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1 **The soluble sperm factor that activates the egg: PLCzeta and beyond.**

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Abstract

PLCzeta(ζ) initiates Ca^{2+} oscillations and egg activation at fertilization in mammals, but studies in mouse eggs fertilized by PLC ζ knockout (KO) sperm imply that there is another slow acting factor causing Ca^{2+} release. Here, I propose a hypothesis for how this second sperm factor might cause Ca^{2+} oscillations in mouse eggs.

Egg activation is caused by increases in cytosolic Ca^{2+} , and in mammalian eggs (MII oocytes) the sperm triggers a prolonged series of repetitive transients, or oscillations, in the cytosolic free Ca^{2+} concentration (Swann & Lai 2016, Sanders & Swann 2016). These Ca^{2+} oscillations are driven by increased inositol 1,4,5-trisphosphate (InsP_3) production which causes cycles of Ca^{2+} release from the InsP_3 -receptor (IP_3R). Since the 1990s we have known that mammalian sperm contain a soluble protein 'sperm factor' (or sperm-oocyte-activating-factor- SOAF), that can trigger Ca^{2+} oscillations after gamete fusion (Swann and Lai, 2016). Its existence inside the sperm can explain why intracytoplasmic sperm injection (ICSI) mimics fertilization in causing Ca^{2+} oscillations in mouse and human eggs (Jones 2018, Kurokawa & Fissore 2003). It is now widely acknowledged that this sperm factor in mammals is the sperm-specific protein phospholipase PLCzeta(ζ) (Swann & Lai 2016, Jones 2018). Key evidence includes the finding that microinjection of PLC ζ cRNA or protein causes prolonged sperm-like Ca^{2+} oscillations in all mammalian eggs studied (Swann & Lai 2016), and that functionally disruptive mutations in PLC ζ alone lead to male factor infertility and egg activation failure in humans in IVF and ICSI (Escoffier *et al.* 2016).

Recently two groups have reported the phenotype of PLC ζ knock out (KO) mice. They both found that injecting PLC ζ KO mouse sperm into eggs (hence ICSI) fails to trigger any Ca^{2+} oscillations (Hachem *et al.* 2017, Nozawa *et al.* 2018). This shows that PLC ζ accounts for the Ca^{2+} signals and egg activation after ICSI. However, during *in vitro* fertilization (IVF) and mating with PLC ζ KO males some eggs are activated at fertilization and embryo development still occurs (Hachem *et al.* 2017, Nozawa *et al.* 2018). Success rates of IVF are lower and litter sizes are smaller with PLC ζ KO males but the result contrasts with what happens with ICSI. The reason why IVF leads some

45 eggs to activate with PLC ζ KO sperm is because there are ~3 large Ca²⁺ oscillations that occur
46 about 40 mins later than expected when compared to wild type sperm (Nozawa *et al.* 2018). The
47 existence of these delayed Ca²⁺ oscillations with PLC ζ KO sperm has been reproduced in my lab
48 (Fluks, Parrington and Swann unpublished). The late Ca²⁺ oscillations with PLC ζ KO sperm lead to
49 delayed egg activation, including cortical granule exocytosis which is required to block extra sperm
50 entry (Nozawa *et al.* 2018). This means that many such zygotes fail to develop because they are
51 polyspermic. Overall, the data suggest that PLC ζ initiates the Ca²⁺ oscillations at fertilization,
52 accounting for most of the Ca²⁺ spikes, but that during IVF the sperm has another mechanism for
53 promoting later Ca²⁺ oscillations in the mouse (Jones 2018). Two characteristics of this secondary
54 mechanism is that it is delayed after gamete fusion, and that it is active in IVF and not with ICSI.

