

Cell Surface Changes in the Egg at Fertilization

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SUMMARY

An egg changes dramatically at fertilization. These changes include its developmental potential, its physiology, its gene expression profile, and its cell surface. This review highlights the changes in the cell surface of the egg that occur in response to sperm. These changes include modifications to the extracellular matrix, to the plasma membrane, and to the secretory vesicles whose contents direct many of these events. In some species, these changes occur within minutes of fertilization, and are sufficiently dramatic so that they can be seen by the light microscope. Many of these morphological changes were documented in remarkable detail early in the 1900s by Ernest Everett Just. A recent conference in honor of his contributions stimulated this overview. We highlight the major cell surface changes that occur in echinoderms, one of Just's preferred research organisms.

Despite the diversity in molecular mechanisms at fertilization among organisms, one can extract a few overlying principles. . .

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Mol. Reprod. Dev. 76: 942–953, 2009. © 2009 Wiley-Liss, Inc.

Published online 5 August 2009 in Wiley InterScience
(www.interscience.wiley.com).
DOI 10.1002/mrd.21090

Received 5 June 2009; Accepted 24 June 2009

INTRODUCTION

Ernest Everett Just championed the cell surface as a driving force of development. His detailed observations of fertilization, the changes in the egg cell surface in response to sperm or parthenogenic activation, and his descriptions of the fast and slow blocks to entry of multiple sperm (polyspermy) are each unparalleled for his time. While much of what he posited for developmental regulation is now attributed to the nucleus, the precise deductions and careful arguments he laid out in his book, *The Biology of the Cell Surface* (Just, 1939a), forced a serious consideration of cell surface contributions to many activities of the cell and the embryo in development. Perhaps prescient of his arguments, elucidated in the later part of the 20th century, is the important role the cell surface plays in transmitting information from other cells and the environment to the nucleus. One only needs to consider the cell surface receptors for growth factors (e.g., EGF, FGF, TGF- β), for wnts, or of notch activity to appreciate Just's philosophy in today's research arena.

Just was equally influential for his role in understanding and contending for the proper conditions for one's research specimens. He is attributed for deducing the best culture conditions for early embryonic development, for microscopic observations, and for experimental perturbations (*Basic Methods for Experiments on Eggs of Marine Animals*, Just, 1939b). This work alone influenced a myriad of investigators of the time, and enabled him to see biological changes in living material that more accurately reflected the in vivo mechanisms. His advice for studying the best species for the problems at hand still holds true. For example, one of his favorite research specimens for studying fertilization was the sand dollar *Echinarachnius parma*, found near the Marine Biological Laboratories in Woods Hole, Massachusetts (USA). Large, clear, and readily available, this cell was an excellent specimen for watching the changes of the egg cell surface during fertilization. It was with this and other specimens that he deduced what is now known as the fast block to polyspermy, a nearly instantaneous change in the electrical potential at the plasma membrane that blocks the fusion of

subsequent sperm with the egg following fertilization. The specimen's clarity further allowed him to describe, in remarkable detail, the changes in the cortex of the egg during secretion of the cortical granules (CGs) and the formation of the fertilization envelope (FE). As a consequence, Just was able to examine fertilization from a comparative perspective, appreciating both the commonality and diversity of this important process at fertilization.

EGGS AND THE DIVERSITY OF CHANGES AT FERTILIZATION

One might conclude that the jobs of an egg between different species are much the same; despite variations in shapes, sizes, and organization of this cell in different organisms, the molecular mechanisms are analogous. After all, many other important and diverse developmental features are shared. For example, the limbs of the fly, the bird, and the mammal are clearly different on morphological inspection, but the molecular mechanisms that drive formation of these structures are remarkably similar (Shubin et al., 2009). So too with the eyes of these and other organisms: vastly different morphology, with a remarkably shared molecular mechanism (Jonasova and Kozmik, 2008; Shubin et al., 2009).

But eggs are driven to differ (Evsikov and Marin de Evsikova, 2009). First, eggs are fertilized at different times of meiosis: some are competent early in meiosis, others remain stalled in metaphase I or II of meiosis, while some can only do so as haploid products of meiosis. The reproductive strategies and molecular mechanisms of various organisms are so diverse that even the shapes and sizes of eggs are different! Consider the round sea urchin egg, the elongated insect egg, and the . . . well, the classically egg shaped bird egg. The sizes range enormously as well: mouse and human eggs are about 80–100 μm in diameter, frog eggs are 1 mm, emu eggs are 15 cm (really, I have one on my desk), and the elephant or whale egg—only 120 μm . These individual cells also differ at their surface. Eggs from flies and fish, for example, are covered with a tough chorion containing only a single site where sperm may access the egg (the micropyle). Mammals, on the other hand, have a thick zona pellucida through which sperm must penetrate whereas nematodes have but a thin egg coat. Indeed, genes involved in the reproductive process are among the most rapidly diversifying genes of an organism, thus rapid genetic drift in reproductive genes can be used to computationally identify genes important for reproduction (Swanson and Vacquier, 2002).

We have followed the philosophy of Just, and selected an organism well suited to study the question at hand. We have also used one of Just's favorite specimens, the echinoderm egg, to address the questions of cell surface molecular changes at fertilization. Here we summarize the changes in the egg cell surface in sea urchins eggs, with emphasis on the formation of the FE as a paradigm for changes in the extracellular matrix (ECM).

