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Multifunctional scaffolds in eggs: sites for localization, signal transduction and meiotic spindle polarity

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TABLE OF CONTENTS

1. ABSTRACT

Molecular scaffolds in the mammalian egg are capable of tethering specific proteins involved in regulation of early development. Scaffolds can take the form of cytoskeletal elements, or involve proteins such as MARCKs or RACKSs during important cellular transitions in the egg. Moreover, with each cellular transition (i.e. germinal vesicle breakdown, meiosis I, meiosis II, etc) comes an extensive rearrangement of architectural elements within the cell. To accomplish this regulatory elements in signaling pathways should be in close molecular proximity to other discrete signaling pathways both to increase the speed of chemical reactions and to promote crosstalk. Crosstalk between signaling pathways is essential to modulate downstream effectors as one pathway can trigger activation/inhibition of another. It also is important to sequester or restrict access to various signaling enzymes for later use. These requirements create both morphological and biochemical heterogeneity, and likely necessitate the use of molecular scaffolds. This review examines the body of literature suggesting cytoskeletal elements serve to meet the aforementioned requirements in the mammalian egg.

2. INTRODUCTION

The fertilization-competent egg transforms into the zygote through concerted biochemical and structural changes predetermined by maternal stores that are spatially localized and poised to interact immediately upon fertilization in the absence of gene transcription (1). In effect, the egg is a cell destined for death, but redirected by the developmental program that includes fertilization, and subsequently early development initiated by the sperm through a series of sequential signal transduction events. Mediation of these transitions could suggest that various signaling elements requisite for normal development are spatially localized and/or tethered to prominent scaffolds in the eggs at each key cellular transition (i.e. germinal vesicle breakdown, fertilization, cleavage, etc). Molecular scaffolds can manifest themselves in several forms in the egg and may include: 1) submembrane microdomains enriched with specific phospholipids and membrane-bound proteins (2-4); 2) the three cytoskeletal filament systems such as the spindle apparatus, the cortical actin network, or even the intermediate filament network, and; 3) high affinity binding proteins such as MARCKs or RACKs that

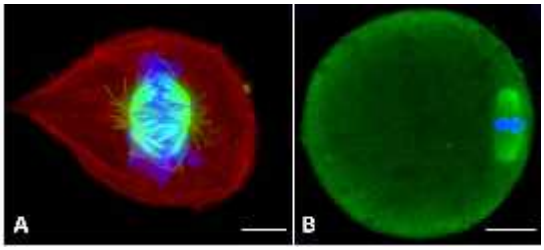

Figure 1. Laser scanning confocal micrographs highlight differences between mouse embryonic fibroblast (A) undergoing mitosis and female germ cells (B) which undergoes meiosis. (A) The somatic cell assembled a centrally positioned spindle apparatus. Filamentous actin is labeled with fluorophore-conjugated phalloidin (pseudocolored red), microtubules are pseudocolored green, and DNA is blue. Scale is 5 micrometers. (B) In contrast to somatic cells, female germ cells undergo meiosis. During the second meiotic division prior to fertilization, the spindle apparatus becomes polarized in the cell to aid in the establishment of the second polar body. Microtubules are pseudocolored green, and DNA is blue. Scale is 20 micrometers .

may act to sequester enzymes such as protein kinase C (PKC). Here we review several lines of evidence from studies focused on meiosis of the female gamete and polarity where relevant, supporting evidence taken from somatic model systems indicate that cytoskeletal elements can operate as molecular scaffolds to tether various signaling agents during cellular transitions. Unfolding of the developmental program subsequent to the meiotic process initiates specialized cellular division in the form of mitosis and these cells demonstrate marked differences in overall morphology.

Comparative analysis at the immunocytochemical level between mouse meiotic MII- and somatic cells undergoing mitosis highlights these fundamental differences (Figure 1 [A-B]). Chief among these differences is the asymmetrically positioned, polarized spindle apparatus of the MII egg juxtaposed to the plasma membrane near the cortical actin network in an area referred to as the actin cap. The MII spindle is considered "acentrosomal" as the mammalian egg is devoid of centrioles, but rather, contains centrosomal material, and is purportedly "barrel" shaped. Furthermore, the mammalian egg, with the exception of the immediate vicinity of the spindle apparatus, is encompassed by microvilli and cortical granules. In contrast, mouse embryonic fibroblasts which undergo mitosis possess a centrally positioned spindle apparatus and is considered centrosomal with two centrioles by which microtubules emanate radially to form a pointed, bipolar spindle apparatus.

