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RESEARCH ARTICLE

The egg membrane microdomain-associated uroplakin III-Src system becomes functional during oocyte maturation and is required for bidirectional gamete signaling at fertilization in *Xenopus laevis*

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ABSTRACT

In *Xenopus laevis*, sperm-egg interaction promotes partial proteolysis and/or tyrosine phosphorylation of uroplakin III (UPIII) and the tyrosine kinase Src, which both localize to the cholesterol-enriched egg membrane microdomains (MDs). Here we show that sperm promote proteolysis and/or tyrosine phosphorylation of UPIII and Src in MDs isolated from ovulated and unfertilized eggs (UF-MDs). An antibody against the extracellular domain of UPIII interferes with these events. Inhibition of fertilization by anti-UPIII antibody is rescued by co-incubation with UF-MDs. This suggests that, like MDs in intact eggs, the isolated UF-MDs are capable of interacting with sperm, an interaction that does not interfere with normal fertilization but rather augments the ability of sperm to fertilize eggs pretreated with anti-UPIII antibody. This unexpected effect of UF-MDs on sperm requires UPIII function in UF-MDs and protein kinase activity in sperm. MDs isolated from progesterone-treated mature oocytes, but not ovarian immature oocytes, are similarly functional as UF-MDs. The anti-UPIII extracellular domain antibody binds more effectively to the surface of mature than immature ovarian oocytes. We propose that the structural and functional competency of the UPIII-Src signaling system in MDs is strictly regulated during oocyte maturation and subsequently in sperm-mediated egg activation and fertilization. The fertilization-related signaling properties seen in UF-MDs can be partially reconstituted in MDs of human embryonic kidney 293 cells (293-MDs) expressing UPIII, Src and uroplakin Ib. However, 293-MDs expressing a proteolysis-resistant mutant of UPIII are less functional, suggesting that the availability of UPIII to protease action is important for MD function.

KEY WORDS: Fertilization, Gamete interaction, *In vitro* reconstitution, Membrane microdomains, Oocyte maturation, Proteolysis, Signal transduction, Src, Tyrosine phosphorylation, Uroplakin III

INTRODUCTION

Recent reverse genetic approaches (e.g. gene knockout) have begun to disclose that the principal features of fertilization, as the key event of sexual reproduction involving the two gametes, namely egg and sperm, are in fact governed by molecular machinery of surprising conservation. One example is the mammalian sperm protein Izumo1 (Inoue et al., 2005) and the plant pollen protein generative cell-specific 1 (Mori et al., 2006), both of which are single-transmembrane-domain proteins essential for gamete fusion at fertilization. Against this background, the study of fertilization has become a field that concerns how these essential molecules act in the cellular processes of fertilization.

We previously showed that uroplakin III (UPIII), a single-transmembrane-domain protein that is expressed in the unfertilized egg of the frog *Xenopus laevis*, serves as a target of sperm-derived protease known to be essential for successful fertilization, and that pretreatment of unfertilized *Xenopus* eggs with an antibody that recognizes the extracellular sequence of UPIII is inhibitory to fertilization (Mahbub Hasan et al., 2005; Sakakibara et al., 2005). We also demonstrated that UPIII is a substrate of the egg cytoplasmic tyrosine kinase Src (Sakakibara et al., 2005), the activation of which is required for sperm-induced activation of phospholipase Cγ and transient increase in intracellular calcium concentration (Sato et al., 1999, 2000, 2001). Another line of evidence demonstrates that plasma membranes of *Xenopus* eggs are composed of at least two distinct compartments that differ in their behavior under sucrose density gradient ultracentrifugation of egg extracts prepared in the presence of the non-ionic detergent Triton X-100 (Sato et al., 2002). UPIII and Src are highly concentrated in one of the membrane substructures: namely, low-density, detergent-insoluble membrane fractions, in which cholesterol and ganglioside GM1, both of which are canonical 'lipid raft' markers, are also enriched. The physiological importance of such specific membrane substructures as membrane microdomains (MDs) is suggested by the fact that methyl-β-cyclodextrin interferes with the localization of Src to the MDs and inhibits egg fertilization (Sato et al., 2002), and that UPIII physically interacts with GM1 in the MDs and externally added GM1 inhibits normal egg fertilization (Mahbub Hasan et al., 2007). Moreover, we showed that activation of Src could be reconstituted *in vitro* by the use of MDs that are isolated from unfertilized eggs (Sato et al., 2003). These results suggest that the MD-associated UPIII-Src system plays a pivotal role in gamete adhesion/fusion and subsequent developmental activation (Mahbub Hasan et al., 2011).

In this study we attempted to explore three major issues. First, we examined whether MDs that were prepared from unfertilized eggs (UF-MDs) are capable of interacting with sperm in signaling reconstitution and *in vitro* egg activation assays. Second, we prepared transfected cultured cells expressing wild-type or mutant UPIII (UPIII-RR/AA), in which possible proteolysis target residues

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are mutated, and performed experiments using their MDs (293-MDs). Third, we investigated when and how MDs of *Xenopus* eggs/oocytes become functionally competent for fertilization. Taking these findings together, we discuss the developmental formation, acquisition of functional competence and physiological relevance of *Xenopus* egg/oocyte MDs, and propose a model to explain how the UPIII-Src system acts in fertilization.

RESULTS

Sperm-dependent tyrosine phosphorylation of UPIII and Src can be reconstituted in MDs isolated from unfertilized eggs

To examine fertilization-related signaling function of MDs *in vitro*, we isolated MDs from ovulated, unfertilized *Xenopus* eggs (hereafter UF-MDs) and performed an *in vitro* kinase assay in the absence or presence of either sperm or cathepsin B, a tryptic protease that can promote the parthenogenetic activation of *Xenopus* eggs (Fig. 1D). Sperm stimulated tyrosine phosphorylation of the MD-associated UPIII and Src (Fig. 1A, lanes 1 and 2). When UF-MDs were preincubated with an antibody that recognizes the extracellular domain of UPIII (anti-UPIII-ED antibody), the sperm-induced tyrosine phosphorylation did not occur (Fig. 1A, lane 3). Two other antibodies, namely anti-UPIII C-terminus (UPIII-CT) and control IgG did not show any such effect (Fig. 1A, lanes 4 and 5). Cathepsin B caused massive proteolysis of UPIII (Fig. 1B, lanes 2 and 4), but did not promote an elevation in tyrosine phosphorylation of UPIII (Fig. 1B, lane 2), whereas it did stimulate tyrosine phosphorylation of Src (Fig. 1B, lane 2). Under the same conditions, preincubation of UF-MDs with the anti-UPIII-ED antibody inhibited both proteolysis of UPIII and tyrosine phosphorylation of Src (Fig. 1B, lane 3). Preincubation of UF-MDs with the anti-UPIII-CT antibody resulted in an increase in tyrosine phosphorylation of UPIII and Src in the presence of cathepsin B (Fig. 1B, lane 4). These results suggest that sperm interact with UF-MDs in a UPIII-dependent manner. The Src-specific inhibitor PP2 and the additional chemical inhibitors GDPβS, LY294002 and MβCD, all of which have been shown to inhibit normal fertilization of *Xenopus* eggs (Mammadova et al., 2009; Sato et al., 2000; our unpublished results), inhibited the tyrosine phosphorylation of Src in response to either sperm or cathepsin B (Fig. 1C).

