COMPOSITION, REGULATION, AND FUNCTION OF THE CYTOSKELETON IN MAMMALIAN EGGS AND EMBRYOS

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1. ABSTRACT

The cytoskeleton of the mammalian egg and early embryo is highly unique when compared to the cytoskeleton of their somatic cell brethren. Although all three cytoskeletal systems, actin filaments, microtubules, and intermediate filaments, are present as early as the unfertilized egg; each system has adapted features that allow the egg and early embryo to meet the strict demands of the developmental process. The major demands placed upon eggs and embryos are developmental transitions (i.e., fertilization, compaction, blastocyst formation, germ layer formation and gastrulation), each of which must be traversed in order for the embryo to form a new individual. To successfully complete all of the necessary processes during early development, eggs and embryos must call upon many signal transduction mechanisms, cytoskeletal components, and genes that are both unique to embryogenesis and ubiquitous among many types of somatic cells. It is the goal of this review to provide some current details into the mechanisms that drive early development primarily focusing on the cytoskeletal components eggs and embryos have adapted to promote embryogenesis.

blastocyst formation, and establishment of germ layers in the egg cylinder in rodents or germinal disk in most other mammals). Exactly how these extraordinary morphological changes take place within the embryo at each developmental transition has been a mystery ever since investigators began studying embryogenesis decades ago (1-6. and references therein). However, over the past 5-10 years or so, investigations have begun to uncover the mechanisms that drive these developmental transitions, a knowledge that has led to a better understanding of mammalian embryogenesis as a whole. While some of these investigations have focused on mechanisms that control cell cycle regulation, signal transduction events, and components that initiate differentiation, many investigations have focused on the structural components that actually perform the architectural changes underlying each developmental transition. These structural components are specifically defined by the cytoskeleton and cytoskeletal-associated proteins of each cell within the embryo. The goal of this review is to examine current knowledge of the composition, regulation, and function of the cytoskeleton during early embryogenesis with special focus on the function of the cytoskeleton and its regulation at each developmental transition.

2. INTRODUCTION

The early stages of mammalian development are marked by distinct, very predictable developmental transitions that begin at fertilization and continue through embryogenesis. Each transition is defined by an exquisite remodeling of the embryo at both the intracellular and intercellular levels that allow the egg or embryo to continue through its developmental program. While some of these transitions are common among many phyla (e.g., fertilization and gastrulation), particular developmental transitions are unique to mammals (e.g., compaction,

2.1. From egg to implantation: A brief synopsis

The developmental process begins in the oviduct where a newly ovulated egg awaits a single sperm. At this stage, the unfertilized egg is arrested at meiotic metaphase II and is poised for fertilization, a developmental transition that results in the activation of numerous intracellular signals responsible for remodeling the egg into a single-celled embryo called a zygote. Once formed, the zygote then follows an echelon of instructions that trigger cell growth, proliferation, differentiation, and cell death, all of
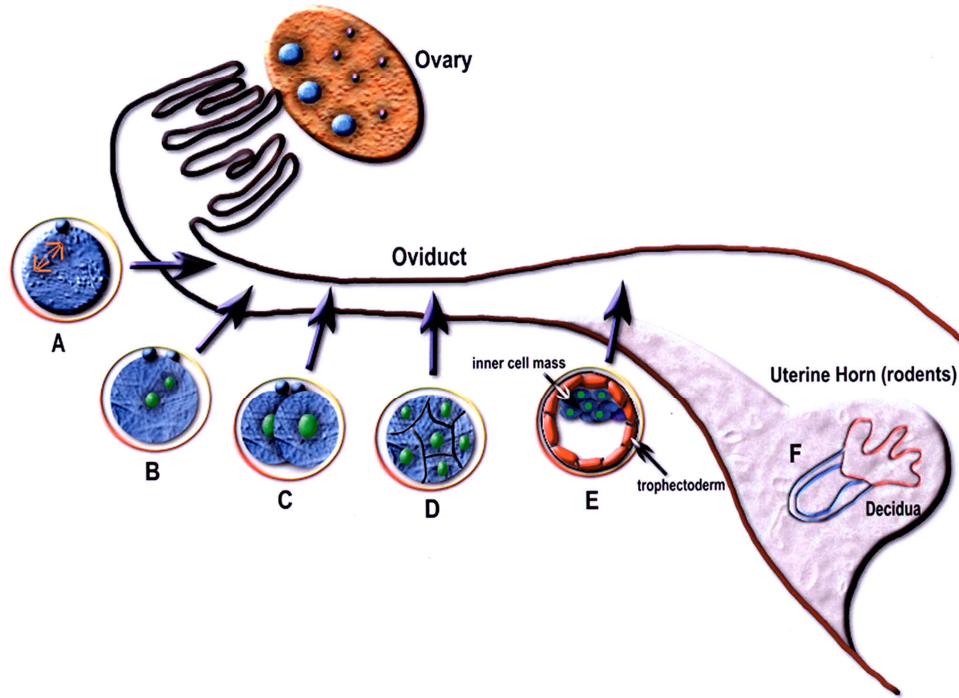
Figure 1. Fertilization and development of the embryo in a mammalian reproductive tract (drawn is an example of a rodent reproductive tract). Upon release from the ovary, the egg undergoes meiotic resumption (**A**). If sperm is present, the first developmental transition, fertilization, will occur in or near the ampula within the oviduct (first large arrow). After fertilization, the egg proceeds down the oviduct developing, first into the one-cell embryo known as the zygote (**B**), which eventually divides into the two-cell (**C**), and four-cell embryo. At the eight- to 16-cell stage, juxtaposed rounded blastomeres flatten (compact) on to one another forming a morula (**D**), which is the first developmental transition unique to mammals. The hallmark of the morula-stage is the onset of both embryonic and cellular polarity, which is first observed in the outside cells of the embryo. As the blastomeres divide further, junctional complexes, membrane pumps (e.g., Na+/K+ ATPase), and channels begin to function in the trophectoderm (red), while the inner cell mass cells (dark blue) remain undifferentiated. Intercellular spaces fill with fluid resulting in a fluid-filled blastocoel at which time the embryo becomes a blastocyst (**E**). As the blastocyst develops further, it enters the uterine horn, hatches, and implants into decidual tissue (**F**).