Polarity innate to the MII arrested egg underscores an intuitively attractive perspective that scaffolds may account for the intracellular heterogeneity of biochemical signaling pathways that mediates asymmetric division since a number of signaling enzymes have been demonstrated to be enriched at the spindle apparatus. Indeed, mounting evidence corroborates the efficacy of scaffolds to: 1) position signaling pathways; 2) sequester signaling agents or substrates and; 3) permit cross talk among distinct signaling pathways (5-8).

3. CELLULAR SCAFFOLDS ESTABLISH CELLULAR POLARITY AND COLOCALIZE SIGNALING ENZYMES

Numerous reports (8-10) have identified colocalization of signaling enzymes, and some of these enzymes have been implicated in cellular polarity itself (5). Scaffolds function to increase efficiency of chemical reactions by tethering various cellular components, and distinct signaling enzymes are often associated with scaffolds. It comes as no surprise that cell polarity determines the location of specific signaling enzymes that are restricted to a given scaffold, and this can have profound implications on a given cell (11). Take, for example, a polarized cell model such as a brush border expressing intestinal epithelial cell (12) (Figure 2 A). Intestinal epithelial cells have an apical and basolateral polarity with respect to not only morphology, but also brush-border expressing proteins such as villin, sucrose-isomaltase, and ZO-1, among others, (13) many of which are associated with cytoskeletal elements (Figure 2 B). It also is well known that the Par3/Par6/aPKC (atypical PKC) complex associates with apically localized tight junctional complexes associated with the actin cytoskeleton (Figure 3), while on the other side of the coin basal scaffolds position aPKC together with elements of the protein kinase A signaling pathway. In this state, the apically polarized ZO-family scaffold has the ability to both sequester aPKC and position other independent signaling pathways. Intriguingly, investigators have shown enrichment of ZO proteins in an area presumed to be the MII spindle apparatus in the mammalian egg (14). This may suggest that ZO-1, and perhaps other junctional proteins, such as the plakin binding protein, Kazrin (15-20), are enriched along a prominent scaffold in preparation for later use.

4. A BRIEF SYNOPSIS OF THE EGG TO ZYGOTE TRANSITION

Initiation of fertilization launches a number of a structural and biochemical changes within the egg as the single sperm bores through the zona pellucida, undergoes fusion, and subsequently forms the zygote. This cellular transition is pivotal; the egg is preprogrammed to eventually die in the event that fertilization does not occur. Key to the argument presented in this review, that cellular scaffolds may provide architecture to mediate dramatic changes within the egg, is the notion that fertilization is dependent on maternal mRNA, and other maternal components. Furthermore, chromatin is highly condensed in the form of chromosomes, and gene transcription has been shown to be absent from the time of the MII arrest through the first few hours after fertilization (1). To manage the dramatic morphological and biochemical reprogramming immediately following the initiation of fertilization, signaling enzymes necessarily initiate, propagate, amplify, modify, and terminate time-dependent

