Chapter 14

Focused Proteomics on Egg Membrane Microdomains to Elucidate the Cellular and Molecular Mechanisms of Fertilization in the African Clawed Frog *Xenopus laevis*

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Abstract Involvement of protein tyrosine kinase-dependent signal transduction (PTK signaling) in fertilization was initially demonstrated by studies using sea invertebrates: namely, an increase of tyrosine phosphorylation in egg or embryo proteins is shown to occur within minutes after gamete interaction. Among vertebrate species so far studied are fish, frog, and some mammalian species in which the importance of PTK signaling for fertilization or activation of development has been shown. In this review chapter, we summarize our experimental data that explore the role played by the tyrosine kinase Src in fertilization of the African clawed frog *Xenopus laevis*. In addition, we introduce our recent approaches that focus on the structure and function of egg membrane microdomains (MDs), where the Src PTK signaling machinery is organized. Finally, we propose a hypothesis that gamete membrane interaction at fertilization is accompanied by mutual signaling cross-talk between egg and sperm using the egg MDs as scaffolds and discuss the versatility of our hypothesis in general understanding of the sexual reproduction mechanism.

Keywords Cross-talk between gametes • In vitro reconstitution • Membrane microdomains • Signal transduction • Tyrosine phosphorylation

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14.1 Src PTK Signaling and Fertilization

Protein tyrosine phosphorylation was initially discovered as an intracellular phenomenon to be associated with malignancy of cancer cells (Hunter 2009). The discovery was made in the process of research on the molecular function of a gene product of Rous sarcoma virus, that is, v-Src, the memorably first example of so-called cancer gene or oncogene. Such a breakthrough finding, in conjunction with another epoch-making discovery of the first example of cellular cancer gene or proto-oncogene, c-Src (hereafter Src), has led many researchers to study the physiological and pathological importance of protein tyrosine phosphorylation catalyzed by Src and other protein tyrosine kinases (PTKs) (e.g., Abelson kinase, epidermal growth factor receptor/kinase, insulin receptor/kinase) (Abram and Courtneidge 2000; Hunter and Cooper 1985; Jove and Hanafusa 1987; Thomas and Brugge 1997). Now, it is well established that a variety of cellular functions involve this kind of posttranslational modification of proteins. Under this background, fertilization is one of the earliest and pioneering as well as contemporary subjects in the PTK research field (Kinsey 2013; Sato et al. 2004; Sato 2008).

Dasgupta and Garbers (1983) published the first report on protein tyrosine phosphorylation in the fertilization study: they demonstrated that PTK activity toward synthetic peptide substrates is present in unfertilized sea urchin eggs and continues to increase during early embryogenesis. Given that early embryos of sea urchin, similar to rapidly proliferating cancer cells, undergo several cycles of very fast cell division, that is, cleavage, it seems to be natural that they employ high PTK activity. Further detailed studies by Kinsey's group and some other researchers (including us) using sea urchins and other animal species, however, highlighted facts of specific importance in this cellular system, that PTK activity is rapidly and transiently activated in response to fertilization, or more precisely, gamete interaction (Abassi and Foltz 1994; Ciapa and Epel 1991; Kamel et al. 1986; Ribot et al. 1984; Sato et al. 1996; Wu and Kinsey 2000), and that the activated PTK may be responsible for sperm-induced increase(s) in calcium concentrations within the fertilized eggs, whose occurrence is believed to be indispensable for the subsequent initiation of embryonic development, in other words, "egg activation" (Giusti et al. 1999; Kinsey and Shen 2000; Runft and Jaffe 2000; Sato et al. 2000).