which ultimately lead to the formation of the organism (Figure 1). The initial sets of instructions within the newly fertilized egg are signal transduction events that begin with a dramatic increase in intracellular free calcium, which eventually propagates into calcium oscillations (7,8). Free calcium ions bind and activate calcium-dependent enzymes such as calcium sensitive protein kinase C isotypes and calcium/calmodulin dependent kinases (9-15) as well as indirectly activate other targets such as small GTP-binding proteins like rabphilin-3A (16). These signals in turn regulate subsequent fertilization events such as metaphase-anaphase transition, second polar body emission, cortical granule exocytosis, pronuclear formation, and activation of the zygotic genome.

Soon after fertilization the early embryo begins to take shape. At about 16-20 hours post fertilization the zygote divides into two, relatively equal-sized embryonic cells (blastomeres) that are almost perfect spheres. Except for normal episodes of cell division and typical G1-G1 growth, blastomeres at this early stage of development are rather quiescent, apolar, and without distinct function, save totipotency. However, this situation soon changes at about the 8-16 cell stage when juxtaposed, rounded blastomeres

alter their shape for the first time by undergoing compaction, the first developmental transition unique to mammals. Compaction is unique because it is at this stage that the spherical blastomeres flatten onto one another resulting in a morula (i.e., ball of cells). This uniqueness is compounded as the changes in blastomere shape completely remodels embryonic environments. The new shape dictates the creation of a polar embryonic environment; one where cells on the inside of the ball only see an inside environment, while cells on the outside of the ball see both an inside and outside environment (5, 17-19). Eventually, the outside blastomeres themselves also acquire a distinct cellular polarity as plasma membranes bathed in the outside environment (i.e., the apical membrane) take on a different structure and function than the plasma membrane adhered to adjacent cells (basolateral membrane; 5, 20).

It is compaction and the establishment of new embryonic and cellular environments that enable the embryo to undergo further remodeling, a process driven primarily by the polarized, outside cells. Soon after compaction as these polarized, outside cells undergo a few more rounds of division, they differentiate into a fully functional epithelial layer, known as the trophectoderm.
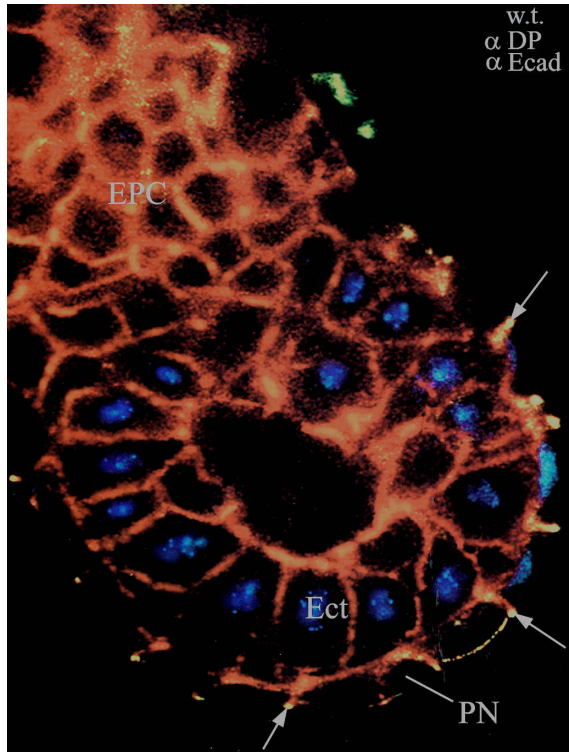
Figure 2. An E5.0 day embryo was dissected from its deciduas and processed for whole mount immuonfluoresence. Antibodies used were: anti-desmoplakin (yellow/green), anti-E-cadherin (red), and DAPI (DNA, blue). Desmoplakin is found only in extraembryonic tissues at this stage of development. Ect, primitive ectoderm (The germ layer that gives rise to the embryo itself); PN, primitive endoderm (extraembryonic tissue); EPC, ectoplacental cone (extraembryonic tissue). Bar = 20 micrometers. Reproduced from Gallicano *et al*. (26) with permission of Academic Press, Inc.

Their differentiation is easily marked by the formation of intercellular junctions such as desmosomes and tight junctions (5, 21-26), expression of simple epithelial keratins 8 and 18 (20, 27, 28), loss of cytoskeletal sheets (29, 30), and Na/K ATPase pumps cleverly positioned on the basolateral membranes. It is the responsibility of the Na/K ATPase pumps in the differentiating trophectoderm to push sodium into interstitial spaces of the morula, which ultimately forms a fluid filled cavity due to osmolarity compensation of water diffusing into the same interstitial spaces. The constant influx of sodium and water expand the fluid-filled cavity (known as cavitation), which eventually becomes the blastocoel. Once the blastocoelic cavity is expanded and the trophectoderm has differentiated, the embryo is considered to be at the blastula stage of development, which is considered the second stage unique to mammalian development.