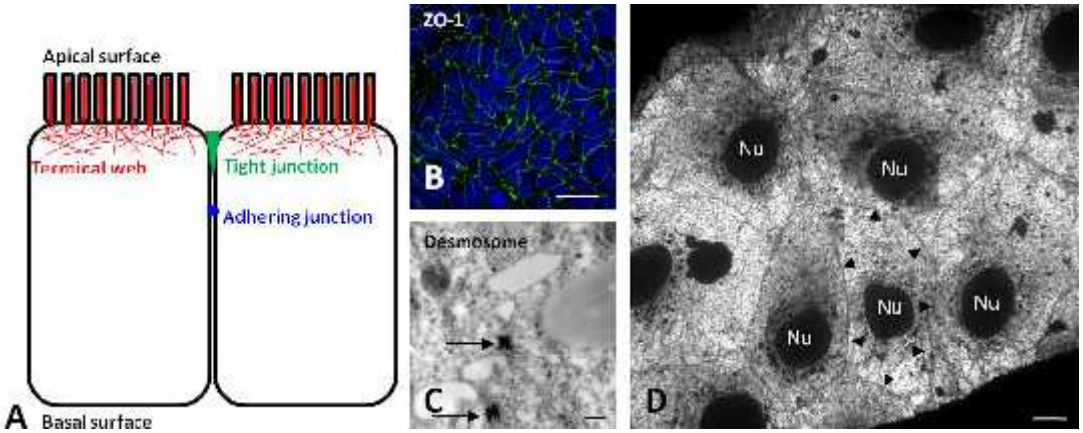

Figure 2. Brush border expressing intestinal cells represent a polarized model epithelium. (A) The cartoon illustrates the general morphology and polarity of Caco-2 epithelium. Microvilli at the apical surface are formed by bundles of actin that extend into the terminal web (red). The polarity of the epithelium is established by tight junctions (green), and underlain by adhering junctions (blue). (B) The ZO-1 micrograph was prepared from a 21 day-confluent monolayer. ZO-1 is in green, while DNA is blue. Scale bar is 30 micrometers. (C) Desmosomes, which connect to the intermediate filament network, are also present between cells. The arrows point to presumed adhering junctions in a transmission electron micrograph. Scale bar is is 250 nanometers. (D) The detergent-extracted whole mount electron micrograph shows cytoskeletal elements within, and between cells. Electron dense nuclei are black (Nu). The edge of a typical cell is indicated with black arrowheads. Scale is 10 micrometers.

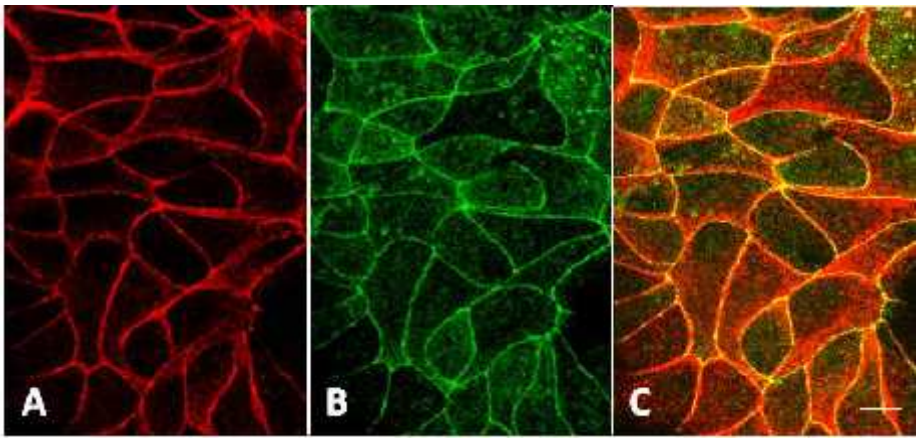

Figure 3. (A) In polarized intestinal epithelial cells, rhodamine-phalloidin labels actin filaments (pseudocolored red). (B) The active form of PKC zeta, that is p-PKC zeta an aPKC, appears to be enriched at intracellular junctions (pseudocolored green). (C) The overlay image shows colocalization of actin and p-PKC zeta due to overlap of red (phalloidin labeled) and green (p-PKC zeta labeled) pixels in yellow. The scale is 10 micrometers.

signals. Thus, it can be speculated that scaffolds, maintaining an architecture to meet these requirements, serve to spatially and temporally position or sequester signaling enzymes to permit the elegant choreography to unfold at this pivotal cellular transition (7, 8).

At the apex of a signaling hierarchy is the calcium (Ca^{2+}) signal initiated by the sperm which serves as a co-factor for activation. There are a number of Ca^{2+}-dependent enzymes that have been implicated in the rise of intracellular-free Ca^{2+} ($[Ca2^{+}]_i$) and some include PKC, maturation-promoting factor (MPF), calcium/calmodulin-dependent protein kinase II (CaMKII) and MAPK. What's more, in the mammalian egg the rise in $[Ca^{2+}]_i$ is not a single spike, but rather an oscillatory event that occurs over the course of several hours (21), and has been postulated to provide a means for repeated activation of Ca^{2+}-dependent enzymes.

