are directed towards different epitopes. Furthermore, monoclonal antibodies produced against hJUNO partially inhibited the intercellular adherence between HEK-hJUNO and K562-Iz-EGFP cells (Fig. 1D). Indeed, in presence of anti-hJUNO mAb, the percentage of HEK-hJUNO cells adhering to K562-Iz-EGFP cells dropped from 43.9% to 17.4% with FJ4F5, and 14.8% with FJ8E8, respectively. This reflects an inhibitory effect of 60% and 66%, respectively. No inhibition of binding was observed with Ts9, a mAb directed to CD9 that is expressed on HEK cells (data not shown).

Finally, the binding of anti-hJUNO mAb to HEK-hJUNO cells is partially inhibited by pre-incubation (15 min) with r-hIZUMO soluble protein (Fig. 1E). A maximal inhibition that does not exceed 80% for FJ4F5 and FJ8E8 and 45% for FJ2E4 is reached at a concentration of 1.12/µg/ml r-hIZUMO. If the cells are incubated first with the anti-hJUNO mAb for 15 min, the binding is not reversed by the addition of r-hIZUMO soluble protein for 15 min (data not shown).

JUNO is present at the surface of human oocytes and progressively expressed during oocyte maturation

Using anti-hJUNO mAb FJ2E4, FJ8E8 and FJ4F5, we demonstrated the presence of JUNO protein at the plasma membrane of human oocytes (Figs 3 and 4). The distribution of the fluorescent signal appeared heterogeneous on fresh M2 oocytes (Fig. 3A–B), whereas it was homogeneous and finely punctuated when the oocytes were fixed before

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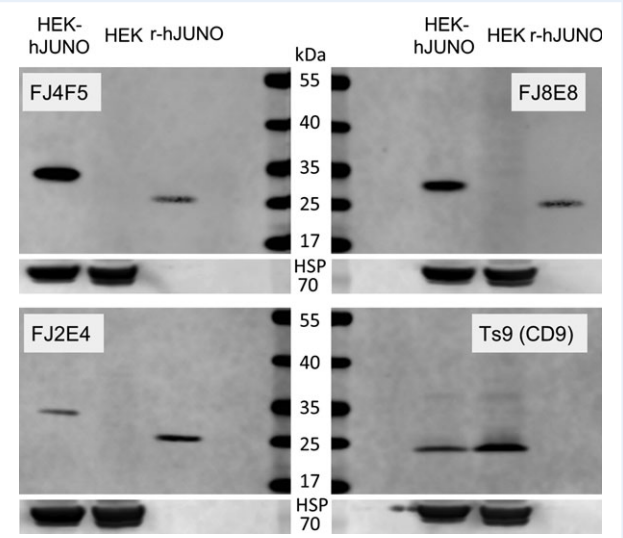

Figure 2 Western blot of HEK, HEK-hJUNO extracts and soluble r-hJUNO protein with anti-human Juno antibodies. A band above 25 kDa apparent molecular weight is visible with the three anti-human JUNO mAbs in HEK-hJUNO extracts. Despite the same amount of cellular material, the intensity of the bands varies between antibodies. The soluble r-hJUNO protein is also recognised by the three mAbs with varying intensity which does not parallel the recognition of the protein found in cellular extracts. The control mAb Ts9 neither labels human Juno in the cellular extracts nor in soluble recombinant protein lanes. In the cell lines lanes, extracts from 10^5 cells were deposited and 100 ng was deposited in the r-hJUNO protein lanes. Proteins and extracts were separated by 5–15% SDS-polyacrylamide gel electrophoresis under non-reducing conditions.

staining (Fig. 3F–G). This suggests a relocation of the protein induced by specific binding to the mAb.

Next, we tested the expression of JUNO protein on immature and incompetent oocytes; it was also detected on the oocyte membrane as early as the GV stage (Fig. 4).

An analysis of the staining intensity was carried out on fresh oocytes in order to quantify JUNO expression according to the oocyte maturity. In this manner, the level of expression of JUNO protein was significantly higher at the surface of M2 and M1 oocytes as compared to GV ($24.1 \times 10^6 \pm 7.4 \times 10^6$ and $26.7 \times 10^6 \pm 4.5 \times 10^6$ vs. $14.2 \times 10^6 \pm 9.0 \times 10^6$, respectively, $P = 0.0085$). When comparing groups, we found a significant difference between M2 oocytes and GV, as well as between MI oocytes and GV ($P = 0.0062$ and $P = 0.0135$, respectively). The difference between M2 and M1 oocytes was not significant ($P = 0.5177$). These results suggest that JUNO protein expression levels increase at the plasma membrane during the course of oocyte maturation.