THE EVENTS

Despite the diversity in molecular mechanisms at fertilization among organisms, one can extract a few overlying

principles upon which investigators can build (including the rule of diversity!). The major, conserved molecular events reviewed here are (1) all eggs are activated by calcium release into their cytoplasm from storage within cellular organelles, and (2) all eggs have an ECM that is modified following fertilization. Originally described in 1847 and called the FE (Derbès, 1847), this ECM of the fertilized sea urchin egg has also been referred to as the fertilization membrane. Although the term “membrane” was used by Just and others, it is not a biological membrane at all as it has no bilayer and no lipids. Thus, the original term (FE) for this extracellular structure is more accurate and will be used here.

Just studied the FE, and in 1919 described its formation in the sand dollar, *E. parma* (Just, 1919). In this publication, he systematically itemized the major observations of the time with respect to where this structure comes from, and what each change yields in terms of successful fertilization and early development. The description stands today for its accuracy and clarity. Just selected *E. parma* because the egg was readily available, is large, and is optically clear. Fortunately, FE formation is also slow enough in this echinoderm to allow for optimal observation.

The FE is constructed at fertilization on an extracellular scaffold called the vitelline layer—the nascent ECM of the egg (Tegner and Epel, 1973; Eddy and Shapiro, 1976; reviewed in Wong and Wessel, 2006a). This ECM is analogous to the zona pellucida of mammals, the vitelline layer of frogs, and the chorion of fish. As in other animals, the echinoderm egg ECM is able to bind sperm in a species selective manner. This echinoderm structure contains large glycoproteins that are mostly undefined, however. One strong molecular candidate in the vitelline layer for specific binding of sperm is egg bindin receptor (EBR; bindin is the protein on sperm necessary and sufficient for binding to eggs; Kamei et al., 2000; Kamei and Glabe, 2003). EBR was first identified in sea urchin eggs based on its affinity for sperm, its rapid sequence divergence, and its localized accumulation in the vitelline layer. Other vitelline layer components may also function in sperm interaction, and/or for interacting with EBR.

A second essential role for the vitelline layer (vl) is to scaffold the formation of the FE. One essential component for assembly appears to be an isoform of rendezvin (RDZ-vl). RDZ-vl is one splice variant of a single gene, the alternative products (RDZ-cg) accumulating instead in the CGs (Wong and Wessel, 2006b). RDZ-vl contains CUB domains, protein interaction folds shown to be important in the formation of the FE. RDZ-vl appears to interact with its sibling splice variants, RDZ-cg, likely through their abundance of CUB domain. RDZ-cg originates from the egg secretions at fertilization, and the observation that the two siblings meet up again after fertilization led to its gene name *rendezvin* (RDZ, -cg for the CG form and -vl for the vitelline layer sibling form; see Table 1).

A remarkable feature of the FE of sea urchins is how it is constructed. This structure, up to four times greater in surface area than its vitelline layer scaffold, forms within 60 sec after fertilization. It assembles from individual molecules—not as a prefabricated structure (Weidman et al., 1985, 1987)—at a rapid speed, yet its mature form

TABLE 1. Major gene products involved in cell surface changes at fertilization

Protein	Residence in egg	Domains	Function
β -glucanase	Cortical granule	Glucanase	?
Cortical granule serine protease (CGSP1)	Cortical granule	Serine triad	<ul style="list-style-type: none"> • Separates VL from egg surface • Regulation of OVOP, Udx1
Extracellular transglutaminase (eTG)	Egg surface	Transglutaminase	<ul style="list-style-type: none"> • Transamidation of SFE9 to RDZ-vi
Nuclear transglutaminase (nTG)	Nucleus, egg surface	Transglutaminase	<ul style="list-style-type: none"> • Transamidation of SFE9 to RDZ-vi • Regulation of Udx1?
Urchin dual oxidase 1 (Udx1)	?	Peroxidase + NAD(P)H oxidase	<ul style="list-style-type: none"> • Synthesis of hydrogen peroxide • Neutralization of hydrogen peroxide?
Ovoperoxidase (OVOP)	Cortical granule	Peroxidase	<ul style="list-style-type: none"> • Dityrosine cross-linking FE proteins, (e.g., RDZ-vi, PLN, SFE-1, SFE-9)
p160	Vitelline layer	CUB	<ul style="list-style-type: none"> • Anchors vitelline layer to egg surface
EBR1	Vitelline layer		<ul style="list-style-type: none"> • Putative sperm receptor
Proteoliasin (PLN)	Cortical granule	LDLrA	<ul style="list-style-type: none"> • Structural protein • Tethers OVOP to the FE
SFE-1	Cortical granule	LDLrA	<ul style="list-style-type: none"> • Structural protein of the FE
SFE-9	Cortical granule	LDLrA + NHK repeat	<ul style="list-style-type: none"> • Structural protein of the FE
Rendezvin (RDZ)	Vitelline layer, cortical granule	CUB \pm PYQ repeat	<ul style="list-style-type: none"> • Structural protein of the VL • Structural protein of the FE

is capable of creating a substantial barrier to both sperm and to macromolecules larger than 40 kDa (Wong and Wessel, 2008). In comparison, the glomerular matrix of mammalian kidneys (also an ECM, not a lipid bilayer membrane) takes many days to form (depending on the animal), the ECM is stationary during its formation, and it forms a molecular cut off to passage of about 69 kDa (e.g. Chang et al., 1975; Deckert et al., 1993; Patari-Sampo et al., 2006). This contrast in assembly time and ontogeny of each ECM lends greater appreciation of the logistical challenges and the efficient solution evolved by the egg.