One such enzyme, PKC, has been shown to act downstream of the Ca^{2+} signal. The PKC family consists of 11 isotypes categorized in three broad categories, conventional, novel and atypical, by their sequence homology and co-factor requirements for activation. The conventional PKCs (cPKCs) consist of PKC alpha, -beta I, -beta II, and gamma and require Ca^{2+}, diacylglycerol (DAG), and negatively-charged phospholipids for activation. The novel (nPKCs) isotypes are collectively PKC delta, -epsilon, -theta, and –mu, and are calcium independent, but require DAG and negatively charged

phospholipids for activation. Finally, atypical PKCs (aPKCs) include human PKC iota, also known as PKC lambda in mouse, and the zeta isotype which are activated solely by negatively charged phospholipids. Numerous reports have demonstrated this activation at a biochemical and/or immunocytochemical level in mouse (22, 23) and rat eggs (24, 25), albeit with subtle species-dependent differences. Once PKC has been activated in the egg, it has been demonstrated to have a number of roles including the maintenance of spindle stability (26-28), cortical granule exocytosis (29, 30), and initiating the emission of the second polar body (31, 32).

How does PKC activate to engage in these essential roles? Mounting evidence (33-38) suggests that the zeta isotype of phospholypase c (PLC zeta) supplied by the sperm initiates an initial Ca^{2+} rise culminating in the activation of Ca^{2+}-dependent enzymes; Once PLC zeta is in the egg, it can hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce inositol triphosphate (IP_3) and DAG. Cytosolic IP_3 can subsequently release Ca^{2+} from intracellular stores, while DAG can serve as a separate co-factor for activation of PKC at the plasma membrane. Translocation of PKC to the cell cortex, in part through the action of RACK1 (39), can, but not always, results in activation of the kinase (40-42) as PKC is in an ideal location to interact with its co-factors (i.e. DAG and phosphatidylserine).

Another key player during this cellular transition is CaMKII. At present there have been 4 subunits identified and those include the alpha, beta, gamma and delta subunits; and all except for the beta subunit, have detectable mRNA levels assessed by quantitative RT-PCR (43) in the egg during early development. In the egg CaMKII acts downstream of the rise in $[Ca^{2+}]_I$, and this Ca^{2+} bound to calmodulin leads to activation of the kinase (44). Once activated CaMKII has been implicated in the metaphase II to anaphase II transition (44). Recent investigations utilizing mammalian eggs provide evidence that the gamma-III isotype of CaMKII plays a role in resumption of the cell cycle and recruitment of maternal stores (43, 45), while others previously demonstrated a role in the establishment of the bipolar spindle apparatus in human somatic cells (46). Furthermore, the CaMKII signaling pathway appears to engage in crosstalk as it was demonstrated to co-localized with, and potentiate MAP kinase on architectural elements in the egg (9).

5. ARCHITECTURAL ELEMENTS ACTING AS SIGNALING HUBS

5.1 CaMKII, the spindle apparatus, and the metaphase to anaphase transition

A hallmark paper by Lorca and colleagues demonstrated that CaMKII in *Xenopus* extracts is necessary and sufficient to release cytostatic arrest and initiate cyclin B degradation machinery (47). These mechanisms may be conserved as later work by Johnson and coworkers (44) employing not only biochemical, but also immunocytochemical analysis during egg activation demonstrated that CaMKII localizes with the spindle apparatus. The tightness of the binding was assessed by employing detergent extraction, a technique which removes the soluble components while leaving behind the detergent-resistant cytoskeleton and only those tightly associated proteins, and CaMKII was shown to be associated with the detergent-resistant spindle apparatus prior to activation and at various time points post-activation indicating that CaMKII is tethered to microtubules during meiosis. Further, immunocytochemical data revealed that after egg activation CaMKII becomes highly enriched at the midzone microtubules of the forming second polar body. This may be universal in mammals as it also has been demonstrated in activated rat eggs (48). What is the importance of the colocalization of CaMKII with midzone microtubules? Since CaMKII appears associated with the meiotic spindle, midzone microtubules, and subsequently enriched in the area presumed to be the contractile ring, it appears as if the spindle apparatus, serving as an architectural element to position signaling enzymes, carries out multiple functions (i.e. remodeling of the spindle apparatus to aid in second polar body formation) on the spindle, a molecular scaffold. The evidence provided in the preceding passages indicate that indeed molecular scaffolds position or localize independent signaling pathways, and further, restrict certain pathways spatially and temporally.