The blastocyst at this stage contains about 32-64 cells, which comprise two distinct populations, 1) the outer cells that have differentiated into a primary epithelia (trophectoderm), and 2) the undifferentiated inner cell mass cells (ICM). While the cells of the ICM will give rise to the embryo itself, the trophectoderm is responsible for setting up and regulating implantation and placental development, a complete description of which can be found in comprehensive reviews by (31) as well as (32).

The blastocyst stage of development is relatively short-lived, though, as further survival of the embryo is reliant upon its implantation into the uterus. However, it must be noted that prior to implantation the blastocyst must undergo a procedure relatively common to most species; the embryo must "hatch" from its shell of extracellular matrix material known as the zona pellucida. Hatching involves the release of a trypsin-like protease, which digests the zona pellucida. The embryo eventually squeezes through the zona pellucida by way of a small digested hole, which can be less than half the size of the embryo. Recent data have shown the importance of the actin filament network in this process and will be discussed later. Once hatched, further development of the blastocyst is uninhibited and the embryo is free to implant into the uterine lining, invade into the maternal deciduas, and undergo egg cylinder formation.

It is during implantation that the next important developmental transition in mammals is recognized as the cells of the ICM differentiate giving rise to two distinct germ layers, the primitive endoderm and primitive ectoderm. Although general morphology of each layer can be distinguished at the level of the light microscope by the trained eye, recent investigations have begun to uncover new markers that identify both layers with greater accuracy. Primitive endoderm can be identified by the presence of desmosomal markers (Figure 2) (26, 33), the expression of VE-1 (34), bands of tonofilaments (24, 26), the formation of a brush-border on apical membranes (20, 35), and anatomical location of the endoderm layer surrounding the primitive ectododerm observed by conventional electron microscopy (33, 35). One of the easiest methods for identifying germ layers is by immunocytochemistry using antibodies against desmosomal components. There is a complete lack of desmosomes in the primitive ectoderm, while the primitive endoderm and trophectoderm contain numerous small desmosomes (Figure 2) (26, 33). It is the formation of these germ layers that dictate the future body plan of the embryo. Without these distinct layers the formation of the egg cylinder (in rodents) or germinal disk (most other mammals) would not be possible, and in turn, the next extremely important stage in mammalian embryogenesis, gastrulation, would not be possible, and as many of the readers know "It is not birth, marriage, or death, but gastrulation, which is truly the most important time in your life. (Lewis Wolpert, 1996)

3. WHAT DRIVES EARLY MAMMALIAN DEVELOPMENT THROUGH EACH DEVELOPMENTAL TRANSITION?

With respect to the unique behavior of blastomeres during early development, the obvious must be noted; they are cells, and like their somatic brethren, they must follow the rules of all cells that continue through the cell cycle. These rules are dictated by certain cell growth and proliferation housekeeping genes, which are found in
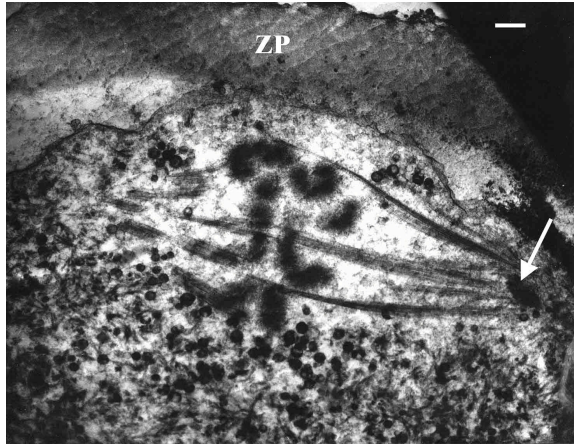
Figure 3. Electron microscopic view of an intact (not detergent-extracted) mouse egg, obtained by intermediate voltage electron microscopy (IVEM). Two distinct cellular regions are visible; the region of the meiotic spindle apparatus, including one spindle pole (arrow), and the cytoplasmic region. ZP, zona pellucida. Bar = 2.0 micrometers

both somatic cells and blastomeres. However, because the egg and early embryo face and overcome specialized challenges and functions during development, exemplified by each developmental transition, a specialization within the blastomeres of the early embryo must exist to meet these demands. Numerous studies, many of them quite recent, have focused on the structural aspect of the early embryo primarily because the hallmark of each developmental transition is a dramatic change in the shape of blastomeres, which in turn alter the shape of the embryo. Regulation of cellular structure (cytoarchitecture) within any cell is dictated primarily by cues from the cytoskeleton, and it appears that early embryogenesis is no different in this regard. What *is* extraordinary about the cytoarchitecture within early mammalian embryos are the exquisite cytoskeletal specializations that have evolved to meet these challenges.

All eukaryotic cells contain a cytoskeleton, a complete description of which can be found in most cell and molecular biology textbooks. In brief, the cytoskeleton is comprised of three independent types of networks, actin filaments, microtubules, and various types of intermediate filaments. Each cytoskeletal network interacts with a plethora of cytoskeletal-associated proteins, which in some cases delineate the function of a cell. A prime example can be seen in neurons where function is dictated by a distinct configuration of neurofilaments and mictrotubules within axons. *A priori*, similar to the cytoskeleton in cells of adult organisms, the cells of the early mammalian embryo also contain all three cytoskeletal components, which can also delineate the function(s) of embryonic cells. Unfortunately, the number of investigations delving into cytoskeletal components, function(s), and regulation in somatic cells is far greater than the number of similar investigations in mammalian embryos. However, thanks to the advent of novel technologies and ingenuity, these types of investigations on the cytoskeleton during mammalian development have intensified.