CALCIUM AND SECRETION

The major mass of the FE originates from the CGs. Egg CGs are made during oogenesis. They first bud from the Golgi apparatus and accumulate throughout the cytoplasm. At meiosis, the population as a whole is then mobilized and trafficked to the plasma membrane (Wessel et al., 2002). In sea urchins, the secretory vesicles at the egg cortex are stably attached to the plasma membrane for hours to weeks prior to release. These secretory vesicles are tethered to the plasma membrane via members of the SNARE complex (Conner et al., 1997; Coorsen et al., 1998; Wong et al., 2007). Proteins of the SNARE complex contribute to membrane dynamics, specifically in the interactions and membrane fusion of cytoplasmic organelles (Südhof and Rothman, 2009). Only upon exposure to cytoplasmic calcium will the contents of these CGs be secreted, thereby contributing the major proteins essential to FE assembly.

The regulation of calcium release into the cytoplasm of eggs is one of the few conserved pathways of animal fertilization. Unlike neurons, whose activating calcium is generally harvested from outside the cell via voltage-sensitive and/or ligand-sensitive calcium channels found in the plasma membrane of the presynaptic bouton, eggs store their own calcium within the endoplasmic reticulum (Lee et al., 2006; Parrington et al., 2007). Release from this compartment requires a transient opening of a calcium channel in the membrane. Gating of these diverse channels is directly regulated by a specific ligand; the ligands include inositol trisphosphate (IP₃), nicotinic acid adenine dinucleotide phosphate (NAADP), and cyclic ADP-ribose (cADPr) (Parrington et al., 2007).

The major mechanism of calcium release in eggs is by IP₃, which is derived from phosphatidylinositol 4,5-bisphosphate (PIP₂) found in the membrane (Fig. 1). This enzymatic reaction is catalyzed by an isoform of phospholipase C (PLC). Activation of PLC thus appears necessary and sufficient for calcium release at egg activation in many animals. However, the upstream signaling that leads to PLC activation varies greatly among species. Mammalian eggs, for example, appear to receive a specialized isoform of PLC (PLC-zeta) via the sperm (Lee et al., 2006; Parrington et al., 2007) whereas other eggs appear to rely on kinase- or G-protein couple receptor pathways to initiate PLC activity (Jaffe et al., 2001; Townley et al., 2009). Following release into the cytoplasm, the calcium is then re-sequestered into the endoplasmic reticulum by an ATP-dependent calcium pump, similar to the Sarco/endoplasmic reticulum Ca²⁺-ATPase pump (SERCA) pump of skeletal muscle cells.

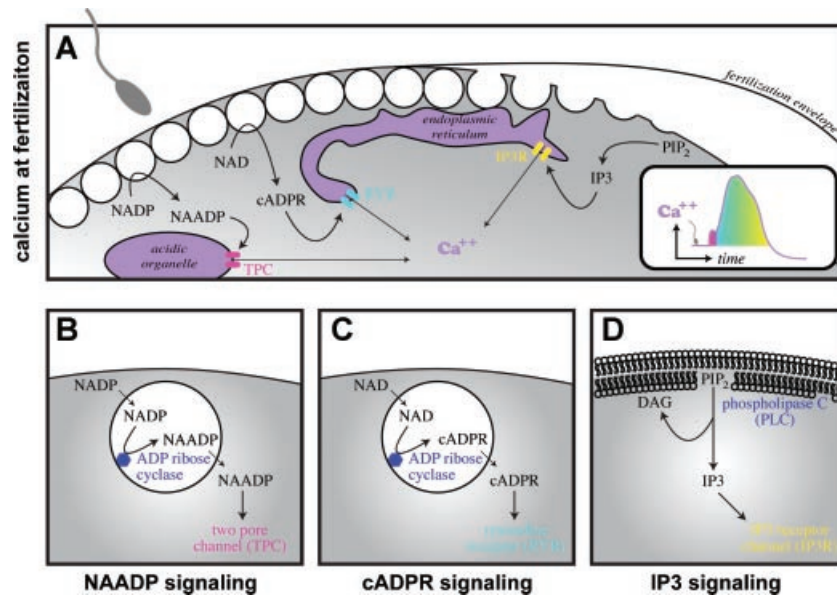


Figure 1. Calcium signaling pathways used by the sea urchin at fertilization. (A) Overview of the three major mechanisms of calcium release from intracellular stores (endoplasmic reticulum and acidic organelles). Each secondary messenger is shown in the approximate chronological order of function after sperm fusion (left) to fertilization envelope formation (right), consistent with the calcium wave (inset). The source of each messenger is detailed below, including NAADP (B), cyclic ADP-ribose (C), and inositol 1,4,5-trisphosphate (D).