55
56 In looking for PLC ζ -independent mechanisms for Ca²⁺ oscillations we need to consider previous
57 data gather from mammalian zygotes. First, all previous studies have shown that without sperm-
58 egg membrane fusion in IVF there are no Ca²⁺ oscillations (Swann & Lai 2016). So, it's reasonable
59 to assume that a second mechanism for Ca²⁺ release involves a sperm factor that is either soluble
60 and enters the egg by cytosolic diffusion, or that it is introduced by the sperm membrane into the
61 egg plasma membrane by two-dimensional diffusion. For either option I will describe it as a sperm
62 factor. It has been suggested that the PLC ζ -independent sperm factor may be 'cryptic' because it
63 is only apparent when PLC ζ is absent (Jones 2018). Whilst this is true from an observational point
64 of view, it does not mean it is inactive during normal fertilization. In fact, it is difficult to see how a
65 second factor could only arise when PLC ζ was not present. As far as we know PLC ζ is only active
66 in eggs, so a lack of PLC ζ would not be evident until after gametes have fused. Clearly, gene
67 expression in spermatogenesis cannot compensate for future events, hence the second factor
68 should operate in IVF with wild type sperm. In hindsight we can see evidence of a secondary
69 mechanism because it was previously found that ICSI causes a shorter duration of Ca²⁺
70 oscillations than IVF in mouse zygotes⁵. If the secondary factor operates in normal IVF it also gives
71 it a selective advantage for it to persist in the presence of PLC ζ . One attractive idea is that this

72 factor is a 'primitive' factor from a role in egg activation in species earlier in the vertebrate lineage
73 (Nozawa *et al.* 2018).

74

75 Previous studies restrict the options for how any factor can trigger Ca^{2+} oscillations in the absence
76 of PLC ζ . For example, one could propose that the second factor promotes Ca^{2+} influx into the egg,
77 perhaps by the insertion of sperm derived Ca^{2+} channels into the egg membrane. However, there
78 are many ways to increase Ca^{2+} influx into unfertilized mammalian eggs and none of them trigger
79 Ca^{2+} oscillations without PLC ζ . An updated version of the ' Ca^{2+} conduit' idea remains implausible
80 (Swann & Lai 2016). The second factor cannot work via messengers such as NAADP, or cADPR,
81 since these also fail to trigger Ca^{2+} oscillations in mouse eggs (Swann & Lai 2016). A sperm protein
82 called PAWP has been suggested to trigger Ca^{2+} oscillations in eggs, but the key data on PAWP is
83 not reproducible (Sanders and Swann, 2016). Furthermore, PAWP is supposed to cause Ca^{2+}
84 oscillations during ICSI, but we now know that PLC ζ accounts for these Ca^{2+} oscillations. Another
85 study has suggested that extramitochondrial citrate synthase is the second sperm factor in
86 mammals (Kang *et al.* 2020). However, the phenotype of extramitochondrial citrate synthase KO
87 sperm at fertilization is apparently the same as PLC ζ KO sperm, with delayed Ca^{2+} oscillations
88 (Kang *et al.* 2020). This result is difficult to rationalize because these citrate synthase KO sperm
89 still contained PLC ζ and the initial Ca^{2+} oscillations should not be delayed. In addition, we have
90 found that citrate synthase protein injection into mouse eggs does not trigger Ca^{2+} release
91 (Sanders and Swann, unpublished observations). From what we know about how to cause Ca^{2+}
92 oscillations in mouse eggs, we can conclude that the second factor is either making InsP_3 , or else
93 directly stimulating the IP_3Rs .