As described, compelling evidence suggests that egg-associated PTK signaling serves an important role in sperm-induced egg activation of nonmammalian species. As well appreciated in somatic cell systems, PTK signaling usually requires the binding of extracellular ligands to their cell-surface receptors. Therefore, it has been thought that gamete interaction at fertilization may act as a ligand-like signal to stimulate an egg-surface receptor so that the intracellular PTK signaling is triggered. On the other hand, eggs of mammalian species (e.g., mouse) and some other nonmammalian species (e.g., bird, newt) seem to employ sperm-derived factor(s), which would be incorporated into the egg cytoplasm, for the sperm-induced calcium responses: molecules identified so far include phospholipase C ζ (Swann and Lai 2013) and citrate synthase (Iwao 2012). This observation may reflect that gamete membrane interaction-mediated and gamete membrane fusion-mediated egg activation systems employ their specific molecular machinery and, perhaps more importantly, some other

differences of fundamental importance that are found between these species: aquatic or terrestrial life, and external or internal fertilization.

In this review article, we explain why we analyze egg membrane microdomains (MDs) and summarize briefly our achievements, with a special focus on the finding of the egg MDs as structural and functional platform for sperm-induced Src PTK signaling. Then, we introduce our current research projects, evaluating the hypothesis that Src and the other MD-associated molecules constitute a signaling network for successful gamete interaction and activation of development. About 10 years ago, we published one review article in the journal *Proteomics* on our focused proteomics project on *Xenopus* egg MDs (Sato et al. 2002a). Therefore, a part of this review article can be considered as updated information for the MDs projects.

14.2 Characterization of Src as a Mediator of Gamete Interaction and Egg Activation

It was 1996 when we published the first paper on the *Xenopus* egg Src (hereafter xSrc) and its possible involvement in fertilization signaling (Sato et al. 1996). In that paper, we showed data about chromatographic fractionation of membrane-associated proteins and in vitro protein kinase assay, by which we could detect an elevation of the activity of xSrc in response to fertilization. Our further studies demonstrated that pharmacological (e.g., use of inhibitors; Sato et al. 1999, 2000, 2001) or molecular biological inhibition of xSrc (i.e., expression of kinase-negative mutant of xSrc; manuscript in preparation) impairs the ability of *Xenopus* eggs to undergo calcium reactions and egg activation in response to sperm (Sato et al. 1999) and that *Xenopus* eggs can be activated in a Src-dependent manner by artificial egg activators that interacts with the egg surface (i.e., RGD peptide and cathepsin B; Sato et al. 1999; Mahbub Hasan et al. 2005) and by hydrogen peroxide that may directly activate xSrc (Sato et al. 2001). These results suggest that xSrc acts between gamete interaction/fusion and an increase in intracellular calcium concentration at fertilization (Fig. 14.1). In support of this, phospholipase C γ , whose activation leads to the hydrolysis of phosphatidylinositol-4,5-bisphosphates and production of the intracellular second messengers diacylglycerol and inositol-1,4,5-trisphosphate (direct activator for intracellular calcium release from endoplasmic reticulum), was shown to be a substrate of the activated xSrc (Sato et al. 2000, 2001, 2003).

Other targets of the activated xSrc include Shc (Aoto et al. 1999), hnRNP K (Iwasaki et al. 2008), lipovitellin 2 (Kushima et al. 2011), and pp40, whose identity is not yet demonstrated (manuscript in preparation) (Fig. 14.1). All these proteins and PLC γ are, however, cytoplasmic proteins resembling xSrc, so that they could not be directly involved in the gamete interaction and subsequent Src activation. Given that xSrc seems to be involved in the upstream signaling for egg activation and perhaps other cellular functions for embryonic development (e.g., translational control of maternal mRNAs), our goal has shifted to understand the mechanisms of gamete interaction and subsequent xSrc activation. Under these circumstances, we became interested in analyzing the egg plasma membrane, or more specifically,

Fig. 14.1 Molecular mechanisms of fertilization and activation of development in *Xenopus laevis*. Shown are photographs for a *X. laevis* egg before (left) and after (right) fertilization. Note that cortical contraction in the pigmented area of the egg (animal hemisphere) is evident after fertilization. Successful gamete membrane interaction and fusion leads to a transient increase in intracellular Ca^{2+} concentration in the egg, by which a series of events, collectively called “egg activation,” takes place. The egg activation events include meiotic resumption, block to polyspermy, nuclear fusion, and initiation of embryonic development. Studies from our research group and others have demonstrated that tyrosine kinase-dependent signal transduction serves a pivotal role in connecting gamete membrane interaction and Ca^{2+} -dependent egg activation

membrane microdomains and their associated molecules. In the following sections, we introduce membrane microdomains and describe our achievements were obtained by the study on this subject.