JUNO is essential for human fertilisation

To assess the involvement of JUNO protein in human fertilisation process, anti-hJUNO mAbs (FJ4F5 and FJ8E8) were used in several *in vitro* fertilisation assays. As mentioned above, in zona-free conditions, multiple spermatozoa are able to fuse with the plasma membrane of human oocytes and therefore penetrate into the cytoplasm (Fig. 5A). Although 100% (7/7, $n = 3$ experiments and 13/13, $n = 6$ experiments) of human oocytes were fertilised in the CD55 and CD53 control groups, respectively (with a mean of 12.5 and 7.4 fused sperm per oocyte, respectively), none (0/11, $n = 6$ experiments) of the human oocytes inseminated in presence of anti-hJUNO mAb were fertilised (Fig. 5). These results indicate that, as in mice, JUNO is involved in gamete membrane adhesion/fusion during fertilisation. Since the

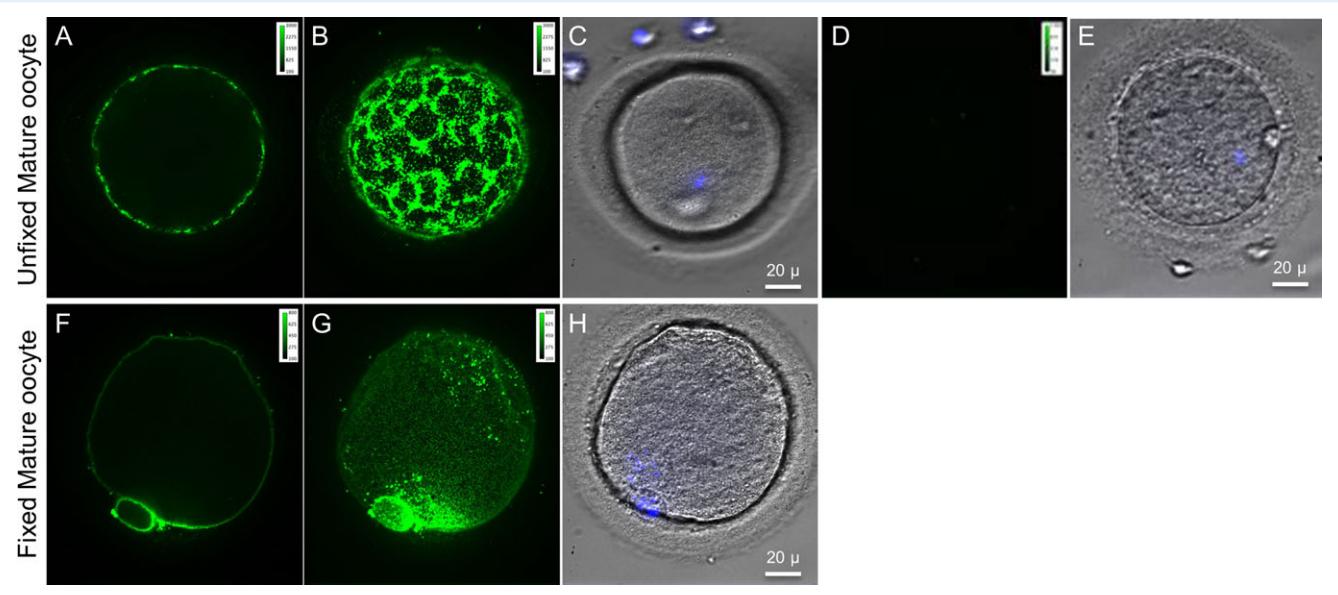

Figure 3 Expression of JUNO protein at human oocyte surface. The use of anti-human JUNO mAb (results presented with FJ4F5), revealed by a goat anti-mouse Alexa Fluo® 488 antibody, showed the presence of JUNO protein on the membrane of human mature metaphase 2-stage (M2) oocytes. The fluorescent signal was heterogeneous with molecules organised in patches around the surface of fresh oocytes (Panels a, b, c); while it appeared homogeneous and finely punctuated when oocytes were fixed prior to staining (Panels f, g, h). The omission of the primary antibody was used as a negative control (Panels d and e). Confocal section of the equatorial plane (a, d, f). Superimposition of a mean of 90 sections corresponding to nearly half of oocytes used (b, g). Transmission image superimposed with Hoechst fluorescent signal (c, e, h).