UF-MDs facilitate sperm fertilization of anti-UPIII antibody-treated eggs

To further examine the function of UF-MDs, we performed an *in vitro* egg activation assay (Fig. 2A). As we showed previously (Sakakibara et al., 2005), when unfertilized eggs were preincubated with the anti-UPIII-ED antibody, the rate of successful egg activation induced by sperm, as judged by the occurrence of cortical contraction, was reduced to less than 15% (Fig. 2B,D). Under these conditions, co-incubation of the anti-UPIII-ED antibody with UF-MDs resulted in a rate of successful egg activation of more than 75% (Fig. 2C,D). This recovery was lost when UF-MDs were preincubated with cathepsin B (Fig. 2D). Eggs that were preincubated with either the intact or the cathepsin B-treated MDs alone showed a normal rate of sperm-induced activation (Fig. 2D). These results suggest that UF-MDs interact with the anti-UPIII-ED antibody in a UPIII-dependent manner.

We next investigated whether sperm interact with UF-MDs under these experimental conditions (Fig. 2E). We assumed that UF-MDs would interfere with fertilization through their interactions with sperm. However, UF-MD-treated sperm were able to fertilize eggs as successfully as untreated sperm (Fig. 2F). In addition, when UF-MD-treated sperm were used for the insemination of eggs that had been pretreated with the anti-UPIII-ED antibody, the rate of successful egg activation recovered to more than 60% (Fig. 2F). This recovery was not seen when cathepsin B-treated UF-MDs were used for sperm treatments (Fig. 2F) and was also lost when UF-MD-treated sperm were prepared in the presence of either anti-UPIII-ED antibody or a synthetic peptide containing a GRR sequence in UPIII (GRR peptide),

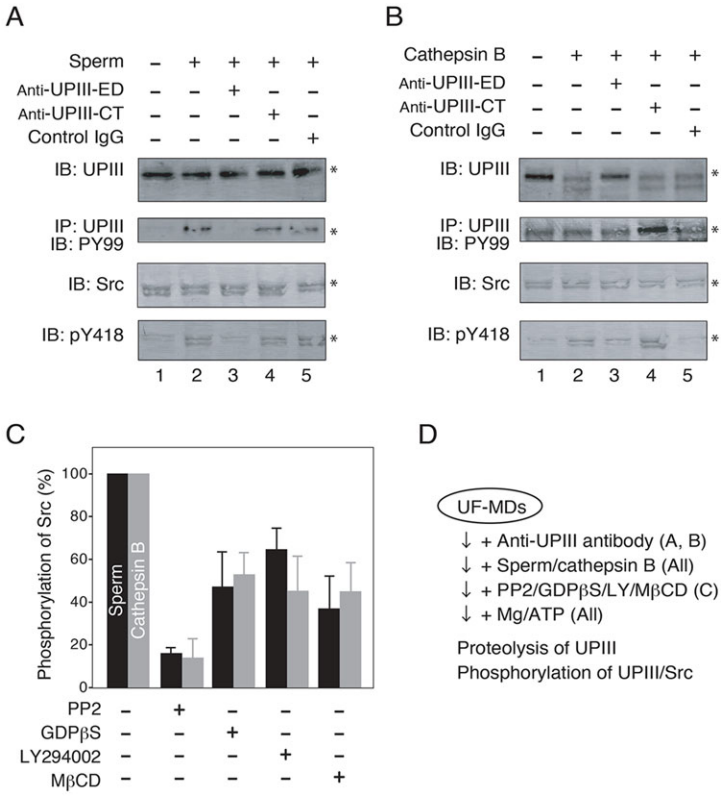

Fig. 1. Sperm and cathepsin B promote tyrosine phosphorylation of UPIII and Src in MDs isolated from unfertilized eggs (UF-MDs). (A,B) UF-MDs (5 μg protein) were treated with either (A) sperm (10^6 cells/ml) or (B) cathepsin B (5 U/ml) in the absence or the presence of anti-UPIII-ED IgG, anti-UPIII-CT IgG or control IgG (each at 40 μg/ml). After treatment, the mixtures were subjected to an *in vitro* kinase assay and analyzed for the tyrosine phosphorylation of UPIII and Src. IB, immunoblotting; IP, immunoprecipitation; PY99, anti-phosphotyrosine antibody; pY418, anti-phosphotyrosine 418 of Src. Asterisks indicate the positions of UPIII or Src. Data shown are representative of three independent experiments. (C) UF-MDs were preincubated with either sperm or cathepsin B, as in A and B, in the absence (control) or presence of 5 μM PP2, 20 μM GDPβS, 10 μM LY294002 or 25 mM MβCD. After the pretreatment, the mixtures were analyzed for tyrosine phosphorylation of Src as in A and B. Data shown are mean±s.d. of three independent experiments. For all data, $P<0.01$ compared with levels in the control (sperm or cathepsin B alone). (D) Schematic representation of the experiments in A-C, with common and specific treatments in each experiment indicated in parentheses (arrows indicate the flow of experimental manipulations).

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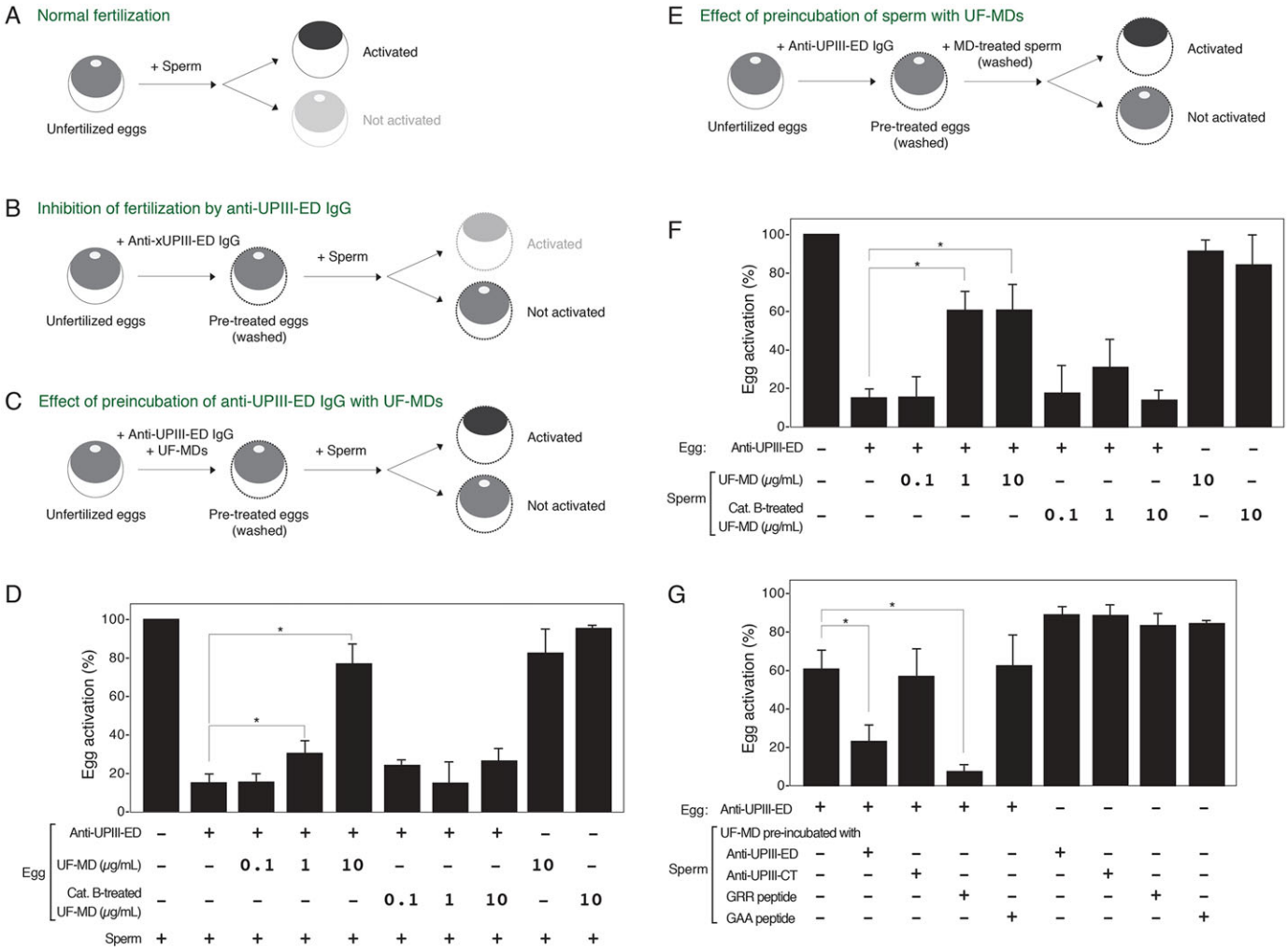