14.3 Focused Proteomics on *Xenopus* Egg MDs: Achievements and Problems

14.3.1 Rationale to Study MDs for Exploring the Mechanism of Fertilization

A growing body of knowledge indicates that cellular plasma membranes consist of mixtures of heterogeneously organized substructures, whose specific identities depend on the composition of lipids, proteins, and their associated carbohydrates (Brown and London 1998; Simons and Ikonen 1997; Simons and Sampaio 2011). These membrane substructures are also called as membrane subdomains or microdomains: we prefer to use the term “microdomains” (MDs) because it would reflect the real scale of these membrane substructures (i.e., diameters of submicrometers or a few micrometers) (Pike 2006).

Experimentally, conventional biochemical fractionation methods are used to isolate MDs; hallmarks for obtaining MDs are such criteria as insolubility under certain cell extraction methods (e.g., resistance to detergent extraction) and tendency to float under ultracentrifugation (i.e., low density). The resulting low-density and detergent-insoluble membranes (LD-DIMs) are often regarded as lipid/membrane “rafts” and are enriched in cholesterol and some specific subsets of components (e.g., sphingolipids, gangliosides, signaling proteins). Several lines of evidence indicate that MDs constitute a platform/scaffold for capturing/sensing extracellular signals (e.g., growth factors, environmental stimuli), as well as for transmitting the signals into the cytoplasm and nucleus, by which a variety of cellular functions are exerted (Simons and Ikonen 1997; Simons and Toomre 2000).

In the fertilization research field, Kitajima and colleagues demonstrated first the presence of the sea urchin sperm MDs and their possible involvement in fertilization (Ohta et al. 1999). Publication of this leading report and our data showing that *Xenopus* egg fertilization involves Src PTK signaling (see foregoing) led us to examine the presence and physiological importance of MDs in *Xenopus* eggs. For a more detailed introduction to MDs, please also refer to our recent publication (Mahbub Hasan et al. 2011).

14.3.2 *Xenopus* Egg MDs Projects: Achievements and Problems

14.3.2.1 Discovery of Egg MDs as an Important Resource for Fertilization Study

In 2002, we published the first report on *Xenopus* egg MDs (Sato et al. 2002b), in which we demonstrated the following: (1) extraction of unfertilized eggs in the presence of Triton X-100 and subsequent ultracentrifugation under stepwise gradients of sucrose concentration yield LD-DIMs fractions (see above) that are enriched in cholesterol, the GM1 ganglioside, and most importantly for us, xSrc; (2) the LD-DIMs fractions that are prepared from fertilized eggs contain at least three tyrosine-phosphorylated proteins, two of which are xSrc and PLC γ ; (3) a similar and more augmented pattern of protein tyrosine phosphorylation is seen in the LD-DIMs that are prepared from hydrogen peroxide-activated eggs; and (4) in the LD-DIMs of eggs that are activated by the calcium ionophore A23187, no increase in protein tyrosine phosphorylation is observed. These results suggest that the LD-DIMs fractions contain MDs, in which sperm-induced Src PTK signaling is operating (Fig. 14.1).