A conserved calcium-sensitive protein of secretory vesicles is synaptotagmin (Chapman, 2006; Südhof and Rothman, 2009). This protein is associated with neuronal synaptic vesicles and egg CGs by a transmembrane domain, and appears to change conformation upon binding calcium. In ways that are still not understood, calcium-bound synaptotagmin enables the zippering of vesicle- and target membrane-SNARE proteins (*v*- and *t*-SNAREs, respectively) within a complex to facilitate membrane fusion (Südhof and Rothman, 2009). This catalyzed event is essential to overcome the energy barrier of two negatively charged, opposing membranes coming into close proximity and eventually merging. Following complete membrane fusion, the content of the CGs is released into the vitelline layer scaffold where the proteins can intermingle and assemble to form the FE.

Surprisingly, the CG membrane and the plasma membrane of quiescent sea urchin eggs are hemifused, e.g., the cytoplasmic membrane leaflets are fused (continuous) to one another while the complementary leaflets of each bilayer remain separate (Wong et al., 2007). This hemifused membrane state is stable while the eggs are stored in the animal, often for weeks or longer, and may reflect a normal intermediate of secretory vesicle fusion. The intermediate state of these secretory vesicles may be selected for due to their increased stability and minimal delay to exocytosis, two properties also selected for in hemifused, fast-release populations of synaptic vesicles in neurons (Südhof and Rothman, 2009).

pH ACTIVATION OF ENZYMATIC ACTIVITIES

The pH of the CG lumen is relatively acidic, approximately pH 4.5, as a result of a proton pump in the CG membrane (Decker and Kinsey, 1983; Saroussi and Nelson, 2009). Yet the optimal pH for activity of the enzymes stored in the CG is a slightly alkaline pH 8 (Deits and Shapiro, 1985; Haley and Wessel, 1999). Thus, the enzymes appear to be stored in an inactive form. Just prior to complete membrane fusion, however, the lumen of these CGs is neutralized as protons are pumped from the granules into the cell cortex, and then quickly moved across the plasma membrane to the surrounding seawater (Paul et al., 1976; Smith et al., 2002; Morgan and Galione, 2007). This local proton flux accomplishes two feats: it temporarily reverses the voltage difference across the plasma membrane, contributing to the fast electrical block to polyspermy (Jaffe, 1976), and it primes content enzymes in the CGs for activation. The timing of luminal alkalination is critical for the rapid assembly of the FE since a delay in activities could, for example, slow the activities necessary for the block to polyspermy.

Three enzymes are affected by the pH shift that occurs at the cortex at fertilization. A serine protease, CGSP1 (see Table 1), and ovoperoxidase are stored as inactive enzymes in the CG (Deits and Shapiro, 1985; Somers et al., 1989; LaFleur et al., 1998; Haley and Wessel, 1999), thereby blocking precocious activity on other content proteins. With a shift in pH comes isomerization of the enzyme, resulting in autoactivation of the protease (Haley and Wessel, 1999) and

ovoperoxidase (Deits and Shapiro, 1985, 1986); both catalytic activities peak in the range of normal seawater (7.4–8.0). Conversely, the structural proteins that form the FE do not appear to undergo a pH-dependent change. This is deduced from studies in which eggs can be activated in media at pH 5, and the protein interactions necessary for FE formation function quite normally (Haley and Wessel, 2004a). The third enzyme affected by the pH shift is transglutaminase, which is briefly activated by the local acidification outside the plasma membrane (Wong and Wessel, 2009). This short burst of cross-linking activity may regulate other enzymes required for egg activation, and may promote the FEs assembly.

PROTEASE ACTIVITY AT FERTILIZATION

The protease of the CGs is a trypsin-like serine protease, CGSP1. Hagstrom first identified its activity in the 1950s using protease inhibitors, observing an aberrant phenotype in the activated sea urchin egg (Hagstrom, 1956). The most obvious phenotype of a protease-null egg is abnormal FE formation—although the FE still forms with a normal molecular constituency, it remains attached to the cell surface at numerous sites (Haley and Wessel, 2004a). Thus, the FE does not completely separate from the egg and as a consequence, numerous sperm are able to fuse with activated eggs of this phenotype, leading to death of the nascent embryo. In these cases, sperm appear able, at least initially, to penetrate the FE even with its otherwise normal molecular phenotype.

Surprisingly, CGSP1 has only a few substrates. Indeed, the only proteins of the CG contents cleaved by the protease are other enzymes, ovoperoxidase (Haley and Wessel, 2004a) and β -1,3 glucanase (Epel et al., 1969). Proteolysis of ovoperoxidase by CGSP1 causes a decrease in ovoperoxidase activity of approximately 15%, and an increase in β -1,3 glucanase activity of approximately the same (Haley and Wessel, 2004a). It is not clear if these are significant enough changes in activity to impact early events in embryogenesis—the molecules cleaved by β -1,3 glucanase are not even known. In the presence of CGSP1 activity, ovoperoxidase appears to be more selectively localized to the FE; the specific sites or targets for cross-linking may be different when it is first cleaved by the protease. CGSP1 also appears essential to cleave proteins off the cell surface of the egg. These proteolytic targets appear to include the surface receptors for sperm, and a protein that links the egg cell surface to the vitelline layer (Carroll and Epel, 1975). This linker protein, called p160 to reflect its mobility by SDS-PAGE, contains several CUB domains at its amino-terminus, and a transmembrane domain inserted in the plasma membrane (Haley and Wessel, 2004a). The CUB domains are homophilic protein interaction domains (Bork and Beckmann, 1993; Romero et al., 1997) that, in the case of p160, may interact with the vitelline layer isoform of the protein Rendezvin (Wong and Wessel, 2006b). Cleavage of p160 by the protease enables the nascent vitelline layer and forming FE to lift off the cell surface (Haley and Wessel, 2004b) via the hydration force of the highly