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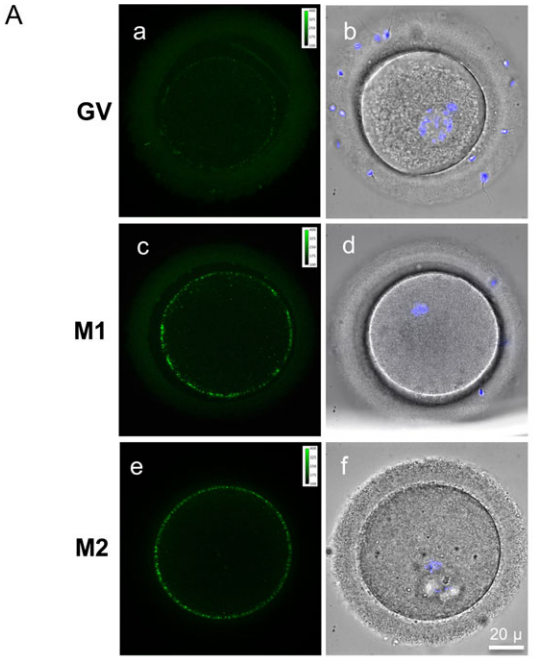
A



B

	GV^{x,y} (12)	M1^{x,z} (6)	M2^{y,z} (15)	*P** value
Fluorescent Intensity (mean±sem)	14.2 x 10^6 ± 9.0 x 10^6	26.7 x 10^6 ± 4.5 x 10^6	24.1 x 10^6 ± 7.4 x 10^6	0.0085

Figure 4 JUNO is progressively expressed at the plasma membrane during oocyte maturation. (**A**) JUNO immunostaining was performed on germinal vesicle (GV), metaphase 1-stage (M1), and mature metaphase 2-stage (M2) oocytes. JUNO was present at the GV stage but weakly expressed (a, b), whereas the fluorescent signal increased at the M1 and M2 stages (c–f). Confocal section of equatorial plane (a, c, e). Transmission image superimposed with blue (Hoechst) fluorescent signals (b, d, f). (**B**) The fluorescent intensity was significantly higher at the M1 and M2-stage oocyte surfaces compared to the germinal vesicle: *x*—Mann–Whitney test VG vs. M1 $P = 0.0120$. *y*—Mann–Whitney test VG vs. M2 $P < 0.0001$. *z*—Mann–Whitney test M1 vs. M2 $P = 0.1676$. *Kruskal–Wallis test.

mAbs inhibit the adhesion of HEK-hJUNO to K562-Iz-EGFP cells (Fig. 1D), they are likely to prevent gamete fusion in the same manner, by blocking the interaction of JUNO with IZUMO1.

Discussion

This study demonstrates for the first time the presence of JUNO protein on the plasma membrane of human oocytes and provides evidence of its implication in human fertilisation. For producing mAbs against human JUNO, we took advantage of an interaction between the putative human JUNO and mouse Izumo1 to purify human JUNO for mice immunisation. The structure of the human JUNO/IZUMO1 complex has been recently reported and critical amino acids in the interaction surface between the two molecules have been identified (Aydin *et al.*, 2016; Ohto *et al.*, 2016). In agreement with structural data, mutations of three IZUMO1 amino acids (W148, H157, R160) completely abolished the interaction between mouse Izumo1 transfected cells and mouse oocytes (Ohto *et al.*, 2016). Interestingly, these three amino acids are conserved between mouse and human which could explain the mouse Izumo1/human JUNO interaction. Three mAbs were selected and their recognition of human JUNO was assessed by immunofluorescence with transfected cells and western blot of cell extracts and recombinant JUNO protein. In experiments performed with two mAbs we could show: (1) an inhibition of cellular interactions between cells transfected with mouse Izumo1 and human JUNO and (2) an inhibition of binding of these mAbs on HEK cells expressing human JUNO by the soluble recombinant human IZUMO1 protein. Using increasing concentrations of IZUMO1 protein, we observed in our experimental conditions that this inhibition reached a plateau of ~80%. The reasons for this limit remain elusive. A possible interpretation would be that a proportion (~20%) of surface JUNO molecules does not bind IZUMO1 due to a particular conformation or interaction with other proteins. Experimental data provided in this work do not allow a precise determination of the epitopes recognised by the mAbs. However, the inhibition of mAb binding by pre-incubation with IZUMO1 protein and the inhibition of cellular interactions suggests that IZUMO1/JUNO molecular interactions are not, or only partially, compatible with the mAb binding, indicating that upon mAb binding, the IZUMO1/JUNO interaction is compromised.