Fig. 2. UF-MDs can augment the ability of sperm to fertilize eggs that have been pretreated with anti-UPIII-ED antibody. (A-C) Schematic representations of the *in vitro* egg activation experiments performed in D. Unfertilized eggs were stripped of the jelly layer and pretreated in the absence (A) or presence (B,C) of anti-UPIII-ED antibody (40 μg IgG/ml) and UF-MDs (0.1-10 μg/ml). After the pretreatments, eggs were washed and then treated with 10^6/ml sperm. Successful egg activation was scored by the occurrence of contraction of the pigmented area in the animal hemisphere of eggs. (D) Pretreatment of eggs with anti-UPIII-ED antibody resulted in greater than 80% inhibition of sperm-induced egg activation. Such an inhibitory effect of the antibody was effectively negated by co-incubation with UF-MDs at more than 1 μg/ml. UF-MDs that had been pretreated with cathepsin B (5 U/ml) (Cat. B-treated UF-MDs) did not show any such effect. UF-MDs alone, irrespective of the pretreatment with cathepsin B, did not show any effect on egg activation. (E) Schematic representation of *in vitro* egg activation experiments performed in F and G. Unfertilized eggs were pretreated with anti-UPIII-ED antibody as in B and then subjected to insemination with sperm that had been pretreated in the absence or presence of UF-MDs (0.1-10 μg/ml) and washed (MD-treated sperm). (F) Pretreatment of sperm with UF-MDs at more than 1 μg/ml was shown to partially negate an inhibitory effect of the anti-UPIII-ED antibody. Such an effect was not seen when UF-MDs that had been pretreated with cathepsin B (5 U/ml) (Cat. B-treated UF-MD) were used. UF-MDs alone, irrespective of the pretreatment with cathepsin B, did not show any effect on sperm ability to activate normal eggs. (G) As shown in F, an egg activation rate of ~60% was obtained when anti-UPIII-ED antibody-pretreated eggs were inseminated with sperm that had been pretreated with UF-MDs (10 μg/ml). Under these conditions, co-pretreatments of sperm with either an anti-UPIII-ED antibody (40 μg/ml) or a synthetic GRR peptide (100 μM) again caused a significant reduction in the egg activation rate. Neither an anti-UPIII-CT antibody (40 μg/ml) nor a synthetic GAA peptide (100 μM) showed such an effect. In addition, activation of normal (i.e. antibody-free) eggs was not affected by any of these sperm conditions. *P<0.01, compared with control. Data are the mean±s.d. of three independent experiments.

an inhibitor of tryptic protease (Fig. 2G). These results demonstrate again that UF-MDs interact with sperm in a UPIII-dependent manner. This interaction somehow allows sperm to activate eggs with cell surface UPIII that has been blocked with antibody.

Bidirectional signaling between UF-MDs and sperm

We proposed that UF-MDs trigger signal transduction events in sperm by which the sperm acquire the ability to activate UPIII-blocked eggs. To verify this possibility, we prepared UF-MD-treated sperm in the presence of various protein kinase inhibitors and examined the ability of the resulting sperm to activate UPIII-blocked eggs. Genistein, PP2 and staurosporin were inhibitory, whereas an inactive analog of PP2 (PP3) was not (Table 1). It should be noted that, whereas PP2 and genistein were also inhibitory to eggs (Sato et al. 1998, 2000), an inhibitory effect of staurosporin was specific to sperm: preincubation of unfertilized eggs with staurosporin even at 10 μM did not show any effect (supplementary material Table S1). These results suggest that sperm protein kinase activity is important for the ability of UF-MD-treated sperm to activate UPIII-blocked eggs. Preincubation of intact sperm with PP2 or staurosporin also resulted in a certain degree of failure to activate intact eggs (Table 1). Taking these findings together, we suggest that sperm protein kinase

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Table 1. Requirement of sperm protein kinase activity for the ability of UF-MD-treated sperm to activate UPIII-blocked eggs

Treatment	Number of eggs activated/total tested	
	Control	UPIII blocked
Sperm alone		
1×10^5/ml	14/30	2/30
3×10^5/ml	18/30	6/30
1×10^6/ml	30/30	5/30
3×10^6/ml	30/30	8/30
Sperm+UF-MDs (3 μg/ml)		
1×10^5/ml	11/30	5/30
3×10^5/ml	21/30	19/30
1×10^6/ml	30/30	24/30
3×10^6/ml	28/30	24/30
Sperm (3×10^6/ml)+UF-MDs (3 μg/ml)+inhibitors		
Staurosporin		
1 μM	6/30	1/30
0.1 μM	7/30	2/30
Genistein		
50 μM	13/30	10/30
5 μM	27/30	29/30
PP2		
10 μM	8/30	3/30
1 μM	27/30	25/30
PP3		
10 μM	25/30	19/30
1 μM	24/30	23/30
Sperm (3×10^6/ml)+inhibitors		
Staurosporin		
1 μM	3/30	ND
0.1 μM	7/30	ND
Genistein		
50 μM	11/30	ND
5 μM	30/30	ND
PP2		
10 μM	8/30	ND
1 μM	17/30	ND
PP3		
10 μM	22/30	ND
1 μM	26/30	ND

Unfertilized eggs were pretreated with anti-UPIII-ED antibody, washed and subjected to *in vitro* egg activation with sperm that was untreated or treated with UF-MDs and several protein kinase inhibitors at the indicated concentrations. Activation was scored by the occurrence of cortical contraction by 30 min after activation treatment. ND, not done.

activity is necessary for normal fertilization at the gamete membrane interaction level.

Isolation and characterization of MDs from human embryonic kidney 293 cells expressing UPIII, UPIb and Src

We next examined whether the sperm-interaction and signaling function of MDs can be reconstituted in cultured somatic cells by performing transient transfection experiments using human embryonic kidney (HEK) 293 cells. We employed plasmids expressing Src and either wild-type UPIII (UPIII-WT) or a mutant (UPIII-RR/AA) into which we introduced a protease-resistant Ala-Ala sequence in place of the protease-sensitive Arg-Arg sequence in the extracellular juxta-transmembrane domain. We also transfected a plasmid expressing UPIb, a binding partner for UPIII, by which membrane localization of UPIII-WT is made possible (Mahbub Hasan et al., 2007). Transfected cells were harvested, fractionated and analyzed for protein expression and fertilization-related signaling functions.

As in the case of UPIII-WT, the levels of protein expression and MD localization of UPIII-RR/AA were higher in cells co-expressing UPIb (Fig. 3A, compare lanes 3 and 5 with lanes 2 and 4). It should be noted that the anti-UPIII-ED antibody binds to UPIII-RR/AA as effectively as it binds to UPIII-WT, indicating that the RR/AA mutation does not affect antibody binding. As we reported previously (Mahbub Hasan et al., 2007), co-expression of Src with UPIII-WT and UPIb resulted in negative regulation of Src activity (Fig. 3B, lane 3). Such negative regulation of Src was also evident in cells expressing UPIII-RR/AA and UPIb (Fig. 3B, lane 5). These results show that molecular interactions among UPIII (wild-type or RR/AA mutant), UPIb and Src are reconstituted properly in HEK293 cells.