The functional importance of MDs in fertilization is suggested by the studies using methyl- β -cyclodextrin (M β CD), a drug that causes disruption of the cholesterol-dependent membrane structures. We found that unfertilized *Xenopus* eggs that are preincubated with this substance fail to undergo tyrosine phosphorylation of the LD-DIMs-associated proteins, intracellular calcium release, and other egg activation events (Sato et al. 2002b). Such inhibitory effect of M β CD could be canceled by the addition of excess amounts of cholesterol; therefore, it was not simply caused by

Fig. 14.2 *Xenopus* egg microdomains (MDs) project. (a) Our ongoing snapshot-type MDs project includes conventional, differential proteome analysis (b shows example images), analysis of post-translational modifications such as phosphorylation, molecular targeting analysis, and preparation and characterization of monoclonal antibody library (c shows example images). These experiments involve the use of MDs that are isolated from fully grown, immature oocytes at the germinal vesicle stage (GV oocytes), mature and unfertilized oocytes/eggs at the second meiotic metaphase (MII oocytes), and fertilized embryos, all of which are called iEMD (isolated egg MDs). (b) Proteome patterns for MDs-associated proteins in GV oocytes (*left*) and MII oocytes (*right*), both of which are separated by two-dimensional gel electrophoresis (first dimension, isoelectric focusing at the pI range of 4.0–6.6; second dimension, SDS-polyacrylamide gel electrophoresis) and visualized by silver staining. (c) In panels shown in a, indirect immunofluorescent experiments are performed to verify the binding of monoclonal antibodies to the surface of unfertilized *Xenopus* eggs. In b, the binding of the antibody to egg proteins is examined by immunoblotting. (d) Our ongoing MDs project also includes in vitro reconstitution of signal transduction events associated with fertilization. In this project, iEMD are subjected to in vitro treatment with sperm, cathepsin B, Src, or some others, and analyzed for their biochemical (e.g., tyrosine phosphorylation of MDs-associated proteins) as well as cell biological responses (e.g., ability of the sperm to fertilize eggs)

toxicity of the substance. Moreover, we found that the addition of sperm to the LD-DIMs, which were isolated from unfertilized eggs, caused an increase in tyrosine phosphorylation of proteins that are present in the LD-DIMs. These results argue the idea that LD-DIMs contain MDs, in which both the receptor for sperm and the signaling machinery for sperm-induced Src activation are pre-organized.

The aforementioned data have led us to consider two major directions of the MDs project (Sato et al. 2002a) (Fig. 14.2). The first subproject is to identify novel fertilization-related components by characterization of MDs-associated molecules. A major achievement in this subproject is the identification of a type I transmembrane protein uroplakin III (UPIII) that is thought to be involved in gamete interaction,

which may involve the action of sperm-derived protease, and regulation of xSrc activity, in that UPIII may be involved in the negative regulation of xSrc in unfertilized eggs (Mahbub Hasan et al. 2005, 2007; Mammadova et al. 2009; Sakakibara et al. 2005a) (Fig. 14.1). The second subproject is to examine in vitro reconstitution of the fertilization signaling events with the use of isolated MDs. We have succeeded in reconstituting sperm-induced egg activation events such as tyrosine phosphorylation of xSrc and PLC γ , calcium responses, and resumption of the meiotic cell cycle by using isolated, unfertilized egg MDs and cyostatic factor-arrested unfertilized egg extracts (Sato et al. 2003, 2006). In the remaining part of this subsection, we describe achievements and current problems in these two subprojects.

14.3.2.2 Characterization of UPIII as a Novel Component of Fertilization

UPIII was originally identified by mass spectrometric analysis of a prominently tyrosine-phosphorylated 30-kDa protein that is present in the LD-DIMs fractions of fertilized eggs (for more detail on UPIII and other UP family proteins, see Mahbub Hasan et al. 2011). Coexpression of UPIII and xSrc in human embryonic kidney cells results in tyrosine phosphorylation of UPIII. These results suggest that the cytoplasmic part of UPIII acts as a target of xSrc in fertilized *Xenopus* eggs, although physiological relevance of the phosphorylation is unknown. It was also shown that treatment of *Xenopus* eggs with cathepsin B, a mimetic enzyme of the sperm-derived protease that can promote parthenogenetic activation of the eggs, causes partial degradation of UPIII and activation of xSrc. These two events were inhibited when eggs are pretreated with an antibody that is raised against the extracellular domain of UPIII. More importantly, the same antibody also effectively inhibits sperm-induced egg activation. These results suggest that the extracellular part of UPIII acts as a sperm receptor, by which it transmits the sperm signal into the egg cytoplasm (via xSrc activation) (Fig. 14.3). One potential mechanism of UPIII-xSrc connection is that, in unfertilized *Xenopus* eggs, the molecular complex consisting of UPIII and UPIb, a well-known binding partner of UPIII, suppresses the activity of xSrc, and that the proteolysis of UPIII at fertilization leads to the liberation of the activated xSrc. This idea is suggested by studies using the overexpression systems of culture cells (Mahbub Hasan et al. 2007).