Recent work by Backs and colleagues (2010) demonstrate a role for CaMKII gamma in the metaphase II to anaphase II transition. Although others have shown CaMKII associated with the metaphase spindle the authors did not show this enrichment, perhaps due to the CaMKII knockout. In this study Backs and coworkers generated a conditional null mutant for the CaMKII gamma gene and it was noted that female homozygous null mutants were unable to produce offspring potentially indicating the importance of this gene in proper egg development. Furthermore, RT-PCR analysis of CaMKII gamma in wild type (+/+) eggs demonstrated a maternal mRNA population, whereas knockout mutants had no CaMKII gamma and importantly it was noted that none of the other CaMKII isoforms were upregulated. These observations both demonstrate the effectiveness of the knockout and further indicate the importance of CaMKII gamma during early development. However, of particular interest are micrographs depicting the spindle apparatus during stages subsequent to egg activation. Compared to wild type eggs, CaMKII gamma-III knockout eggs' spindle apparatus appear slightly elongated, and the general microtubule morphology appears disrupted (2010). Intriguingly, what was described is reminiscent of a result from a study by Na and Zernicka-Goetz (2006), although the phenotype reported by Backs appears attenuated comparatively. However, the focus of the study by Na and Zernicka-Goetz was to investigate the role of CDC42 during asymmetric cortical spindle migration and provide evidence that PKC zeta may play a role as a downstream signaling enzyme involved in microtubule organization (49). Taken together, these results may highlight a need to assess whether CaMKII has the potential to influence microtubule organization and asymmetric positioning through crosstalk among these distinct signaling pathways. Moreover, these data permit one to speculate that molecular scaffolds

position independent signaling pathways to permit crosstalk.

5.2 The PKC family and architectural elements: a tale of cytoskeletal promiscuity

The present body of literature contains adequate reviews detailing the involvement of PKC during early development (10); however, to date few explicitly communicate its involvement from an angle considering the three cytoskeletal systems as molecular scaffolds, that is considering the actin filaments, intermediate filaments, and microtubule networks, all of which are known to interact with the PKC family in the mammalian egg at various cellular transition.

Filamentous actin (f-actin) is composed of globular actin (g-actin) monomers twined in a helical arrangement with a diameter ranging from 5-9 nm. Actin monomers are divided into three classes: alpha, beta, and gamma isotypes. Historically noted by others (50), and more recently brought to light (51), the f-actin network has received considerable attention because of its alleged collaboration with the mitotic spindle apparatus during blastomere division; studies conducted in Bement's laboratory examining the role of the actin cytoskeleton in the mitotic spindle (52) prompted investigations focused on the role of actin filaments during female division in the mammalian counterpart (53). For instance, other studies have shown that mouse eggs require alpha and gamma cytoplasmic actin isotype for continued development and both appear to differentially localize at each cellular transition (54). The authors provide evidence at the immunocytochemical level that cytoplasmic actin isotype first become polarized in anaphase I oocytes as beta actin appears differentially enriched within the actin cap, and later at telophase I and the first cytokinesis beta-actin is enriched at the presumptive cleavage furrow. Later, during analysis of preimplantation embryos, it was demonstrated that beta, but not the gamma isotype is specifically localized between cell-cell boundaries of blastomeres. Microinjection of gamma-specific antibodies suggested that the gamma isotype may establish early polarity. For starters, this microinjection, compared to anti-alpha- and anti-beta-actin caused severe perturbation to the morphology of the oocyte as a whole including blebbing and high mortality rates. Moreover, one-third of the oocytes injected with low amounts of anti-gamma-actin antibody underwent symmetric division. Finally, these oocytes contained disorganized spindle microtubules, and half remained arrested in MI. Of note, at fertilization PKC alpha, beta, and gamma are recruited to the cortex. Here cPKCs can interact with another actin crosslinking protein, MARCKS proteins (55, 56). Once activated, cPKCs phosphorylate MARCKS subsequently disassembling the cortical actin network in preparation for cortical granule exocytosis (57). Moreover, PKC is known to interact with the apical actin network in polarized intestinal epithelial cells as detailed in preceding sections. It is thus compelling to investigate the possibility that specific PKC isotypes differentially interact with each actin isotype, as total PKC alpha was shown associated with the contractile ring (58-60), and later at the 8-cell stage PKC alpha, -gamma, -delta, and –mu are enriched at cell-cell boundaries. The study by Brockmann *et al.* is supported by evidence for the Arp2/3 complex in establishing polarity in the egg as the Arp2/3 complex nucleates g-actin (61), and further inhibition of the actin-nucleating proteins Arp2/3 via the specific inhibitor CK666 resulted in symmetric division. Whether or not these mechanisms engage in cooperate crosstalk perhaps in part through the action of PKC, or act as redundant mechanisms remains elusive. Together these data indicate that actin isotypes can function as molecular scaffolds to maintain not only a structural polarity, but also polarize specific signaling pathways in space and time.