We next examined whether HEK293 cells expressing UPIII, UPIb and Src have the ability to reproduce the fertilization-related signal transduction events at the cell level and at the isolated MD level. At the cell level, neither sperm nor cathepsin B promoted tyrosine phosphorylation and/or proteolysis of UPIII (wild-type and RR/AA mutant) and Src (Fig. 3C), demonstrating that extracellular signaling function is not appropriately reconstituted in these cells. By contrast, cathepsin B caused massive proteolysis of both constructs of UPIII at the isolated MD level (termed 293-MDs; Fig. 3D, lanes 2 and 6). When 293-MDs were incubated with cathepsin B in the presence of anti-UPIII-ED antibody, proteolysis of UPIII-WT and UPIII-RR/AA was blocked to a similar extent (Fig. 3D, lanes 3 and 7). However, when 293-MDs were incubated with cathepsin B in the presence of anti-UPIII-CT antibody, only the proteolysis of UPIII-RR/AA was effectively blocked (Fig. 3D, lanes 4 and 8). These results suggest that the extracellular domain of UPIII-RR/AA is more resistant to cathepsin B-induced proteolysis than that of UPIII-WT, whereas the intracellular domains of UPIII-WT and UPIII-RR/AA are equally susceptible to cathepsin B-induced proteolysis. Under these conditions, however, we did not observe any increase in tyrosine phosphorylation of Src and UPIII (Fig. 3D). Tyrosine phosphorylation of UPIII and Src was not observed under any condition, suggesting again that signaling function is not fully reconstituted in this artificial UPIII-Src system. In addition, sperm did not promote any of the signaling events in the 293-MDs (supplementary material Fig. S3).

Reconstitution of the fertilization-related signaling function in 293-MDs

To verify further the fertilization-related function of 293-MDs containing either the wild-type or RR/AA mutant UPIII, as well as UPIb and Src, we performed *in vitro* egg activation experiments. First, we examined whether the 293-MDs interact with anti-UPIII-ED antibody. Unfertilized eggs were preincubated with a mixture of anti-UPIII-ED antibody and 293-MDs and then inseminated with normally prepared sperm. We also examined the effect of 293-MDs isolated from control HEK293 cells (mock transfected). Eggs pretreated with anti-UPIII-ED antibody plus control 293-MDs or with anti-UPIII-ED antibody alone showed rates of successful egg activation of less than 15%. By contrast, eggs pretreated with anti-UPIII-ED antibody plus 293-MDs containing UPIII-WT or UPIII-RR/AA showed rates of successful egg activation of more than 35%, demonstrating that UPIII proteins in these 293-MDs were interacting with anti-UPIII-ED antibody. Thus, it appears that MDs containing UPIII block the inhibitory function of anti-UPIII-ED antibody.

We next examined, as in the work shown in Fig. 2, the effect of preincubation of sperm with 293-MDs on the sperm-induced activation of anti-UPIII-ED antibody-treated eggs (Fig. 4A).

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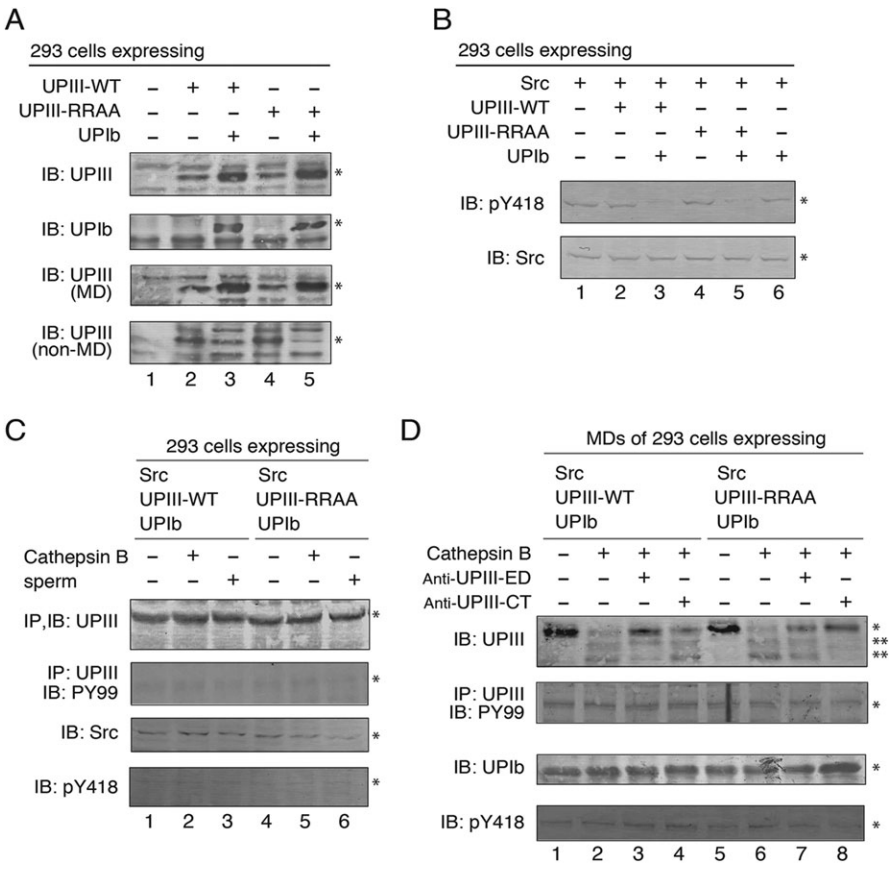

Fig. 3. Isolation and characterization of MDs from HEK293 cells that co-express wild-type or RR/AA mutant UPIII with UPIb and Src. (A) HEK293 cells were transfected with the indicated combinations of expression plasmids (UPIII-WT, UPIII-RR/AA and UPIb). Following transfection, cells were extracted, fractionated (top two panels, whole cell extracts; bottom two panels, MD and non-MD fractions) and analyzed for the expression of recombinant proteins by immunoblotting. (B) HEK293 cells were transfected with the indicated combinations of expression plasmids (Src, UPIII-WT, UPIII-RR/AA and UPIb). Following transfection, cells were extracted and analyzed for expression as well as tyrosine phosphorylation of ectopically expressed Src by immunoblotting. (C) HEK293 cells expressing either Src/UPIII-WT/UPIb or Src/UPIII-RR/AA/UPIb were left untreated (control) or treated with 5 U/ml cathepsin B or 10^6/ml sperm. After treatment, cells were extracted and analyzed for expression as well as tyrosine phosphorylation of UPIII and Src as in Fig. 1. (D) MDs were prepared from HEK293 cells expressing either Src/UPIII-WT/UPIb or Src/UPIII-RR/AA/UPIb and subjected to an *in vitro* kinase assay in the absence or presence of the indicated combinations of cathepsin B (5 U/ml), anti-UPIII-ED antibody (40 μg/ml) and anti-UPIII-CT antibody (40 μg/ml). Single asterisks indicate the positions of proteins of interest; double asterisks indicate the products of partial proteolysis of UPIII.

293-MDs containing UPIII-WT, but not those containing UPIII-RR/AA, were shown to recover significantly in terms of the rate of successful egg activation (Fig. 4B). As in the case of UF-MDs, 293-MDs containing UPIII-WT exhibited normal fertilization (Fig. 4B). The recovery effect by 293-MDs could be negated by their co-incubation with GRR peptide and anti-UPIII-ED antibody (Fig. 4C). These results demonstrate that the fertilization-related signaling function is at least partly reconstituted in 293-MDs, in a manner that depends upon the availability of the MD-associated UPIII to sperm-derived protease(s).