3.1. The cytoskeleton during fertilization

The act of fertilization relies heavily on the cytoskeleton. As previously noted, the egg is poised for fertilization by a single sperm, which, during normal fertilization, fuses to the plasma membrane of the egg (for a highly detailed review of sperm-egg fusion see 36). Initially, the head of the sperm is engulfed by microvilli on the egg surface, followed by entry into the cortical region of the egg. The cytoskeletal backbone of microvilli is comprised of actin filaments and appears to be similar to microvilli found in most cells containing a brush border. Once in the egg cortex, the sperm head becomes enmeshed in an intricate array of filaments (37). One novel approach for investigating the interaction of the cortical cytoskeletal network and the sperm head in mammalian eggs was developed and refined by Webster and McGaughey, (37) who made cortical patches and platinum replicas of eggs before, during and after sperm penetration. In that study it was found that cortical cytoskeleton in mammalian eggs is organized into two distinct networks: a loose network of actin filaments surrounding localized dense networks composed of non-actin filaments (37). During fertilization, the actin-based loose network is intimately associated with the head and neck of the sperm (37) suggesting that this network may be necessary for normal fertilization. In support of this observation, studies by Maro *et al*., (38) in mouse eggs, as well as Le Guen *et al*., (39) in sheep eggs showed that the cortical actin network was indeed necessary for fertilization to occur normally. Sheep eggs incubated in the actin depolymerizing drug cytochlasin D blocked formation of the incorporation cone (39) and incorporation of the sperm tail (40). However, it must be noted that although the cortical actin network is necessary for fertilization to occur normally, sperm entry was still observed, albeit less efficiently, in eggs where the actin network was disrupted (39).

Another essential function of the cortical actin network is its association with the spindle apparatus (described below). In cow, sheep, and rodent eggs, ultrastructural analyses have repeatedly shown the spindle apparatus attached to the cortical actin network (37, 39, 41; G. I. Gallicano personal observations). This observation was confirmed biochemically when eggs incubated in Cytochlasin D resulted in the migration of the spindle apparatus away from the plasma membrane (39). Longo and Chen, (42) as well as Maro *et al*., (38) also showed that the cortical actin network was responsible for a phenomenon found primarily in rodent eggs known as spindle rotation where the entire spindle apparatus turns 90 degrees to properly orient half the chromosomes for deposition into the second polar body. Prior to egg activation the chromosomes within the spindle lie at roughly a 90-degree angle to the egg surface (Figure 3).

Formation of the second polar body (PBII; the product of unequal cytokinesis in the egg) is also highly reliant on an intact actin network (39). Experiments in which eggs were incubated in actin poisons repeatedly resulted in PBII malformation upon egg activation (41, 42). Recently, the mechanisms that control the actin network during PBII formation were investigated resulting in the
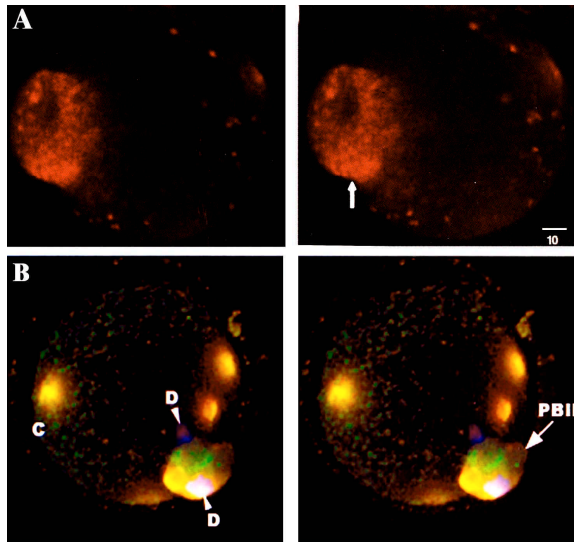
Figure 4. (**A**) Stereoscopic confocal view of a mouse egg transiently (5 min) activated with calcium ionophore and incubated in media for 50 min, followed by fixation and brief incubation in the PKC reporter dye, Rim-1. The PBII is well initiated and is in the process of being emitted (arrow); however, the contractile ring (at the base of the initiated PBII) has not begun to close. PKC is localized with Rim-1, on the lateral aspects (arrow) of the PBII, but not at its apex. (**B**) Stereoscopic confocal view of a mouse egg treated similarly as in (**A**) except following a 55-65 min incubation in media post activation, it was probed with antibodies to CamKinase II (green), calmodulin (red), and DAPI (purple). Yellow represents colocalization of CamKinase II and calmodulin (red and green). CamKinase II is found on the contractile ring at the base of the PBII. D, DNA; C, egg cortex; PBII, second polar body. Bar in **A** = 10 micrometers. Figure 4A reproduced from Gallicano *et al.*, (12) with permission from Wiley and Sons, Inc. Figure 4B reproduced from Johnson *et al.*, (17) with permission from Academic Press, Inc.
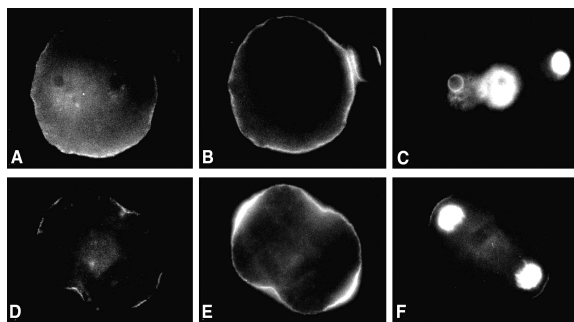