We detected JUNO protein on mature oocytes independently of their origin (i.e. unfertilised M2 oocytes or *in vitro* matured oocytes). Using different procedure parameters, we could observe variable distribution of JUNO at the plasma membrane. For example, on fixed oocytes, the signal was broadly homogeneous and finely punctuated which could indicate the organisational pattern of the membrane microvilli. Whereas, on fresh oocytes, the fluorescent signal appeared patchy and heterogeneous and suggested a relocation of the protein induced by binding to the antibody. This process is thus inhibited by the loss of membrane fluidity induced by paraformaldehyde fixation.

Human JUNO is progressively expressed at the oolemma during oocyte maturation with an expression level significantly higher in M1 and M2 stages compared to the GV stage. As the maturation of the oocyte is a long and progressive process, this allows the oocyte to successfully support fertilisation and the development of the preimplantation embryo. This maturation involves the plasma membrane, the cytoplasm contents and the nucleus. Therefore, we hypothesise that in the human, the fusibility of the oocyte with spermatozoa could be related to the threshold amount of JUNO present on the oolemma. This has already been shown for CD9 tetraspanin, for which the expression level is seen to increase during oocyte maturation in the pig (Li *et al.*, 2004). According to the authors, this indicates that the level of CD9 expression is indicative of the oocyte's competence to be fertilised. However, it must be noted that the protein expression profiles are not entirely conserved from one species to another. In fact, in the mouse, Cd9 and Juno expressions reach their maximum at the GV stage and remain stable in later stages (Komorowski *et al.*, 2006; Suzuki *et al.*, 2017).

The use of functionally inhibiting mAbs during *in vitro* fertilisation assays allowed elucidation of the involvement of JUNO in human gamete fusion. JUNO appears to be essential, as none of the inseminated

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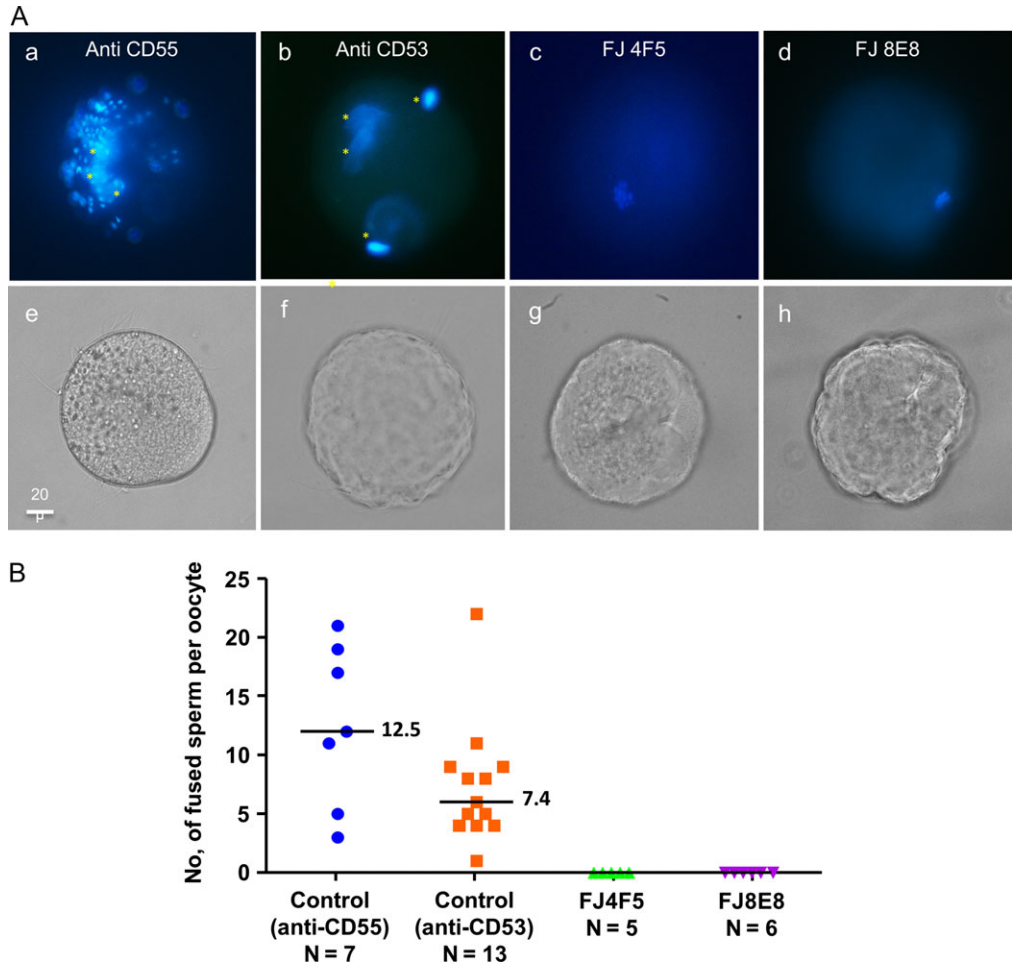