Developmental expression and localization of UPIII in growing and maturing oocytes

In *Xenopus*, ovarian immature oocytes at the germinal vesicle (GV) stage undergo hormone-induced maturation, after which the cell cycle is rearrested at the second meiotic metaphase (MII). MII oocytes undergo passage through the oviduct to become fully competent for fertilization and are subject to ovulation. We examined the timing at which MDs and the MD-associated molecules become competent for sperm-induced signal transduction. First, we analyzed the protein expression and localization of UPIII in the course of oogenesis and oocyte maturation. Immunoblotting experiments demonstrated that UPIII is expressed from the beginning of oogenesis: oocytes at oogenesis stage I showed an almost equivalent level of UPIII expression, as normalized to total amount of proteins analyzed, to those seen at oogenesis stage II and thereafter (~stage VI; Fig. 5A). During *in vitro* oocyte maturation induced by progesterone, the expression level of UPIII was unchanged (Fig. 5B).

Cell surface biotinylation experiments demonstrated that UPIII is exposed on the surface of both immature and hormone-treated mature oocytes to a similar extent (Fig. 5B). However, indirect immunofluorescence experiments demonstrated that anti-UPIII antibody had only a limited or no ability to bind to the cell surface of immature GV oocytes at stage VI; it should be noted that the bright area in the vegetal hemispheres of the oocyte does not reflect antibody-specific fluorescent signals but intrinsic fluorescence of the oocyte (Fig. 5Ca,b). The effective binding of the antibody became apparent at 2 h of progesterone treatment (Fig. 5Cb) and thereafter (6 h post progesterone treatment; Fig. 5Cc). These results suggest that the cell surface organization of UPIII and/or other UPIII-interacting molecules is modulated so as to be properly oriented at MDs during oocyte maturation.

Isolated GV oocyte MDs are not competent for sperm-induced signal transduction

The results presented in Fig. 5C led us to examine whether the function of MDs is modulated during oocyte maturation. We performed *in vitro* kinase assays and *in vitro* egg activation experiments using MDs that were prepared from immature oocytes (GV-MDs) and compared their functions with those of MDs prepared from progesterone-treated MII oocytes (MII-MDs). As shown in Fig. 6A, sperm- or cathepsin B-induced proteolysis and tyrosine phosphorylation of UPIII and Src in GV-MDs (lanes 4-6) were not as evident as in MII-MDs (lanes 1-3). Co-incubation of MII-MDs (Fig. 6B, lanes 2 and 5) or UF-MDs (Fig. 6B, lanes 3 and 6) with sperm and cytostatic factor (CSF) extract promoted the dephosphorylation of mitogen-activated protein kinase (MAPK), an event that reflects the occurrence of calcium-dependent signal transduction in the extract, whereas GV-MDs did not promote such an event (Fig. 6B, lanes 1 and 4). Unlike UF-MDs (Fig. 2) and MII-MDs (supplementary material Fig. S4), GV-MDs did not interact effectively with the anti-UPIII-ED antibody (supplementary

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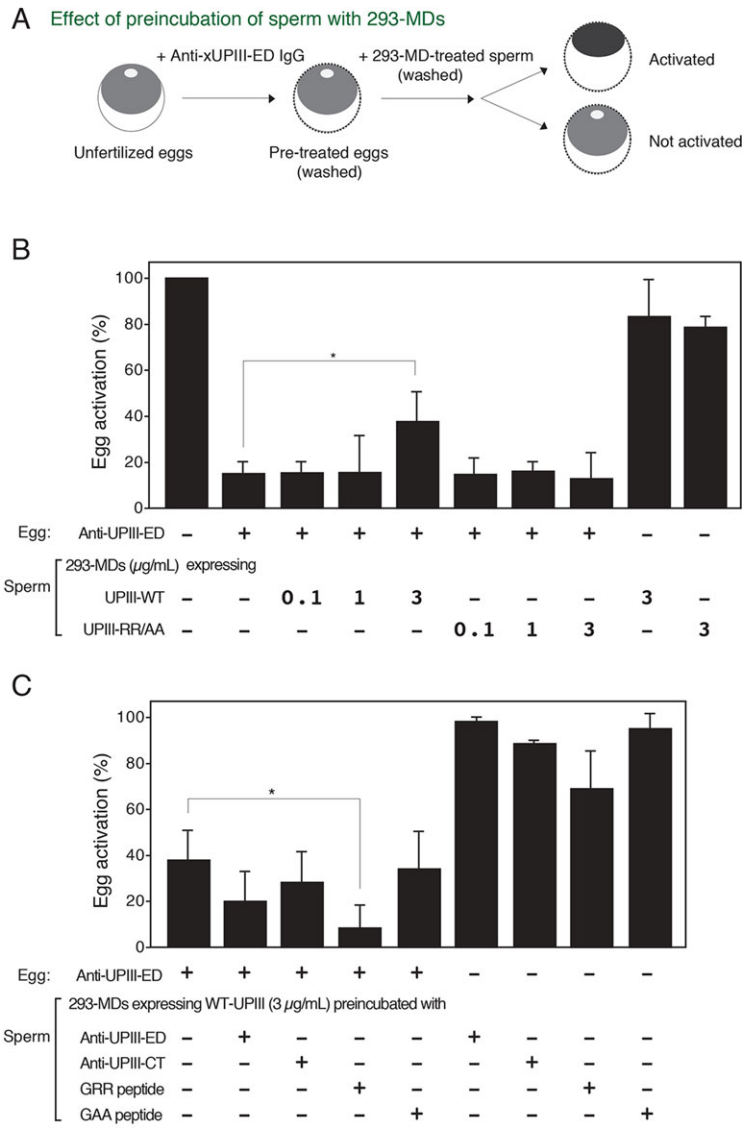

Fig. 4. 293-MDs containing UPIII-WT, but not UPIII-RR/AA, can augment the ability of sperm to fertilize eggs that have been pretreated with anti-UPIII-ED antibody. (A) Schematic representation of the *in vitro* egg activation experiments performed in B and C. Unfertilized eggs were pretreated with anti-UPIII-ED antibody and then subjected to insemination with sperm that had been pretreated in the absence or presence of 293-MDs expressing UPIII (wild-type or RR/AA mutant)/UPIb/Src (0.1-3 μg/ml) and washed (293-MD-treated sperm). Successful egg activation was scored as in Fig. 2. (B) Pretreatment of eggs with anti-UPIII-ED antibody resulted in ~80% inhibition of sperm-induced egg activation as shown in Fig. 2D. The inhibitory effect of the antibody was partly negated by pretreatment of sperm with 293-MDs containing UPIII-WT at 3 μg/ml. 293-MDs containing UPIII-RR/AA did not show such an effect. Treatment of sperm with 293-MDs alone, irrespective of expression of UPIII-WT or UPIII-RR/AA, did not show any effect on egg activation. (C) As shown in B, an egg activation rate of ~35% was obtained when anti-UPIII-ED antibody-pretreated eggs were inseminated with sperm that had been pretreated with 293-MDs containing UPIII-WT (3 μg/ml). Under these conditions, co-incubation of sperm with a synthetic GRR peptide (100 μM) caused a significant reduction in the egg activation rate. Such an effect was not seen when anti-UPIII-ED antibody (40 μg/ml), anti-UPIII-CT antibody (40 μg/ml) and synthetic GAA peptide (100 μM) were used for the co-incubation. In addition, activation of normal (i.e. antibody-free) eggs was not affected by any of these experimental conditions. **P*<0.01, compared with control. Data are the mean±s.d. of three independent experiments.

material Fig. S4). Finally, GV-MDs failed to recover the sperm-induced activation of eggs that had been pretreated with anti-UPIII-ED antibody, whereas MII-MDs did (Fig. 6C). These results suggest that the fertilization-related signaling function of MDs is acquired during the course of oocyte maturation.