As part of the down regulation of the kinase, PKC is cleaved from the portion containing the membrane-binding, Ca^{2+}-binding, and pseudosubstrate domains and this catalytic domain remains constitutively active and is released into the cytoplasm. This released kinase, referred to by some as PKM, has altered substrate specificity and was shown to diffuse into the cell interior to phosphorylate and reorganize the intermediate filament network (22). The number of reports highlighting the action of PKCs on intermediate filaments appears to be increasing. For instance sheer forces appear to induce intermediate filament reorganization through the action of PKC zeta phosphorylation on kertain filaments (62). In Caco-2 cells, both immunocytochemical and immunoprecipitation analysis demonstrate that PKC iota is tethered to the apical intermediate filament network via HSP 70 (63), thus the possibility exists that intermediate filaments serve as scaffolds for PKC to establish polarity or sequester signaling agents.

Many PKC isotypes appear to interact with the prominent scaffolding network known as microtubules in the mammalian egg. Microtubules are the largest diameter of the three cytoskeletal systems with a diameter of approximately 25 nm, and each subunit of a protofilament is composed of a tubulin heterodimer (alpha-beta heterodimer). At present there appears to be laboratory specific jargon regarding the specificity of the antibody employed; in the proceeding text we refer to antibody specific to a phosphorylated, or active form as "p-" followed by the enzyme itself (e.g. an antibody that recognizes a phosphorylated form of PKC delta appears in the text as "p-PKC delta"), whereas pan, or antibody specific to both the phosphorylated and unphosphorylated form appears as "t-".

In both female germ cells and somatic cells, microtubules are the principal component of the spindle apparatus and appear to localize a myriad of signaling enzymes (Table 1). In the mammalian egg a number of PKC isotypes decorate the spindle apparatus (59). During MI t-PKC delta localizes along the length of the spindle apparatus, while p-PKC delta was specifically enriched at the spindle poles (Figure 4), localized with pericentrin and gamma-tubulin (64). t-PKC alpha, -gamma, -delta, and -zeta were shown enriched on the spindle apparatus at MII. Subsequently, the tightness of the binding was assessed by detergent-extraction and only t-PKC zeta and -delta remained associated with the detergent-resistant

Table 1. Localization of various proteins in the egg

Protein	Enriched Localization	Cellular Transition	Reference	Method
ZO-1	Cell contacts	4-cell stage	(14)	ICC
ZO-2	Cell contacts	8-cell stage	(14)	ICC
t-PKC	M, PBII	A	(23)	ICC
PKC alpha	N/A	A	(25)	ICC
PKC beta I	N/A	A	(25)	WB
PKC beta II	M[1], N/A[2], SA[3]	A[1,2], MII[3]	(25)[1,2], (39)[3]	ICC[1,3], WB[2]
PKC gamma	N/A	A	(25)	WB[1]
PKC delta	N/A[1], SA[2]	A[1], MI[2,3]	(25)[1],(71)[2],(72)[3]	WB[1], ICC[2,3]
PKC epsilon	N/A	A	(25)	WB
PKC theta	N/A	A	(25)	WB
PKC mu	N/A	A	(25)	WB
PKC zeta	N/A	A	(25)	WB
PKC lambda	N/A	A	(25)	WB
t-p-PKC	GV, SP, SP & SA	GV, MI, MII	(73)	ICC
p-PKC zeta	SP[1,2,3]	MII	(27)[1], (49)[2], (65)[3]	ICC[1,2], WB
p-PKC delta	SP[1,2]	MII	(27)[1], (64)[2]	ICC[1,2], WB[1,2]
t-GSK3 alpha/beta	SA	MI	(74)	ICC
pGSK3 beta(Ser9)	SP[1]	MII	(27)	ICC[1], WB
Par6	SA	MII	(27)	ICC
RACK1	SA[1], M[2]	MII[1], F[2]	(44)[1], (75)[2]	ICC[1,2]
p-MARCKS	GV&M, SP&MZ, PB	GV&GVBD, MI&MII, 1-cell	(65)	ICC
p-AKT(Thr308)	SP[1,2]	MI[1,2], MII[1]	(76)[1], (77)[2]	ICC[1,2]
p-AKT(Ser473)	SA[1,2]	MI[1,2], MII[1]	(76)[1], (77)[2]	ICC[1,2]
t-CaMKII	SA	MII, A	(44)	ICC, WB
CaMKII gamma	N/A[1,2]	MII, A	(43)[1], (45)[2]	RT-PCR[1,2], WB[1]