charged glycosaminoglycans also released from the CGs (Runnstrom and Immers, 1956; Schuel et al., 1974). Without p160 cleavage, attachments remain between the plasma membrane and the forming FE, perhaps facilitating additional sperm binding to their receptors with subsequent fusion with the egg. A higher frequency of small pieces of the plasma membrane snap off and remain with the FE in the absence of protease activity as well. Thus, the force of hydration that normally separates the FE from the cell surface exceeds the membrane tension of the egg.

SELF-CONSTRUCTION AND PROTEIN INTERACTIONS

Six proteins from five transcripts make up the major mass of the FE. Each of the proteins is derived from the CGs, and once released, autonomously interact to form the geometrically regular array of units that comprise the envelope (Inoue and Hardy, 1971). The structural proteins are each relatively large, from 60 to over 210 kDa, and each protein contains tandemly repeated domains involved primarily in protein interactions (Table 1 and references therein). CUB domains and low-density lipoprotein type A repeats (LDLrA), both known to mediate protein interactions (Russell et al., 1984; Esser et al., 1988; Bork and Beckmann, 1993), are most prevalent in these structural proteins (Table 1). The tandem array of these relatively low-affinity binding motifs significantly enhances the net affinity of each protein for its partner, allowing the assembly of these proteins rapidly, specifically, and with resistance to extreme shifts in pH, salinity, and even chaotropic agents (Shapiro et al., 1989; Wong and Wessel, 2006b).

Each of the structural proteins associates into a unique proteome, interacting with a specific partner protein within the complex. Based on binary protein interaction data from *Strongylocentrotus purpuratus*, it is known that proteins containing LDLrA and CUB domains do not have a preference for one domain over another (Wong and Wessel, 2006b). That said, however, the specific regions that are bound by either LDLrA or CUB are not defined. Currently, the proteome of other echinoderms is being analyzed to assess how specific the assembly process is. We anticipate that, were one component of the complex to change significantly, other compensatory changes respond to accommodate formation of the unit.

Preliminary sequence analysis of orthologs from *S. purpuratus* and *Lytechinus variegatus* suggests that these adjustments may be occurring as domains are extended or lost (Wong and Wessel, 2004). We hypothesize that significant changes have occurred in the genes that encode the FE proteins between species. This stems from the observation that CGs and FE morphologies of different echinoderm species have dramatically different ultrastructural organization (Anderson, 1968, 1974; Wong and Wessel, 2004). While these differences may result from different ratios of contents, or differences in the glycosaminoglycans (Bal, 1970), since the major proteins of the FE are also contents of the CGs, we anticipate that the morphological differences might be explained at least in part by the evolution of the

structural proteins in each respective species. Continued analysis of where the divergence occurs in these structural orthologs will be informative to understand how specific regions are evolving, if other interaction domains have integrated into the genes of these structural proteins, and what selective forces may be acting on their divergence. With the limited information available so far from diverse species such as frogs, mammals, and mollusks, it is clear that the egg ECM is rapidly diverging. Although certain domains may be shared, including ZP, CUB, and LDLrA domains, the proteins and their sequences are otherwise substantially different among species (Shapiro et al., 1989; Swanson and Vacquier, 2002; Wong and Wessel, 2006a).

STABILIZING THE ECM BY ENZYMATIC CROSS-LINKING

Free spawning animals are prevalent in nature, and their early progeny (e.g., zygotes and early embryos) have limited maternal protection against the environment. Thus, selective pressures must be strong for an ECM that is rapidly stabilized to protect the fertilized egg and developing embryo. While the protein interactions that make up the ECM are robust and stable, covalent cross-linking of these interacting proteins is commonly found. For example, the fly and fish have a rugged chorion that is cross-linked by peroxidase-mediated methods (e.g., Margaritis, 1985; Fakhouri et al., 2006), the frog vitelline layer is reinforced by the action of a zinc metalloproteinase (Lindsay and Hedrick, 2004), and by analogy it is thought that the mammalian zona pellucida also undergoes similar protease-dependent changes that enhance its mechanical and biochemical properties following fertilization (Moller and Wassarman, 1989; Shapiro et al., 1989; Wong and Wessel, 2006a). Reinforcing the ECM, however, comes with a cost. Substrates or by-products of some enzymatic reactions can be toxic to the embryo, thus the benefits received by robust stabilization to environmental challenges are likely maintained through tight regulation of the modifying activity. In addition, hatching from this protective barrier must factor into the equation: once the embryo has reached the feeding stage, it must be able to free itself from the confines of this modified ECM without compromising its further development.

The sea urchin egg has two distinct and substantial cross-linking mechanisms, and each is shared with other cell types (Fig. 2). The more ancient and ubiquitous mechanism involves transglutaminase-dependent cross-linking of neighboring glutamines and lysines. This is a simpler system, utilizing a single enzyme present in nearly all organisms including algae, fungi, and metazoans (Lorand and Graham, 2003). The other mechanism present in eggs is a peroxidase-mediated cross-linking. This second process likely arose more recently since the constituent activities are more restricted in phylogeny. A defined cascade of supporting biochemical and enzymatic reactions is necessary for peroxidase activity to occur (e.g., Klebanoff et al., 1979; Shapiro, 1991). These include a peroxidase cross-linking mechanism, and a hydrogen peroxide generating system.