94

95 If the second sperm factor generates InsP_3 this implicates another PLC. There are many other PLC
96 isoforms in mammalian sperm (Parrington *et al.* 2002). However, the other PLCs are about two or
97 three orders of magnitude less active in causing Ca^{2+} release than PLC ζ in eggs (Swann and Lai
98 2016; Mehlmann *et al.* 2001). To be active in eggs they would have to be expressed at >2pg per
99 sperm, and yet there is only 40pg of total protein in a mouse sperm (Mehlmann *et al.* 2001). The

100 second sperm factor could stimulate an egg membrane derived PLC, but this is not consistent with
101 some previous findings. For example, if eggs are imaged using GFP tagged C1 domains, there is
102 no measurable diacylglycerol increase in the plasma membrane for at least two hours of sperm
103 induced Ca^{2+} oscillations in mouse eggs, despite the ability of this probe to respond to other stimuli
104 (Yu *et al.* 2008). Hence, it appears that a plasma membrane derived PLC activity is not stimulated
105 in fertilizing eggs. This is not an issue for PLC ζ which is the only mammalian PLC without a PH
106 domain and it binds to PIP₂ in intracellular vesicles (Fig 1) and not the plasma membrane (Swann
107 & Lai 2016). However, conventional PLCs (β , γ or $\delta 1$ class) locate to the plasma membrane with a
108 PH domain, and one would expect some diacylglycerol increase to occur if they were active at
109 fertilization. If the second sperm factor makes InsP₃ then it probably needs to stimulate another
110 unconventional PLC that is localized on vesicles in the egg. It is not clear whether any other PLCs
111 would match the unusual localization pattern of PLC ζ , but it might be worth investigating the
112 localization of the epsilon or eta class of PLCs in eggs.

113

114 In the absence of data on other PLCs I can suggest an alternative idea, that the second sperm
115 factor acts to sensitize the IP₃R. Strontium ions or thimerosal both stimulate Ca^{2+} oscillations in
116 mouse eggs, and they both appear to act directly to sensitize the IP₃R to release Ca^{2+} (Swann &
117 Lai 2016). The schematic in Fig.1 shows the second factor affecting IP₃R induced Ca^{2+} release
118 following PLC ζ entry. If the target is the IP₃R, or vesicular PIP₂, the protein factor is likely to be
119 soluble and diffuse into the cytosol. To explain why PLC ζ independent Ca^{2+} release is not evident
120 with ICSI, it is possible that the second factor is released from the sperm during their preparation
121 when the sperm is damaged, or when the head is removed. Damaging the sperm membrane is
122 standard practice before ICSI. Plasma membrane damage during cryopreservation may also lead
123 to the loss of the second factor from sperm, which could explain why there was a lack of Ca^{2+}
124 oscillations with most cryopreserved PLC ζ KO sperm in IVF (Hachem *et al.* 2017). The other
125 feature of the second sperm factor is a delayed action. It could be that the synthesis of a second
126 sperm factor protein from RNA in the sperm could account for the >40 min delay before Ca^{2+}
127 transients (Jones 2018). However, the total amount of RNA in a single mouse sperm (0.1pg) is

128 similar to the amount of PLC ζ RNA injected into an egg to cause Ca²⁺ oscillations, and yet sperm
129 RNA is made up of several hundred varieties. Any protein synthesized from sperm RNA would
130 have to be >100 times more potent than PLC ζ which is active at concentrations of less than 10nM.
131 A more realistic idea is that secondary factor is another protein delivered by the sperm. The delay
132 could be because this protein needs to first diffuse and equilibrate throughout the egg and then act
133 indirectly to sensitize IP₃Rs. The second factor may not be active in human fertilization since
134 human eggs are less sensitive to Ca²⁺ release, and for example do not oscillate in response to
135 strontium medium (Lu *et al.* 2018). This could explain why inactivating mutations in human
136 PLC ζ lead to male factor infertility with both normal conception and ICSI (Escoffier *et al.* 2016). The
137 second factor may only be evident in mouse and rat eggs, or possibly in some non-mammalian
138 species that do not appear to use PLC ζ to activate the egg (Swann & Lai 2016).

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140

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142 The author declares that there is no conflict of interest that could be perceived as prejudicing the
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148 KS conceived the ideas and wrote the paper.

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152

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204 **Figure Legend.**

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206 Fig.1. A schematic representation of the hypothesis for PLC ζ and a second factor may act to

207 cause Ca²⁺ oscillations in fertilizing mouse eggs.