Figure 5. Fertilized mouse eggs and early 2-cell conceptuses triple stained with polyclonal anti-tropomyosin CG3 (A, D), rhodaminyl lysine phallotoxin (RLP) for microfilaments (B, E), and Hoechst 33258 for DNA (C, F). (A-C) Fertilized eggs with pronuclei, stained with monoclonal CG3, showing cortical tropomyosin. (D-F) Late anaphase of the first cleavage, stained with monoclonal CG3, showing tropomyosin in the cortex of the cleavage furrow and over the chromosomes. Reproduced from Clayton and Johnson (46) with permission from Academic Press, Inc.

identification of distinct signal transduction pathways, one of which was responsible for initiating the PBII while another controlled PBII emission. Within those signaling pathways at least two kinases were identified: protein kinase C-alpha for the former (PBII initiation) and calcium calmodulin dependent protein kinase II (CamKinase II) for the latter (Figure 4) (9, 12, 15, 43). These investigations have shown that once activated, PKC-alpha forms a ring around the base of the forming polar body (Figure 4) (12) apparently phosphorylating components necessary in the initial formation of the PBII. Johnson *et al.*, (15) confirmed these results and showed further that CamKinase II was localized to the detergent-resistant cytoskeleton of the polar body being emitted. Antagonists of these kinases clearly inhibited second polar body initiation and emission. Although further investigation is needed to undeniably confirm that these kinases directly act on the actin filament network to form the second polar body, the data from the aforementioned studies allows a safe assumption that these kinases function at fertilization by acting upon the actin filament network either directly or through actin-associated proteins to form the second polar body.

One actin-associated protein that is gaining interest during fertilization and subsequent stages of early mammalian development is tropomyosin. In non-muscle cells tropomyosin acts as an actin-binding protein associated with actin filament stabilization (44). Soon after fertilization, at least three isotypes of tropomyosin have been identified (45). Immunofluorescent localization of tropomyosin clearly shows that it resides primarily in the cortex region of newly fertilized eggs (Figure 5). This cortical localization persists until the 8-16 cell stage when it becomes polarized at the apical plasma membrane (Figure 6) (45). It also must be noted that tropomyosin associates with the cleavage furrow late during cytokinesis in mammalian embryos. Its function during cytokinesis in blastomeres is not known; however recent experiments using the yeast *S. pombe* in which the cdc8 gene product, a tropomyosin homologue, was ablated, cytokinesis was disrupted (46, 47). Taken together, these data suggest that in embryos, tropomyosin in association with other actin-associated proteins may function in the contractile role during cytokinesis of blastomeres.

Functions for many other actin binding proteins have been examined as well; however, questions remain for many of them. For example, fodrin, which co-localizes with actin in a region overlying the spindle apparatus, was found localized to a fodrin-actin cap external to the spindle (48). However, the function of this cap remains obscure. It is hypothesized that the cap may aid in meiotic arrest by crosslinking with actin filaments, thereby stabilizing the network and preventing premature cytokinesis. Such speculation is intriguing; however, to test this hypothesis, it will be necessary to construct mice whose eggs contain an inducible form of a fodrin knockout gene thereby removing or inhibiting production of fodrin protein. Alternatively, injection of fodrin inhibitory antibodies into eggs prior to or during fertilization could be used to disrupt the fodrin-actin cap enough to gain a better understanding of this protein.
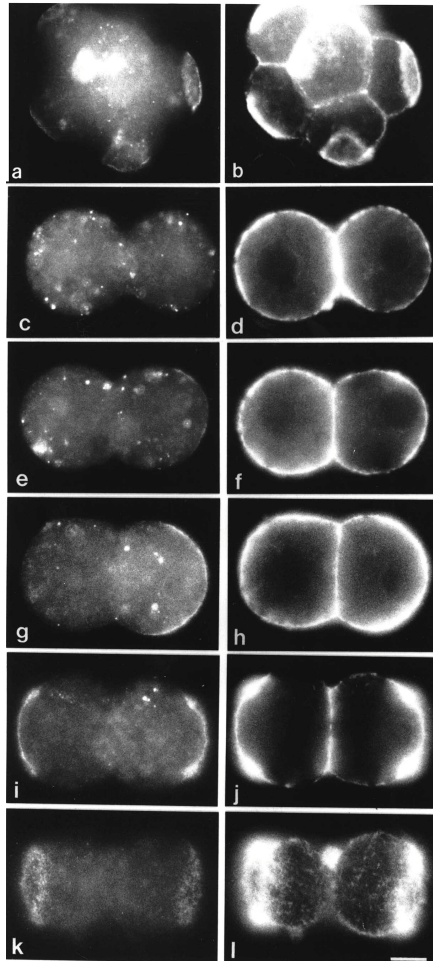
Figure 6. Whole 8-cell conceptuses (**a, b**) or 2/8 pairs of blastomeres (**c-k**) stained with polyclonal anti-tropomyosin (**a, c, e, g, i, k**,), RLP for microfilaments (**b, d, f, h, j, l**). The cells were also stained with Hoechst 33258 for DNA to check the state of the nuclei (not shown in this Figure). (**a, b**) Late 8-cell conceptus, around, 8 h postdivision, showing poles of tropomyosin staining (**a**) colocalized with microfilaments (**b**). There is no apparent tropomyosin localization with the microfilaments at baso-lateral surfaces. (**c, d**) 0-1 h postdivision 2/8 pair showing patchy cortical RLP staining in both blastomeres (**d**), but little cortical tropomyosin (**c**). (**e, f**) 2-3 h 2/8 pair in which the right hand blastomere shows faint cortical tropomyosin (**e**), whereas both blastomeres show complete, though patchy, cortical and basal microfilament staining (**f**). (g, h) 4-5 h 2/8 pair showing cortical tropomyosin in one blastomere (**g**) with coincident solid, i.e., nonpatchy, RLP staining (**h**). (**i, j**) 8-9 h 2/8 pair showing both blastomeres polar with respect to tropomyosin and microfilaments. Note the poles appear to occupy a smaller area of the blastomere surface than those of earlier time points. Note there is no tropomyosin at basolateral surfaces. (**k, l**) 10-11 h 2/8 pair photographed at a different focal plane to show surface concentrations of microfilaments (**l**) and tropomyosin (**k**) staining at the blastomere surface. Bar, 10 micrometers. Reproduced from Clayton and Johnson (46) with permission from Academic Press, Inc.