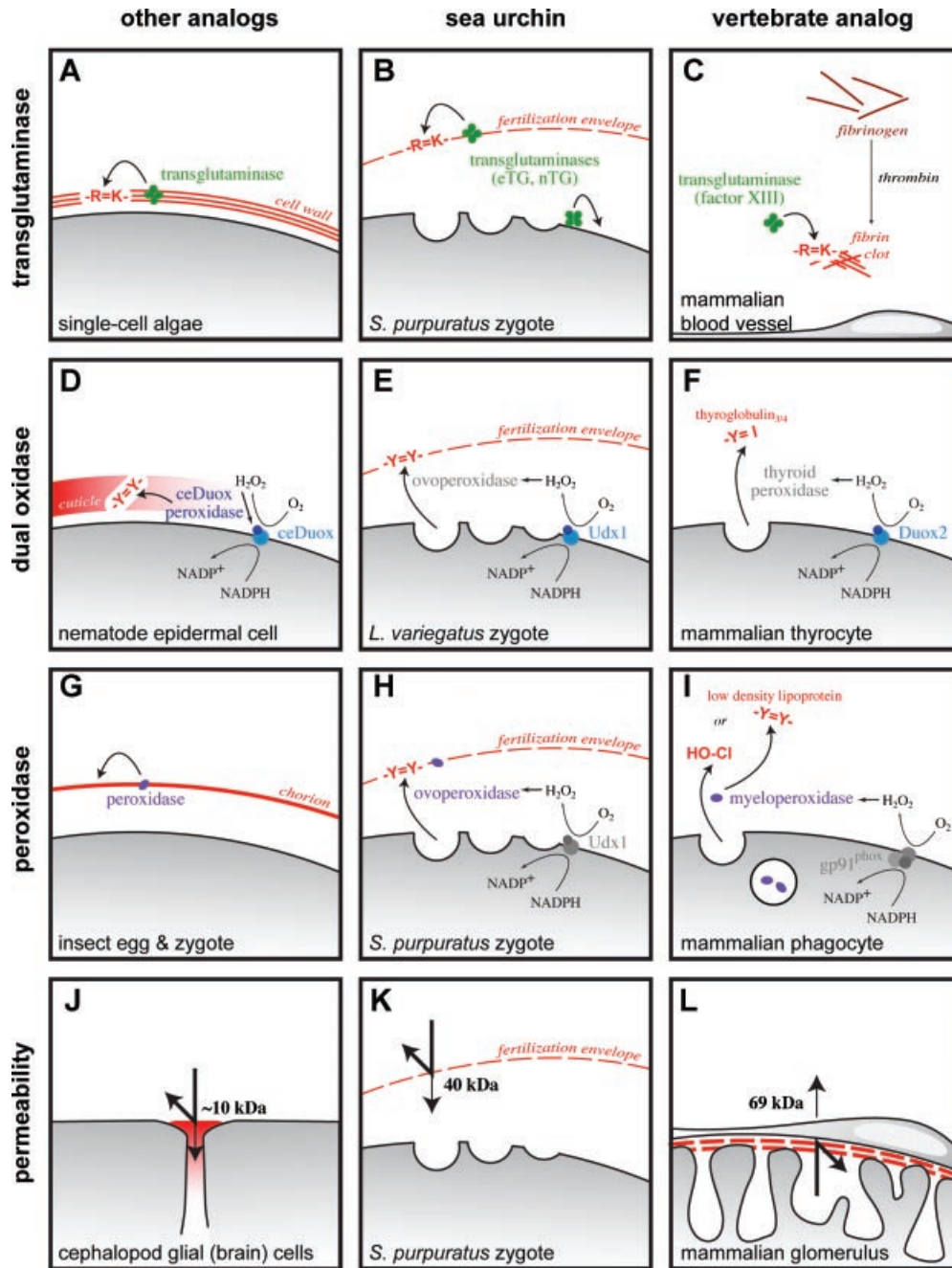


Figure 2. Major interprotein crosslinking events in the sea urchin fertilization envelope, and analogous activities in other eukaryotes. Central column shows the three major protein crosslinking enzymes active in the sea urchin zygote (B, transglutaminase; E, dual oxidase/NADPH oxidase; H, peroxidase), and the permeability barrier established as a consequence of their activity (K). Similar enzyme activities are used by various eukaryotes to modify their respective extracellular matrices, including algae (A), invertebrates (D, G, J) (left column) and vertebrates (C, F, I, L) (right column). For more details, see the following references: (A) Waffenschmidt et al. (1999); (B) Wong and Wessel (2009); (C) Esposito and Caputo (2005); (D) Edens et al. (2001); (E) Wong et al. (2004); (F) Leseney et al., 1999; Dupuy et al. (1999) (G) Li et al. (1996); (H) Foerder and Shapiro (1977); Deits et al. (1984); (I) Heinecker et al., (1993), Bhattacharjee et al. (2001); (J) Abbott and Bundgaard (1992); (K) Wong and Wessel (2008); (L) Chang et al. (1975), Deckert et al. (1993), Patari-Sampo et al. (2006).