Figure 5 Human fertilisation is blocked by anti-human JUNO monoclonal antibodies. Zona-free mature human oocytes were inseminated in the presence of anti-human JUNO mAbs (FJ4F5 and FJ8E8, 20 µg/ml). Oocytes inseminated in the presence of anti-CD53 and anti-CD55 mAb were used as controls. The number of uncondensed sperm heads in the oocyte cytoplasm were recorded 18 h after insemination on a Nikon eclipse E600, obj x40, oil. (**A**) None of the oocytes inseminated in the presence of FJ4F5 and FJ8E8 were fertilised; the Hoechst staining revealed the oocyte in metaphase (c, d), while in the control groups, all inseminated oocytes were polyspermic. On sub-panels a and b, 3 and 4 uncondensed sperm head can, respectively, be seen in the recorded plane (yellow asterisk). Sub-panel, e, f, g and h are light images corresponding to fluorescent pictures. The 'shrinking' aspect of oocytes in f, g and h is due to the Vectashield© Mounting Medium, not used in the CD55 control (e) corresponding to a different set of experiments in which the inseminated oocyte was observed directly in M16 medium, thus explaining the different aspect of the oocyte's light image. (**B**) Number of intra-cytoplasmic decondensed spermatozoa per inseminated oocyte. (-) fertilisation index. *N*: number of inseminated oocytes.

oocytes were fertilised in the presence of anti-hJUNO antibodies. The robustness of this result is based on the monoclonal antibodies we validated with strong evidence. Furthermore, the removal of the ZP presents the advantage of studying the process of gamete membrane fusion without any possible interference of the ZP or cumulus cells.

The interaction of Juno on the oocyte's surface with Izumo1 on the surface of the sperm has been shown to be essential for mouse sperm–oocyte fusion (Bianchi *et al.*, 2014). Our data showing that anti-hJUNO mAbs completely block human fertilisation *in vitro*, together with (i) the presence of IZUMO1 on the head of acrosome reacted-human sperm (Inoue *et al.*, 2005), (ii) the inhibition of human sperm fusion with hamster oocytes in the xeno-species fusion system induced by the anti-human IZUMO1 polyclonal antibody (Inoue *et al.*, 2005) and (iii) the affinity of JUNO and IZUMO1 human proteins (Bianchi *et al.*, 2014; Aydin *et al.*, 2016) allows us to propose that JUNO is an IZUMO1 receptor essential for sperm–oocyte fusion in humans.

Despite the essential role played by JUNO and IZUMO1 in human fertilisation, identification of a mutation affecting one of their genes is still at large (Hayasaka *et al.*, 2007; Yu *et al.*, 2018). However, the highlighting of rare, non-synonymous SNPs in the *JUNO* gene of two infertile patients presenting with *in vitro* fertilisation failure suggests a contribution to the aetiology of this phenotype (Yu *et al.*, 2018). In this study, we further the understanding of underlying molecular mechanisms that drive gamete fusion. This discovery could promote a more detailed exploration of clinical infertility and propose customised care.

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This is a crucial challenge at the time when infertility affects 16% of reproductively active couples worldwide.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' contributions

F.H., Y.Z., E.R. and C.B. designed and realised the method for production of anti-human JUNO monoclonal antibodies. M.C. and C.G. produced the K562-Izumo1–EGFP cell line. J.E.L. and P.Y. expressed, purified and characterised both r-hIZUMO and r-hJUNO and provided us with human IZUMO and JUNO recombinant proteins. C.J., A.Z. and V.B.L. designed and performed the experiments on human gametes. C.J., F.H., J.P.W., C.B. and V.B.L. analysed and interpreted the data. C.J., F.H., C.B. and V.B.L. wrote the manuscript. A.Z., E.R., C.G., J.P.W. and J.E.L. critically revised the manuscript. All authors read and approved the final version of the manuscript.

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Conflict of interest

None.

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