The actin network and its associated components are undoubtedly important for fertilization to occur normally. However, fertilization is also highly dependent on the microtubule cytoskeleton. Once a sperm is firmly ensconced within the egg cytoplasm, two simultaneous microtubule-dependent events must occur to complete fertilization and induce formation of the zygote. The first event entails the expulsion of half the chromosomes into the second polar body via the metaphase-anaphase transition of the spindle apparatus. The second is pronuclear migration. Both events occur in a time-dependent manner and are regulated by signal transduction events.

The spindle apparatus is a wine-barrel shaped structure with fat poles, and in rodents is specifically anchored in the cell cortex so that the chromosomes lie at roughly a 90-degree angle with respect to the plasma membrane (Figure 3). As discussed previously, immediately after fertilization and prior to its separation, the spindle must rotate (about 90 degrees), a process reliant on the actin filament network. Three types of tubulin are found in the spindle, alpha, beta, and gamma (49, 50). The alpha and beta forms of tubulin comprise the filamentous microtubules that emanate from the spindle poles and attach to the chromosomes, while gamma-tubulin resides in the spindle poles (51), and functions in microtubule nucleation (52). Although two other tubulins, delta and epsilon, have recently been found in microtubule organizing centers (MTOCs) of mammalian origin (53), their localization or functions in eggs and embryos have not been determined.

The spindle poles in mammalian eggs and early embryos are, for the most part, analogous to centrosomes or MTOCs in adult somatic cells in that they are capable of nucleating and elongating microtubules. However, there is one exception to the similarity, which has brought on a biological controversy. Spindle poles in mammalian eggs and early embryos do not contain centrioles. What are centrioles and why the controversy? Centrioles are cytoskeletal structures composed of acetylated alpha-tubulin and beta-tubulin subtypes (54-56), which combine to form 9 triplets of tubules arranged in a 9 + 0 circular configuration (unlike axonemes found in sperm tails or cilia which have tubules arranged in a 9 + 2 circular configuration). One centriole is usually found at a $90°$ angle to its partner with each centriole ranging from about 0.3-0.5 micrometers in length. In most cases the centriole is surrounded by a dense cloud of material comprised of proteinacious components, our knowledge of which is quickly expanding (e.g., pericentrin, gamma-tubulin, basonuclin, cennexin, etc.).

Investigations have demonstrated that centrioles disappear from centrosome at or near the pachytene stage of meiosis I early in oogenesis (57, 36) and are absent through the first few cleavage divisions after fertilization (58). They reappear in blastomeres prior to embryo implantation. During early stages of meiosis I, acentriolar centrosomes comprising the spindle poles can be visualized by immunofluorescence using antibodies to pericentriolar material such as gamma-tubulin or the 5051 antibody (36, 59). Recent investigations have revealed that these

acentriolar centrosomes form in immature mouse oocytes from two large gamma-tubulin-positive structures that are multivesiculated and localized in the oocytes cortex (36, 60, 61). As the oocyte matures (still in meiosis I) the multivesiculated structures migrate to either side of the germinal vesicle where they function similarly to MTOCs (36). However, even with these recent findings, the reason for the absence of centrioles remains a mystery. That said, the fact that acentriolar MTOCs exist, and the fact that they seem to function very similarly to MTOCs that contain centrioles (Both can nucleate microtubules, which during M-phase extend towards the condensed chromosomes to form a spindle apparatus) has, consequently, clouded further the already perplexing question of centriolar function.

The primary function of the acentriolar spindle apparatus is seen during the metaphase-anaphase (I and II) transitions where the spindle provides a template for segregating half the egg's homologous chromosomes into the first polar body during meiosis I, followed by segregation of one copy of each chromosome into the second polar body during meiosis II. The final result of these segregations leaves in the eggs, one copy of each chromosome, which is united with the haploid set of chromosomes distributed by the incoming sperm. Although numerous, rather beautiful studies have described the movement of chromosomes as the spindle apparatus separates (see reviews by 60, 62), far fewer studies have defined the intracellular signals that control this event. One reason for the relative paucity of investigations is the difficulty entailed in analyzing incredibly small quantities of material comprising early eggs and embryos. However, with the advent of new technologies as well as some clever retooling of older techniques used for deciphering signaling cascades in cell lines and tissue, the signals involved in regulating the M-phase transition of the spindle are slowly being resolved.