14.3.2.3 In Vitro Reconstitution of Fertilization Signaling by Isolated MDs

Reportedly, cellular and molecular insights into the cell-cycle events (e.g., mitosis, meiotic resumption) have been well documented by studies using cell-free extracts that are prepared from unfertilized *Xenopus* eggs (Murray 1991). In particular, cyostatic factor-arrested unfertilized egg extracts (CSF extracts) are used to reconstitute meiosis and other cell-cycle events, as seen in *Xenopus* oocytes or embryos (Maresca and Heald 2006; Ohsumi et al. 2006). In this experimental

Fig. 14.3 Signaling cross-talk between sperm and egg MDs. A number of gamete membrane-associated components have been implicated in fertilization, some of which seem to be species specific whereas others are of universal importance in a wide variety of species. In *Xenopus laevis*, sperm-induced egg activation involves proteolytic cleavage of UPIII and activation of the tyrosine kinase xSrc, both of which are localized events in the egg MDs. Our recent data suggest that membrane interaction between egg and sperm via the egg MDs also involves modulation of fertilizing property in sperm. Such bidirectional signaling between egg and sperm may be crucial for completion of successful gamete interaction/fusion and initiation of embryonic development

platform, however, the only events to be evaluated are cytoplasmic and nuclear functions. Our idea in the second MDs subproject is to employ the isolated MDs as a resource to reconstitute plasma membrane-associated functions and their interactions with cytoplasmic environments at fertilization (Fig. 14.2). As described earlier, the addition of sperm to the isolated MDs causes an elevation in tyrosine phosphorylation of MDs-associated proteins. This phenomenon actually involves the activation of xSrc as well as phosphorylation of UPIII (Sato et al. 2003, 2006; unpublished results), both of which are the earliest events that are seen in fertilized *Xenopus* eggs. Therefore, we sought to combine the sperm-treated MDs with CSF extracts to reconstitute events of fertilization from the plasma membranes through the cytoplasm. As a result, we have found that the sperm-treated MDs can promote a transient calcium release, dephosphorylation of mitogen-activated protein kinase, and cell-cycle progression as judged by the morphological change in sperm-derived nuclei (Sato et al. 2003). In addition, the isolated MDs that are pretreated with hydrogen peroxide (Src PTK signaling is stimulated), but not those pretreated with A23187 (Src PTK signaling is not stimulated), are shown to promote the aforementioned events. These results are consistent with the idea that egg MDs can be used as an experimental platform to reconstitute membrane-associated, Src PTK-dependent signaling events at fertilization (Sato et al. 2003).

Given this background, there are two major problems to be solved in our MDs project: one is when and how sperm-dependent signaling functions of MDs are established; and the other is how egg MDs interact with sperm and what is the consequence of this interaction.

14.3.3 Ongoing Approaches to Explore the Physiological Functions of MDs

As already described, our MDs project has involved two major subprojects: snapshot analysis of MDs-associated proteins and in vitro reconstitution of MDs functions (Fig. 14.2), and these two subprojects have raised new problems and questions. To explore further these problems and questions, we are now undergoing a panel of experiments. In the following subsections, we describe the aims and current states of those ongoing MDs projects.