The mammalian egg appears to be enriched with a number of proteins requisite for normal development. Many of these proteins are enzymes and appear to be enriched at the spindle apparatus. (Legend: spindle apparatus=SA, spindle poles=SP, germinal vesicle=GV, membrane=M, second polar body=PBII, activation [parthenogenetic or otherwise]=A, metaphase I=MI, metaphase II=MII, fertilization=F, germinal vesicle break down=GVBD, immunocytochemistry=ICC, Western Blott=WB, reverse-transcription polymerase chain reaction=RT-PCR, N/A=not applicable). The superscripts apply to the references used in the table.

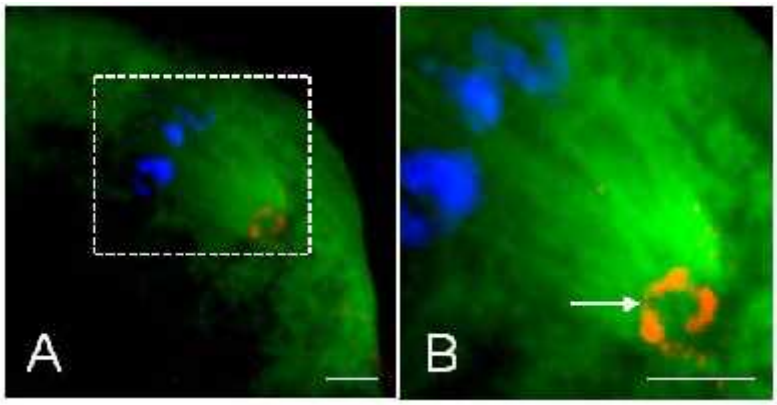

Figure 4. (A-B) In contrast to somatic cells, eggs assemble a barrel-shaped spindle apparatus. The centrosomal area is decorated with p-PKC zeta (orange) in a circular arrangement (white arrow). The chromosomes are blue. Tubulin, which labels microtubules, is shown in green. (A) Low magnification view of the spindle apparatus in a metaphase II arrested egg. The scale is 20 micrometers. The white box in (A) is shown at high magnification in the micrograph (B). The scale (B) is 20 micrometers. Only one spindle pole is apparant due to the angle of the spindle, and the image is represented as a single optical section.

cytoskeleton, and this tightness was confirmed by FRET analysis. FRET analysis revealed a close molecular association between alpha-tubulin and PKC zeta, and alpha-tubulin and PKC delta (59). In addition, an antibody that recognizes both p-PKC delta and p-PKC zeta showed enrichment at the acentrosomal spindle apparatus. Intriguingly, work from Michaut *et al*, provide evidence that p-MARCKS are also localized at the region of the centrosome and appear to be phosphorylated by aPKCs (65). Upon egg activation, as stated previously, cPKCs migrate to the cortex. In contrast, t-PKC zeta remains associated with the spindle and p-PKC zeta remains enriched at the spindle poles. This suggested that PKC zeta may play a role in maintaining the integrity of the spindle apparatus during division. Inactivation of the kinase with a specific, membrane-permeate peptide inhibitor of p-PKC zeta resulted in disruption of the spindle apparatus (27).

The Par6/PKC zeta/GSK3 beta pathway is known to be involved in microtubule stability. When GSK3 beta