It is well known that the M-phase transition is elicited by a rise in intracellular free calcium, which activates and deactivates numerous known and unknown downstream intracellular signaling cascades. Some of the known signals include cytostatic factor (CSF; 63) and cyclins (64, 65), which are deactivated upon fertilization, and PKC and CamKinase II (see above) as well as and rabphilin-3A (16), which are activated as a result of fertilization (please see Figure 1 in 43). Another kinase, MAP Kinase (active in the egg), is neither activated nor deactivated as a direct result of fertilization; however, recent studies have shown MAP Kinase to be an integral component of the fertilization process.

The metaphase-anaphase transition of the spindle requires at least two specific kinases, MAP Kinase and CamKinase II. Both are directly associated with the spindle and both rely on an increase in cytoplasmic calcium concentrations to regulate their activities. CamKinase II is regulated by calmodulin, a calcium binding protein that can interact with other proteins depending on how many molecules of calcium (between 0 and 4) are bound (15). In unfertilized eggs, CamKinase II is tightly associated with the meiotic spindle. Almost immediately after fertilization and the initial rise in intracellular free calcium, calmodulin colocalizes tightly with CamKinase II on the spindle. It was shown elegantly in a permeabilized egg system that at physiological levels, addition of CamKinase II, ATP, calmodulin, and calcium were capable of driving the spindle into an anaphase configuration when calcium and calmodulin were present in a 4 to 1 ratio (15). Eggs incubated in the presence of CamKinase II inhibitors result in the inhibition of the metaphase-anaphase transition both *in vivo* and *in vitro* in the permeabilized egg assay.

The role of MAP Kinase during fertilization; however, is only slowly becoming evident. Known is the fact that as eggs reach meiotic metaphase II, a pool of MAP Kinase becomes active (66-68) and localizes to the spindle apparatus in close proximity to CamKinase II (69). A large pool of inactive MAP Kinase also is present in eggs; however that pool resides in the cytoplasm and does not appear to associate with the spindle apparatus (69). An interesting set of experiments recently demonstrated that the active form of CamKinase II, which is associated with the spindle apparatus, may directly or indirectly associate with the active form of MAP Kinase. It was shown that the level of activity of MAP Kinase was reliant upon CamKinase II activity as eggs incubated in KN93, a CamKinase II inhibitor, or myristolated AIP, an inhibitory peptide specific for CamKinase KII, decreased both CamKinase II activity and MAP Kinase activity (KN93 and myristolated AIP are not an antagonist of MAP Kinase). Based on these data and the fact that MAP Kinase contains CamKinase II consensus phosphorylation sites (GeneBank Accession #'s Z14249 and X58712), an interesting hypothesis was proposed, suggesting that CamKinase II may potentiate MAP Kinase activity preventing its degradation (69). Why would the fertilized egg do this? One potential function for active MAP Kinase is to inhibit pronuclear formation (69). CamKinase II may keep MAP Kinase active to prevent precocious formation of the pronucleus. Yet to be established, however, is whether CamKinase II directly phosphorylates MAP Kinase or phosphorylates other kinases (e.g., MEK, MEKK), which in turn phosphorylates MAP Kinase.

The second microtubule-dependent event that is necessary for normal fertilization and zygote formation is the establishment of the cytoplasmic microtubule network. The primary responsibility of this network is to promote migration of the male and female pronuclei. Interestingly, the cytoplasmic microtubule cytoskeleton is assembled differently in different mammalian species. Most mammalian species rely on the incoming sperm to provide a centrosome (and centriole) for nucleation of cytoplasmic microtubules. Rodents, on the other hand, do not use the sperm centrosome. Instead they rely on a dozen or so cytoplasmic asters (acentriolar MTOCs) randomly located in the cytoplasm of the unfertilized egg for formation of cytoplasmic microtubules (70). In each case, however, as the male and female pronuclei form, microtubules emanating from either the sperm centrosome or cytoplasmic asters quickly fill the cytoplasm and contact the male and female pronuclei. Once bound, minus end-

oriented microtubule motors then move the pronuclei into apposition (reviewed by 70,62) where they wait (unfused) until the zygote enters the first mitotic phase, nuclear envelope breakdown occurs, and the zygote enters the two-cell stage.

What controls the second microtubule-dependent event? Unfortunately, compared to the number of investigations that have studied the signals, which regulate the cytoskeleton during initial episodes of mammalian fertilization, far fewer have studied the intracellular signals that control the latter stages of cytoskeletal evolution in the zygote. In fact, the only intracellular signal that has been studied in any detail is PKC and its catalytic subunit, PKM (9, 11, 71). This kinase, however, has not been shown to seriously affect directly either the microtubule or actin filament networks. Instead, it was shown to associate with and drastically alter the third cytoskeletal network found in mammalian eggs and embryos, the intermediate filament network.