14.3.3.1 Evaluation of UPIII and MDs Functions in Immature Oocytes

In general, ovarian and immature oocytes cannot be fertilized because they are not yet fully competent to undergo sperm-induced developmental activation. It has long been appreciated that hormone-induced oocyte maturation is an event in that oocytes acquire fertilization competence by establishing intracellular conditions (e.g., meiotic cell-cycle stage, subcellular localizations of endoplasmic reticulum and other intracellular components, biochemical properties including the activity of certain protein kinases such as maturation-promoting factor). However, it seems that oocyte maturation also involves an alteration in the structure and function of the oocyte plasma membranes. To access this possibility, we are now analyzing expression, subcellular localization, and molecular interaction of UPIII in the course of oocyte growth (oogenesis) and progesterone-induced oocyte maturation. In addition, we are examining whether sperm-induced PTK signaling can work in the immature oocyte MD. Results so far obtained suggest that oocyte maturation leads to the exposure of the extracellular domain of UPIII on the oocyte surface and that, at a similar timing, MDs become fully competent for sperm-induced PTK signaling (manuscript in preparation).

14.3.3.2 Gain- and Loss-of-Function Experiments on xSrc and UPIII

We have prepared immature oocytes expressing xSrc of either kinase-active or kinase-negative mutants, and we are now examining in vitro maturation of the oocytes and their activation in response to artificial egg activators: the calcium ionophore A23187, cathepsin B, and hydrogen peroxide (manuscript in preparation). We have also constructed mutant UPIII, in which its possible target amino acids for tryptic protease in the extracellular domain are mutated to be proteolysis resistant. We are now characterizing molecular function of the mutant UPIII (named UPIII-RRAA mutant) by using expression systems with human embryonic kidney 293 cells. Results so far obtained demonstrate that the UPIII-RRAA mutant, as does the wild-type UPIII, localizes to plasma membranes/MDs and is capable of inactivating the coexpressed xSrc in 293 cells (manuscript in preparation). In addition, more importantly, it has been shown that the UPIII-RRAA is actually resistant to

proteolysis by cathepsin B. We are now examining the sperm-induced PTK signaling function of MDs that are prepared from 293 cells expressing the UPIII of either the wild type or RRAA mutant. These experiments would not only evaluate the function of the UPIII-RRAA mutant but also suggest that the sperm receptor function can be reconstituted in MDs of the cultured cells.

14.3.3.3 Unbiased Approaches to Identify and Characterize Novel Components

Although the aforementioned two approaches focus on functions of certain well-characterized egg MD-associated proteins (i.e., xSrc and UPIII), the following two approaches are aimed to discover novel fertilization-related components that localize to the egg MDs. The first one is to generate a library of monoclonal antibodies in which plasma membrane-associated components are used as antigens. In *Xenopus*, some egg surface-interacting substances have been reported to act as an egg activator: examples include disintegrin peptides (Iwao and Fujimura 1996; Shilling et al. 1998) and cathepsin B (Mizote et al. 1999). As we mentioned earlier, an antibody that recognizes the extracellular domain of UPIII has been shown to inhibit normal fertilization (Sakakibara et al. 2005a). Therefore, we expect that an antibody(s) of the library, which can bind to the surface (in other words, some known or unknown components essential for fertilization) of unfertilized *Xenopus* eggs, would be able to activate or inhibit egg functions. We are now screening monoclonal antibody clones that satisfy the following criteria: (1) to bind to the egg surface in indirect immunofluorescent experiments, (2) to bind to protein bands in immunoblotting experiments, and (3) to inhibit normal fertilization or to activate the egg in the absence of sperm. The second approach is to identify the molecular basis of polarity in the egg surface. In *Xenopus*, sperm entry point is limited to somewhere in the pigmented area of the egg: the animal hemisphere. Our hypothesis is that this limitation is the result of uneven distribution of sperm receptor or its inhibitory machinery. To access this problem, we are now undergoing comparative analysis of the animal and vegetal hemisphere-associated proteins. Other kinds of differential proteome analysis have been done in our previous study, in that an antibody that recognizes fertilization-induced plasma membrane-associated antigens in *Xenopus* eggs is generated by subtractive immunization (Sakakibara et al. 2005b). Therefore, these two ongoing experiments would also be useful for further evaluation of such an “unbiased” approach to discover fertilization-related egg components.