DISCUSSION

In this study, we employed UF-MDs as an *in vitro* reconstitution system in which partial proteolysis and/or tyrosine phosphorylation of UPIII and Src were tested (Fig. 1D). These signaling events actually occurred in response to sperm or cathepsin B and were

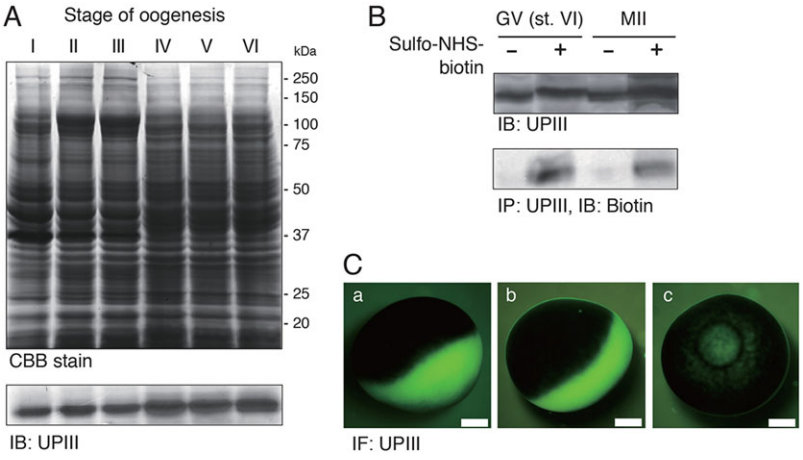

Fig. 5. Expression and cell surface presentation of UPIII during oogenesis and progesterone-induced oocyte maturation. (A) Whole cell lysates (20 μg protein per lane) were prepared from immature oocytes of GV stages I to VI, separated by SDS-PAGE and the gels analyzed by Coomassie Brilliant Blue (CBB) staining (top) and by immunoblotting with anti-UPIII-ED antibody (bottom). Data shown are representative of four independent experiments. (B) Immature oocytes at stage VI and progesterone-treated mature oocytes (MII) were subjected to cell surface biotinylation. After treatment, whole cell lysates (20 μg protein per lane) were prepared and analyzed for the expression (top) and biotinylation (bottom) of UPIII. Data shown are representative of three independent experiments. (C) Immature oocytes (a) and maturing oocytes of 2 h (b) and 6 h (c) progesterone treatment were subjected to indirect immunofluorescence staining with anti-UPIII-ED antibody. Images shown are representative of three independent experiments, as obtained under a fluorescence stereomicroscope. Scale bars: 250 μm.

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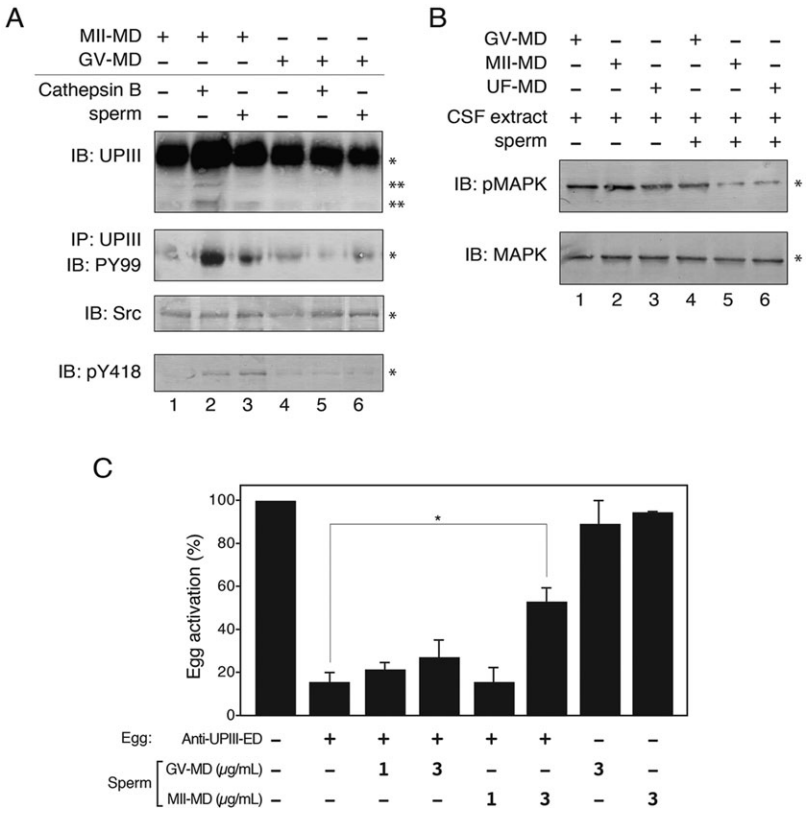

Fig. 6. MDs acquire the fertilization-related signaling function after progesterone-induced oocyte maturation. (A) MDs (5 μg protein) were prepared from immature oocytes (GV-MD) and mature oocytes (MII-MD), then treated with either cathepsin B (5 U/ml) or sperm (10^6/ml), and subjected to an *in vitro* kinase assay as in Fig. 1. Single asterisks indicate the positions of proteins of interest; double asterisks indicate products of partial proteolysis of UPIII. Data shown are representative of three independent experiments. (B) GV-MDs, MII-MD and UF-MDs (each 5 μg protein) were treated with CSF extract (100 μg protein) in the absence or presence of sperm and then analyzed for the phosphorylation of MAPK. Asterisks indicate the positions of phosphorylated (top panel) or total (bottom panel) MAPK. Data shown are representative of two independent experiments. (C) *In vitro* egg activation assay was performed with eggs that were untreated or pretreated with anti-UPIII-ED antibody as in Fig. 2. In this series of experiments, sperm (10^6/ml) that had been pretreated in the absence or presence of GV-MDs or MII-MDs (1 or 3 μg/ml) were used for insemination. **P*<0.01, compared with control using untreated sperm. Data are the mean±s.d. of three independent experiments.

inhibited by pretreatment of the UF-MDs with anti-UPIII-ED antibody (Fig. 1A,B). These results are consistent with our previous data showing that the same antibody inhibits normal egg fertilization (Sakakibara et al., 2005). Chemical inhibitors (PP2, GDPβS, LY294002, MβCD) also inhibited the Src tyrosine phosphorylation (Fig. 1C), suggesting that targets of these inhibitors are involved in this reconstitution system (Mammadova et al., 2009; Sato et al., 2000, 2002; our unpublished observations). The anti-UPIII-CT antibody showed a stimulatory effect on the cathepsin B-induced tyrosine phosphorylation of UPIII and Src (Fig. 1B). We propose that UF-MDs are mixtures of vesicles, in not only normal but also reverse (i.e. inside-out and outside-in) orientation of the lipid bilayers, and of non-vesicle fragments (i.e. inside-out and outside-out orientation), by which intracellular constituents of the MDs, such as the cytoplasmic tail of UPIII, become accessible to cathepsin B. If this were the case, the binding of the anti-UPIII-CT antibody might protect the cytoplasmic tails from proteolysis by cathepsin B, which facilitates preservation of the tyrosine phosphorylation state of UPIII.