Further, because of the damaging features of hydrogen peroxide and its ability to dismutate into free radicals, the enzyme responsible for hydrogen peroxide production would require stringent regulation. Finally, additional meth-

ods to scavenge free radicals in cells likely would be present for this process (e.g., Turner et al., 1986, 1988; Shapiro and Hopkins, 1991). So, at least in comparison to the unimolecular dependence of transamidation, the peroxidase

-dependent mechanism of egg ECM cross-linking is likely a more derived character in the process.

TRANSGLUTAMINASE CROSS-LINKING OF FE PROTEINS

Transglutaminases are found throughout phylogeny and cross-link proteins via an epsilon(gamma-glutamyl)lysine bond. These covalent bonds are generally irreversible, and are regulated by substrate availability, nucleotides, and/or calcium depending on the transglutaminase isoform (Lorand and Graham, 2003). The sea urchin contains two transglutaminases, and the extracellular isoform (eTG) is the main one responsible for cross-linking proteins of the FE (Wong and Wessel, 2009). Activation of the enzyme appears to come from a rapid and transient pH shift—a result of the acid released from the cell cortex to the surface of the egg (Johnson and Epel, 1976; Smith et al., 2002). When this acidification is mimicked *in vitro*, a substantial increase in transamidation that closely matches the *in vivo* pattern is observed (Wong and Wessel, 2009). This shift is unlike the activation steps observed for other, classic transglutaminases such as mammalian factor XIII, activated by proteolysis and involved in blood clotting, or transglutaminase type I, activated by calcium and utilized by epidermal keratinocytes to establish keratin polymers (Lorand and Graham, 2003; Candi et al., 2005; Esposito and Caputo, 2005). Perhaps the pH shock alters eTG conformation to expose the enzyme site. Alternatively, association of the transglutaminase with other proteins may be altered by the pH shift, allowing temporary accessibility to its preferred substrates. This activation step of a cell surface enzyme adds to the common theme that existing proteins in the sea urchin egg are differentially activated by transient exposure to calcium or pH. This role of pH influence of transglutaminase activity might further be examined by altering the proton-release pathway in these eggs (Johnson and Epel, 1976; Morgan and Galione, 2007).

HYDROGEN PEROXIDE SYNTHESIS AND HYDROGEN PEROXIDE-MEDIATED CROSS-LINKING

The other major cross-linking mechanism of the sea urchin FE is dependent on hydrogen peroxide. This cross-linking mechanism requires synthesis of hydrogen peroxide by the egg, enzymatic utility of the hydrogen peroxide for cross-linking proteins, a tethering mechanism for the peroxidase, and likely a scavenging system for extraneous hydrogen peroxide and its free-radical byproducts. As with transamidation, the formation of these covalent bonds between tyrosine side chains is a common cross-linking mechanism used in eggs of other species, as well as in somatic cells (Fig. 2). The mammalian thyroid gland, for example, iodinate the precursor hormone thyroglobulin (Igo et al., 1964; Ekholm, 1990; Dunn and Dunn, 2001) using a process analogous to the one used by the sea urchin egg to cross-link the FE (Fig. 2) (Dupuy et al., 1991, 1999). Because of the potential free-radical damage elicited by hydrogen perox-

ides, however, this mechanism has coevolved with additional regulatory factors to minimize cellular damage.

In the sea urchin egg, hydrogen peroxide is synthesized by the urchin dual oxidase 1 (Udx1) (Wong et al., 2004). Udx1 is a member of the family of dual oxidases, its name arising from its two resident domains: a peroxidase domain and a NAD(P)H oxidase domain (a ferric reductase) (Edens et al., 2001; Lambeth et al., 2007; Sumimoto, 2008). The NAD(P)H oxidase domain takes electrons from the cofactor NADPH and oxidizes water to generate hydrogen peroxide, H₂O₂. This reaction is so robust that the original observation of its activity was mistakenly termed the respiratory burst due to the number of oxygen molecules consumed per second (Warburg, 1908). The initial hypothesis was that oxygen uptake was invested in oxidative phosphorylation and ATP generation in the mitochondria. In sea urchins, nearly 100 nanomoles of hydrogen peroxide is made by Udx1 within the 10-min window of its activity (Wong et al., 2004). The peroxidase domain of the dual oxidase is related to catalase, a class of enzymes generally responsible for the detoxification of hydrogen peroxide into free water and molecular oxygen—a reaction clearly distinct from the dityrosine cross-linking activity occurring through ovoperoxidase in the FE (Wong et al., 2004). Currently, we can only posit that the role of the peroxidase domain in Udx1 is to scavenge excess hydrogen peroxide before they damage lipids or proteins within the cell. This combined activity may thereby create a net diffusion of hydrogen peroxide away from the cell, specifically for use in the FE or against environmental agents. Since the rate of oxygen consumption likely exceeds the reaction rate of the Udx1 peroxidase, the egg also accumulates excess molecular reducing agents such as ovothioliol (Turner et al., 1986, 1988; Shapiro et al., 1989; Shapiro and Hopkins, 1991) and glutathione to further minimize the effects of the hydrogen peroxide production within the cell. A similar bimolecular system is used in the thyroid, where a dual oxidase generates hydrogen peroxide, to be used by an independent thyroid peroxidase to conjugate iodine to thyroglobulin; the role of the dual oxidase's peroxidase domain is not clear (Lambeth et al., 2007).