14.3.3.4 Analysis of Signaling Cross-Talk Between MDs and Sperm or Egg Cytoplasm

In this ongoing project, we analyze the interactions of the egg MDs with sperm and the egg cytoplasm. Regarding the egg MD–sperm interactions, we have preliminary data showing that the isolated egg MDs do not inhibit normal

fertilization when they are present in the insemination media, but rather recover the ability of sperm to fertilize the eggs that are pretreated with the anti-UPIII extracellular domain antibody (manuscript in preparation). In other words, it is suggested that the MD-treated sperm somehow overcome the absence of functional UPIII on the egg surface. In *Xenopus*, no evidence is available with regard to the presence of extracellular MDs- or other membrane-containing vesicles (such as exosomes in the mouse; Miyado et al. 2008; manuscript in preparation). Therefore, we assume that, exactly at the time of gamete membrane interaction and fusion, egg-“associated” MDs act not only as a platform for sperm-induced Src PTK signaling in fertilized eggs but also as an unknown signaling trigger for sperm, by which sperm may acquire the ability to fertilize eggs, in a UPIII-dependent manner (Fig. 14.3). We now assess this “bidirectional signaling between sperm and the egg MDs” hypothesis by analyzing what kind of cellular functions in sperm are modulated after their interactions with the isolated MDs, and what kind of molecule(s) in the sperm surface are involved in the interactions with the isolated MDs. One interesting thought is the involvement of PTK signaling in sperm, which has been well documented in several other aspects of sperm functions (Ijiri et al. 2012).

14.3.3.5 Analysis of Signaling Cross-Talk Between MDs and Egg Mitochondria

We are also interested in functional interactions between egg MDs and organelles such as mitochondria. This interest is based on the fact that serine/threonine-specific protein kinase Akt, whose activation is shown in fertilized *Xenopus* eggs (Mammadova et al. 2009), localizes predominantly to mitochondria and that two mitochondrion-associated proteins of ~40 kDa on SDS-polyacrylamide gel electrophoresis (named pp40) are identified as possible Src substrates at fertilization (manuscript in preparation). These two proteins are also present in MD fractions. In some cell systems, cell-surface receptor/kinases [e.g., epidermal growth factor receptor (EGFR)] and membrane-associated cytoplasmic protein kinases (e.g., Akt, Src) are shown to translocate or localize to mitochondria in response to cell stimuli (Demory et al. 2009; Hebert-Chatelain 2013). Therefore, it will be interesting to examine whether fertilization involves a similar signaling linkage between MDs and mitochondria by way of characterization of these novel Src substrates. Our interest in mitochondria also involves a possible relationship of their known functions and fertilization. In somatic cell systems, mitochondria serve a pivotal role in ATP production and survival control. Thus, this ongoing project will explore the presence of MDs–mitochondria interactions at fertilization and their physiological relevance to the metabolism and viability of developing embryos. In this connection, we have started to analyze quantitative and spatiotemporal regulation of ATP in oocytes and eggs during oocyte maturation and fertilization (Ijiri et al. 2014).

14.4 Summary and Perspectives

Our ongoing egg-MDs project, consisting of snapshot experiments and in vitro reconstitution experiments, molecular targeting approaches and unbiased proteomics approaches, and cell biological approaches involving sperm and egg mitochondria; aim to understand how structure and function of egg MDs are developed and how egg MDs and MD-associated molecules contribute to successful fertilization and subsequent embryogenesis. We believe that the knowledge obtained with this project in itself would contribute to general understanding of the fertilization system. In addition, fertilization system involves, with no exception, membrane interaction and fusion between female and male gametes, in spite of a countless variety of species-specific differences in the fertilization system such as molecules of fertilization (e.g. requirement of Src PTK signaling), systems for egg activation (e.g., physiological monospermy and polyspermy), extracellular environment for fertilization (e.g., internal and external fertilization), and even animal and nonanimal fertilization. Therefore, our approach would be useful, at least in part, for fertilization studies on a wide range of other organisms.

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