The above results suggest that sperm and UF-MDs interact effectively with each other. To explore this interaction further, we performed *in vitro* egg activation assays and examined the effect of UF-MDs on the ability of sperm to fertilize eggs. Our expectation was that the UF-MDs would inhibit fertilization because the MD-treated sperm could not contact the eggs. However, the UF-MDs did not show any effect on normal fertilization (Fig. 2D). Instead, UF-MD-treated sperm was able to fertilize eggs that had been pretreated with anti-UPIII-ED antibody (Fig. 2E; see above), indicating that the inaccessibility of sperm to the antibody-bound eggs was somehow lifted, as MD interaction with sperm causes the sperm to be more effective in fertilizing an egg. Pretreatment of UF-MDs with cathepsin B, anti-UPIII-ED antibody or a synthetic peptide that corresponds to the RR motif in the potential proteolytic site of UPIII inhibited this phenomenon (Fig. 2G). Therefore, we suggest that the recovery effect of the UF-MDs requires protease-accessible UPIII in the MDs. The recovery effect was also reduced by the treatment of sperm with protein kinase inhibitors (Table 1). This reduction seems to have been due to the inhibition of protein kinase activity within sperm because staurosporin, which was not inhibitory to signaling processes in UF-MDs, was as effective as PP2 and genistein (Table 1). These results suggest that protein kinase-dependent signal transduction that is triggered in MD-treated sperm contributes to the acquisition of the ability to fertilize antibody-bound eggs. We hypothesize that such functional acquisition in sperm occurs at the time of gamete membrane interaction, most likely after the acrosome reaction of sperm and just before gamete membrane fusion. Epididymal maturation of mouse sperm involves the reception of epididymosomes, by which Src is incorporated into sperm (Krapf et al., 2012). In bovine sperm, epididymal maturation involves transfer to the sperm of CD9-positive microvesicles containing some proteins for sperm maturation (Caballero et al., 2013; Sullivan et al., 2007).

Taking these findings together, we suggest that the interaction between sperm and UF-MDs promotes bidirectional signal transduction (Fig. 7). Such signal crosstalk might be made possible as a consequence of the sperm-egg membrane interaction. In this context, it is interesting to note that, in the mouse, CD9-containing membrane components called exosomes have been shown to interact with fertilizing sperm before contact with the egg plasma membrane, such that the ability of the sperm to fuse with the egg is bestowed (Miyado et al., 2008), although this observation and accompanying hypothesis have been challenged by other groups (Barraud-Lange et al., 2012; Gupta et al., 2009). In *Arabidopsis*, mutual signaling between egg cell-derived EGG CELL 1 protein and sperm has been shown to be responsible for the cell surface presentation of GCS1/HAP2 that is essential for gamete fusion (Sprunck et al., 2012). These reports, together with our present findings, suggest that gamete

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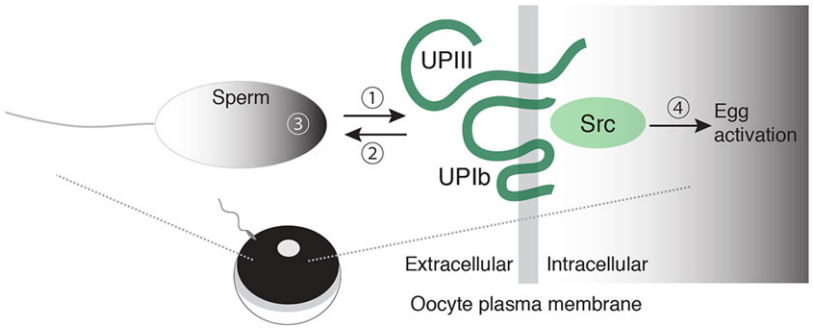

Fig. 7. Bidirectional signal transduction between sperm and egg operates at fertilization in *X. laevis*. Model of involvement of the MD-associated UPIII-Src signaling system at fertilization in *X. laevis*. (1) At the gamete membrane interaction, a sperm-derived protease targets the extracellular domain of UPIII and promotes tyrosine phosphorylation of UPIII and Src (Fig. 1). (2) At the same time, egg/oocyte-associated MD components, including UPIII, interact with sperm (Fig. 2), (3) thereby modulating protein kinase activity in sperm (Table 1). (4) Such bidirectional signal transduction (1 and 2) culminates in successful onset of the fusion between sperm and egg/oocyte, and in sperm-induced egg activation. The UPIII-Src signaling system becomes functional during hormone-induced oocyte maturation (Figs 5 and 6) and its sperm interaction function can be reconstituted to some extent in HEK293 cells (Fig. 3).

plasma membrane interaction acts as a trigger of mutual signal transduction in a wide variety of organisms.

We employed HEK293 cells for reconstituting the UPIII-Src system and for evaluating the function of mutant UPIII (UPIII-RR/AA), in which the protease-targeting RR motif is mutated to di-alanine residues. Co-expression of wild-type or mutant UPIII with UPIb resulted in their membrane- and MD-enriched subcellular localization (Fig. 3A). In addition, Src was maintained in an inactive state under these two conditions (Fig. 3B) (see Mahbub Hasan et al., 2005, 2007). These results indicate that the RR/AA mutant of UPIII is as capable of interacting with Src as wild-type UPIII. The application of sperm or cathepsin B to the transfected cells did not promote fertilization-like responses in UPIII (neither wild-type nor RR/AA mutant) and Src (Fig. 3C), suggesting that the extracellular domain of UPIII is not properly presented on the surface of the transfected cells and/or that the intracellular signaling system for Src activation is not properly reconstituted. In the case of the experiments using MDs (293-MDs), cathepsin B-induced proteolysis of the wild-type UPIII was observed, whereas tyrosine phosphorylation of UPIII and Src was again not observed in these experiments using 293-MDs (Fig. 3D). Importantly, the RR/AA mutant of UPIII was shown to be more resistant to proteolysis than the wild-type UPIII (Fig. 3D). Moreover, 293-MDs containing wild-type UPIII, but not the RR/AA mutant, were shown to rescue the ability of sperm to fertilize eggs that had been pretreated with anti-UPIII-ED antibody (Fig. 4B). These results demonstrate that we were able to reconstitute, to some extent, the extracellular function of the UPIII-Src system in the MDs of HEK293 cells, and that the presence of an intact RR sequence in UPIII is important for the functional reconstitution. With regard to the intracellular functions, we suggest that an appropriate contribution of Src-activating component(s) inside the plasma membrane, possibly PI3 kinase (Mammadova et al., 2009) and/or GTP-binding protein (Sato et al., 2003), is missing in the 293-MDs, although further study is necessary to clarify this issue.

In *Xenopus* oocytes, structural and functional competency for fertilization is acquired by a series of intracellular and extracellular events in response to hormonal stimulation. The intracellular events include the protein synthesis and/or activation of some protein kinases, germinal vesicle breakdown (Dorée et al., 1989; Maller et al., 1989), and the redistribution of cortical granules (Bement and Capco, 1990) and endoplasmic reticulum (Campanella et al., 1984; Charbonneau and Grey, 1984). These events culminate in the secondary arrest of the oocyte meiotic cell cycle (MII), at which the oocytes are subject to ovulation and fertilization (Iwao, 2000; Tunquist and Maller, 2003). During passage through the oviduct, the extracellular surface of the oocytes is also changed. This includes modification of the coelomic envelope with the pars recta-derived acrosome reaction-inducing substance in *Xenopus* (ARISX) (Ueda et al., 2002) and a structural transition from coelomic envelope to vitelline envelope (Gerton, 1986). Subsequently, the oocyte outer surface is deposited with multiple jelly layers, by which the oocyte-derived sperm chemoattractant allurin is retained (Olson et al., 2001). Oocyte maturation and ovulation also involve structural changes in the plasma membrane, such as flattening of the ultrastructure, as a possible result of reorganization of cytoskeletal proteins (Bement and Capco, 1990). However, functional transition of the sperm-interaction machinery in maturing oocytes has not yet been fully documented.