Udx1 is regulated by calcium and protein kinase C (Heinecke and Shapiro, 1992; Wong et al., 2004). It is turned on only after egg activation has occurred, when CG exocytosis is nearly complete. At that stage, ovoperoxidase and the proteins requiring cross-linking for stabilization are localized far away from the plasma membrane, within the assembled, but immature FE. The delay in major Udx1 activity could be a fortuitous consequence of slow PKC activation, of transamidation (Wong and Wessel, 2009), of a delay in CGSP1 activity (Haley and Wessel, 1999), or an isomerization due to the pH sensitivity of the enzyme. Turning off Udx1 activity appears to occur within several minutes, and may be controlled by the re-sequestration of calcium into the endoplasmic reticulum or by more permanent mechanisms including endocytotic internalization (Whalley et al., 1995; Wong and Wessel, 2004) or enzymatic alteration.

The hydrogen peroxide produced at fertilization is utilized primarily by FE-localized ovoperoxidase to cross-link proteins selectively with this ECM (Somers et al., 1989). Only

select proteins are cross-linked in this structure (Wong and Wessel, 2008), perhaps due to steric interference or accessibility of substrates to the active enzyme. One limiting factor is the association of ovoperoxidase with proteoliasin, a structural protein that tethers the enzyme to the FE (Weidman et al., 1985, 1987; Somers et al., 1989). As hydrogen peroxide is the limiting substrate, ovoperoxidase activity also subsides about 10 min after fertilization (Deits and Shapiro 1985), although it has been shown that a subpopulation of this enzyme is still active well into embryogenesis if provided with exogenous hydrogen peroxide and a substrate (Klebanoff et al., 1979).

EPILOGUE

The mature FE is thus a composite of CG-derived proteins adsorbed and covalently attached to the vitelline layer. The biochemical interactions are stabilized by protein interaction domains organized in tandem arrays and by two distinct interprotein cross-linking events. It is little wonder that this structure is resistant to sperm, chemical treatment, and mechanical shearing (Wong et al., 2004). Yet the embryo is still capable of hatching out of it, just as the fish and fly embryo emerge from their chorions and the mammal from its zona pellucida. Among the developmental signals later in embryogenesis that signal for new gene expression is the synthesis and secretion of a hatching enzyme (Lepage et al., 1992; Reynolds et al., 1992). The embryo is then capable of feeding, growth, and free movement.

Were Ernest Everett Just still in the laboratory today, we would know better the impact of his own research, for as we transit through different technical periods of scientific advancement, his original light microscopic analyses would have yielded to electron microscopic images, to biochemical analyses, and finally to a molecular perspective that alludes to his original observation of cell surface changes in the egg at fertilization. One can only imagine what his next research horizon may be in today's world. We would anticipate that it would include the best of experimental conditions, using the best organism, for answering the most important questions of the time.

GLOSSARY

Bindin: The protein on sea urchin sperm necessary and sufficient for binding to eggs in a species-specific fashion.

Cortical granules (CGs): Secretory vesicles of eggs containing contents that modify the egg's extracellular matrix.

Cortical granule serine protease (CGSP1): Originally reported by Hagstrom in 1956, it is the major protease of sea urchin egg cortical granules and after fertilization is responsible both for removing sperm receptors on the surface of the egg and for cleaving the linkages between the FE and the egg cell surface.

CUB: An acronym for the first three proteins found with this protein domain: complement factor C1r, and C1s, in urchin

EGF (uEGF), and BMP1. It is known to be a homophilic interaction domain rich in β -sheets, but dimers and multimers also bind carbohydrates.

Cyclic adenosine dinucleotide phosphoribose (cADPr): A small molecule formed at fertilization that stimulates calcium release from the endoplasmic reticulum.

Egg bindin receptor (EBR): A candidate sperm-binding molecule found in the vitelline layer of echinoderm eggs.

Extracellular matrix (ECM): All eggs have a matrix surrounding them prior to and following fertilization. The ECM is modified following fertilization, and this change restricts subsequent sperm from access to the egg.

Fertilization envelope (FE): The extracellular structure that forms in echinoderms following fertilization. Also described as an early embryonic extracellular matrix.

Inositol trisphosphate (IP3): Inositol trisphosphate, formed from cleavage of PIP2 by PLC and functional in calcium release from the endoplasmic reticulum.

Low-density lipoprotein receptor (LDLr) domain: A protein interaction domain first found in the receptor for low-density lipoprotein particles. It is a prevalent domain in the proteins of the fertilization envelope in echinoderms.

Nicotinic acid adenine dinucleotide phosphate (NAADP): A potent ligand for calcium release in eggs at fertilization, and in other cells throughout the body. It is formed at fertilization and stimulates entry of calcium into the egg cytoplasm.

SERCA: Sarco/endoplasmic reticulum Ca^{2+} -ATPase pump that returns calcium into the endoplasmic reticulum and thereby returns calcium to its resting levels of 1–2 nM.

Soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE): This family of diverse gene products is involved in the regulation of stimulus-dependent secretion, for example, the cortical granules at fertilization.

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