is activated microtubules can be destabilized (66), however, when GSK3 beta is phosphorylated on serine 9 (Ser9) it is inactivated and microtubule stability is maintained. In the mammalian egg, p-PKC zeta probably phosphorylates GSK3 beta, and thereby inactivates the kinase to promote microtubule stability (27). Kalive has recently (2011) provided evidence that p-PKC zeta interacts with GSK3 beta to maintain the spindle apparatus albeit employing mouse embryonic fibroblasts which contain centrosomal spindles (28). The authors employed a permeabilized cell system and systematically flushed in active GSK3 beta protein and subsequently inhibitors to active GSK3 beta and p-PKC zeta in replicate samples. It was demonstrated that after 30 minutes in the presence of active GSK3 beta the general morphology of the spindle apparatus appeared perturbed, and at the one hour time point the spindle apparatus was virtually abolished indicating that active GSK3 beta disrupts spindle stability under these conditions. This disruption via active GSK3 beta was subsequently confirmed by flushing in active GSK3 beta with a specific inhibitor to the kinase and it was found that the spindle apparatus was largely maintained providing further evidence that active GSK3 beta can disrupt the spindle apparatus. Finally, to assess the potential for p-PKC zeta to interact with p-GSK3 beta, the authors methodically inhibited each of the two kinases and assessed the effects on the spindle apparatus. It was reported that inhibition of p-PKC zeta ablated not only the spindle, but also the general localization of both p-PKC zeta and p-(Ser9)GSK3 beta. However, the phenotype was rescued by employing p-PKC zeta inhibitor and subsequently a GSK3 beta-specific inhibitor indicating that p-PKC zeta may inactivate GSK3 beta through phosphorylation on the Ser9 residue leading to microtubule stability.

To determine if the p-PKC isotypes are tethered to the cytoskeleton the authors employed detergent extraction and it was determined that p-PKC alpha, -beta II, -gamma, -delta, -zeta, and -mu, but not -theta, are part of the detergent resistant cytoskeleton. Studies of each of the isotypes indicate that p-PKC zeta, and -delta show the greatest colocalization with p-(Ser9)GSK3 beta. Further, FRET analysis revealed that p-PKC zeta was in close molecular proximity to p-(Ser9)GSK3 beta at both centrosome and at the putative kinetochore region. This was confirmed at the biochemical level for each of the phosphorylated PKC isotypes through comparative studies of metaphase synchronized cell lysates and Western blot analysis followed subsequently by immunopurification with an antibody specific to p-(Ser9)GSK3 beta. As expected p-PKC zeta, and -delta demonstrated the highest relative band intensities indicating the greatest level of interaction with p-(Ser9)GSK3 beta.

Taken together these results provide evidence that mitotic spindle microtubules may serve as a scaffold to localize specific signaling enzymes as both p-PKC zeta and p-(Ser9)GSK3 beta co-localized with, and affect the integrity of the spindle apparatus during analysis of fixed mouse embryonic fibroblasts (NIH 3T3). Given the level of promiscuity that PKC zeta has for the various architectural elements within the cell, that is the various cytoskeletal filament systems, it is not surprising that PKC zeta can modulate processes requiring extensive cytoskeletal reorganization through interactions with small GTPases of the Rho family such as CDC42 (67-70). Moreover, it appears as if distinct PKC isotypes (i.e. p-PKC delta, and p-PKC zeta) are enriched on architectural elements in the event of a complete knockout to potentially serve as a mechanistic “Plan-B” as studies have revealed that PKC delta is highly enriched at the centrosome (28, 59), and involved in meiotic spindle organization (64).

6. CONCLUSION

The mammalian egg contains a number of prominent scaffolds in the form of architectural elements that consist of cytoskeletal networks, or other proteins such as MARCKs and RACKs. These scaffolds are important in maintaining a structural and biochemical heterogeneity innate to the female germ cell undergoing extensive modification. Without these scaffolds the egg cannot proceed through the progressive development program, that is, if one element is perturbed, subsequent time-dependent events are disrupted resulting in abnormal development.

The data presented in this review emphasize the need to further investigate the potential overlapping role of signaling pathways and cytoskeletal networks as many pivotal signaling pathways are differentially enriched in the vicinity of the cytoskeletal networks. Indeed, evidence from independent reports exists suggesting that both CaMKII and PKC localize to prominent molecular scaffolds in the mammalian egg. At the immunocytochemical level there appears to be a robust colocalization of many signaling pathways with architectural elements. Furthermore, experiments such as detergent extraction, FRET analysis, immunopurification, etc., give credence to a “building block” model, whereby various signaling pathways are tethered in close molecular proximity with putative scaffolds to engage in crosstalk, or to sequester/restrict access, to spatially and temporally facilitate signaling cascades through early development.

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Abbreviations: PKC: protein kinase C, CaMKII: calcium/calmodulin-dependent protein kinase II, GSK3: glycogen synthase kinase 3, Par 3: partitioning defective protein 3, MARCKS: myristoylated alanine-rich C-kinase substrate, RACK: receptors for activated C-kinase, ZO-1: zona occludens 1, FRET: fluorescence resonance energy transfer, Arp2/3: actin related proteins 2/3

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