Against this background, we analyzed the expression of UPIII in oocytes at different times of oogenesis (i.e. stages I-VI) and oocyte maturation. UPIII is expressed from the beginning of oogenesis. Expression levels and MD localization of UPIII in fully grown oocytes (Fig. 5A) and mature oocytes are indistinguishable from each other (Fig. 5B). The extent of cell surface presentation of the extracellular domain of UPIII, as judged by surface biotinylation, also seems to be similar in each case (Fig. 5B). However, indirect immunofluorescence experiments demonstrate that the extent of cell surface presentation of UPIII increases over the course of oocyte maturation (Fig. 5C). We suggest that oocyte maturation is accompanied by some conformational change in the UPIII extracellular domain and/or an alteration in interactions involving UPIII and other molecules (e.g. UPIb). Similar examples showing a dynamic alteration in cell surface expression during oocyte maturation include the PMCA Ca^{2+}-ATPase and β1-integrin (El-Jouni et al., 2008; Müller et al., 1993).

In the signal reconstitution experiments, MII-MDs, but not GV-MDs, show the same responsiveness to sperm or cathepsin B as UF-MDs do (Fig. 6A,B). Moreover, *in vitro* egg activation assays demonstrate that MII-MDs, like UF-MDs but not GV-MDs, augment the ability of sperm to fertilize eggs that have been pretreated with anti-UPIII-ED antibody (Fig. 6C). These results suggest that MDs acquire the sperm interaction and signaling functions after oocyte maturation. Therefore, it is attractive to hypothesize that the sperm interaction and signaling functions in the oocyte MDs are strictly modulated during the course of oocyte

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maturation. Further investigations into the functional acquisition of MDs for fertilization signaling are underway.

MATERIALS AND METHODS

Frogs, antibodies, synthetic peptides and other reagents

Adult *Xenopus laevis* were purchased from local dealers. Rabbit polyclonal antibodies against a C-terminal sequence of UPIII (UPIII-CT) and an internal sequence in the non-catalytic domain of Src1/2, and a rabbit antibody against the extracellular domain of UPIII (GST-UPIII-ED) were prepared as described previously (Sakakibara et al., 2005). Anti-phosphotyrosine antibody PY99 and a phospho-specific anti-active Src family kinase antibody pY418 were from Santa Cruz Biotechnology (sc-7020) and Biosource International (4460G), respectively. Polyclonal antibodies against mitogen-activated protein kinase (MAPK; 9102L) and its active form (pMAPK; 9101L) were purchased from Cell Signaling Technology. PP2 was obtained from Calbiochem-EMD Millipore. GDPβS was purchased from Sigma-Aldrich. LY294002 was from Calbiochem-EMD Millipore. Methyl-β-cyclodextrin (MβCD) was purchased from Wako Pure Chemicals. A synthetic peptide that corresponds to residues 177-191 of UPIII (GRR peptide, SSGTIDTWPGRRSGG) and a mutated version (GAA peptide, SSGTIDTWPGAASGG, in which di-arginine residues are mutated to alanine residues) were obtained from Bex Corporation (Tokyo, Japan). Bovine spleen cathepsin B was obtained from Sigma-Aldrich. Sulfosuccinimidobiotin (EZ-Link sulfo-NHS-biotin) was purchased from Thermo Fisher Scientific. A monoclonal antibody against biotin (clone BN-34; B7653) was obtained from Sigma-Aldrich. Protein A-Sepharose was obtained from Amersham Biosciences.

Collection and experimental manipulation of oocytes and eggs

Immature and hormone-induced matured oocytes were prepared as described previously (Sato et al. 1996; Tokmakov et al., 2005). *In vitro* fertilization of ovulated and unfertilized eggs was performed as described (Sato et al., 1999, 2000). For *in vitro* fertilization, we used sperm that were pretreated with jelly water as described previously (Sato et al., 2000), by which sperm achieve active motility and undergo the acrosome reaction effectively and the success rate of egg activation is increased. In some experiments, jelly layer-free eggs were pretreated with a rabbit antibody against GST-UPIII-ED at the concentrations specified in the text. After the antibody pretreatment of 15-25 min, eggs were washed several times with buffer solution and subjected to *in vitro* fertilization. In addition, sperm and/or eggs were sometimes preincubated with MDs at the specified concentrations (see next section), washed as above, and subjected to *in vitro* fertilization.

Preparation of oocyte/egg MDs

Preparation, dialysis and concentration of MDs were carried out as described (Sato et al., 2006). The positions of MD fractions were determined by protein silver stain and immunoblotting with anti-UPIII-ED antibody (supplementary material Fig. S1). Procedures for MD preparation from HEK293 cells were similar to those described previously (see also below) (Mahbub Hasan et al., 2007) (supplementary material Fig. S2).

In vitro reconstitution of fertilization signaling events

Proteolysis and tyrosine phosphorylation of MD-associated UPIII and Src were reconstituted *in vitro* as described previously (Mahbub Hasan et al., 2005; Sato et al., 2003) with some modifications. MDs that had been prepared from GV stage VI oocytes, MII oocytes or from unfertilized eggs (2.5-10 μg protein, equivalent to 30-60 oocytes or eggs) or HEK293 cells (3.3-10 μg protein, equivalent to 1.5-5×10^6 cells) were treated in the absence or presence of either of the following egg activators: sperm (10^6/ml) or cathepsin B (5 U/ml) for 10 min at 21°C. In some experiments, the mixtures of MDs and egg activators were co-incubated with one of the following: anti-UPIII IgG (5 μg/ml), PP2 (5 μM), GDPβS (20 μM), LY294002 (100 μM) or MβCD (25 mM). After incubation, the mixtures were supplemented with 0.5 mM $CaCl_2$, 5 mM $MgCl_2$ and 1 mM ATP, and further incubated at 21°C for 10 min. The reactions were terminated by the addition of excess EDTA and EGTA, and then analyzed in immunoprecipitation experiments. Alternatively, the reactions were terminated by the addition of Laemmli SDS sample buffer (Laemmli, 1970); in this case, the samples were separated by SDS-PAGE on 8% (w/v) gels and subjected to immunoblotting. Dephosphorylation of MAPK was also reconstituted *in vitro* using MDs and cytostatic factor-arrested egg extracts (CSF extracts) according to methods described previously (Sato et al., 2003, 2006; Tokmakov et al., 2002).

Expression plasmids for UPIb, UPIII and Src

Messenger RNAs encoding UPIb, UPIII and Src (*Xenopus* Src2) were purified from *Xenopus* liver using the Quick Prep Micro mRNA Purification Kit (GE Healthcare). Expression plasmids for UPIII and FLAG-tagged *Xenopus* UPIb were constructed using p3xFLAG-CMV-14 vector and pCMV-tag5A (Sigma-Aldrich), respectively, as described previously (Mahbub Hasan et al., 2007; Sakakibara et al., 2005). Preparation of p3xFLAG-CMV-14 containing Src-WT was as described (Iwasaki et al., 2006). For expression of mutant UPIII (UPIII-RR/AA), in which di-arginine residues (Arg187-Arg188) in the conserved juxta-transmembrane sequence were substituted by di-alanine residues (Ala187-Ala188), the pCMV-tag5A/UPIII vector was subjected to PCR with mutagenic primers (5′-3′, mutations underlined): forward, ACATGGCCTGGCGCAGCGAGTGGTGGGATG; reverse, CATCCCACCACTCGCTGCGCCAGGCCATGT.

Transfection of cultured cells and other protein analysis methods

HEK293 cells were used for transfection experiments as described previously (Mahbub Hasan et al., 2007). Immunoprecipitation, SDS-PAGE and immunoblotting were performed according to described methods (Sato et al., 1999, 2000, 2002). Biotinylation of oocyte/egg surface and indirect immunofluorescence to evaluate the cell surface expression of UPIII were as described previously (Sakakibara et al., 2005).

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Competing interests

The authors declare no competing financial interests.

Author contributions

K.S. conceived and designed the experiments. A.K.M.M.H., A.H., Y.M., T.M., S.K., T.W.I. and K.S. performed the experiments. A.K.M.M.H., T.W.I., Y.F. and K.S. analyzed the data. K.S. wrote the paper.

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Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.105510/-/DC1

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