The mere mention of intermediate filaments residing in the egg and early embryo may be a surprise to some readers. Skepticism has arisen primarily because of the disagreements and semantics used in detailing when expression of intermediate filament genes occur during embryogenesis and when intermediate filament proteins are first observed. With regards to gene expression, the evidence is quite clear. The keratin 8 and 18 genes are the first intermediate filament genes expressed in the mammalian embryo (27, 72-74). K8 and 18, like all keratin subtypes of intermediate filament proteins, must pair with each other to make a filament. Each pair of keratins is expressed in distinct tissues. For example, keratin filaments in the basal layer of the epidermis are comprised primarily of K5 and K14, while keratin filaments in simple epithelial tissues such as the trophectoderm, visceral endoderm, and liver are comprised of K8 and K18. Activation of the K8 and K18 genes has been shown to be as early as the compaction stage of embryogenesis; however it is possible that the K8 and 18 genes may be active earlier since K8 and 18 proteins have been detected as early as the 2-4 cell stage (20). However, because maternal mRNA is stored in eggs prior to fertilization, and activation of the zygotic genome, exactly when the K8 and K18 genes become active in the embryos has not been accurately determined.

With regards to when intermediate filament *proteins* are first observed, the disagreements run much deeper. Some investigations have stated that intermediate filament proteins are not seen until compaction or blastocyst formation (27, 73, 75, 76). However, at least three independent laboratories have detected intermediate filaments in oocytes and eggs of many different mammalian species including, mice, hamsters, rats, porcine, bovines, canines, sheep, and humans (10, 20, 30, 77-82). While it is possible that the intermediate filament proteins found at compaction and later stages are synthesized by embryonic genes, it is quite clear that the intermediate filaments residing in oocytes and eggs are maternal, having been synthesized during oogenesis (81-83). A further discrepancy has arisen in the literature

concerning how the intermediate filaments are arranged in eggs and early embryos. While a few investigations have identified individual intermediate filaments within eggs (79-82), the majority of investigations have shown that the physiological localization of intermediate filaments is in a highly crosslinked network, which is completely coated with a layer of one or more proteins. Based on numerous ultrastructural observations the orientation of crosslinked filaments and protein coat give these cytoskeletal structures a sheet-like appearance, hence they have been termed "cytoskeletal sheets" (29, 31, 83, 84).

What evidence exists suggesting that the cytoskeletal sheets are composed of intermediate filaments? The evidence is strong as eggs and early embryos from several mammalian species were subjected to numerous different biochemical and ultrastructural methodologies. One of the first observations made from employing these approaches was the finding of two types of sheets, one with a fibrous composition and the other a more planar composition (29, 77, 83). Each species examined contained only one type of sheet. Eggs from humans, bovines, porcine, canines, and mice contained fibrous sheets, while rat and hamster eggs contained planar sheets (77). The fact that the two types of sheets could be found in either mouse eggs (fibrous) and hamster eggs (planar) greatly enhanced the speed and accuracy of the study primarily because there was a relatively ample supply of eggs from these species upon super ovulation.

To visualize the cytoskeleton in eggs and embryos a unique type of electron microscopy was employed known as embedment-free electron microscopy (EFEM). Since its inception in 1980's in the Penman laboratory at M.I.T. (85), numerous investigators have provided detailed observations of the cytoskeleton within eggs, embryos and somatic cells. This type of microscopy has been repeatedly proven powerful because it allows examination of relatively thick sections (~400nm) thus providing more spatial information within the cell than conventional thin-section electron microscopy. The power of this technique is amplified if cells are first extracted with non-ionic detergents such as Tween-20 or Triton X-100, which remove a large portion of the aqueous cellular components leaving behind the detergent-resistant cytoskeleton.

By using different types of detergents on mouse and hamster eggs it was possible to systematically remove sheet components, which in turn revealed sheet substructure. When mouse eggs were analyzed after detergent extraction in medium containing a relatively weak detergent, Tween 20, sheets had a fibrous appearance; however, detergent extraction in a harsher detergent, Triton-X 100 (removed more proteins that Tween-20), revealed a distinct substructure to the sheets. It was clear that fibrous sheets were composed of 10-11nm filaments lying side-by-side (Figure 7A) (77). In contrast, a discernable difference was not observed upon examination of sheets in hamster eggs after extraction in either Tween-20 or Triton X-100 (Figure 7B). Only when hamster eggs were subjected to a mixed micelle of detergents containing non-ionic and a small amount of ionic detergents was the
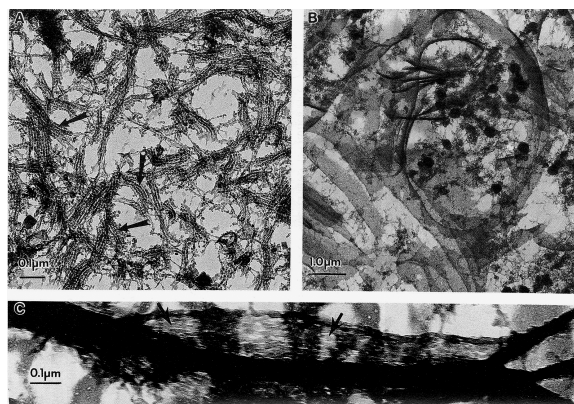
Figure 7. Embedment-free electron microscopic views of sheets in a mouse and hamster eggs extracted with different detergents. (**A**) High-magnification view of a mouse egg extracted with Triton X-100 shows that sheets (arrows) are composed of 10- or 11-nm filaments lying side by side. Note, each filament exhibits a periodicity of 20.6 ± 1.6 nm. (**B**) The sheets in this view of a hamster egg, which is extracted with Triton X-100, do not exhibit a substructure similar to that found in sheets in mouse eggs extracted with Triton X-100. (**C**) Cytoskeletal sheets in a hamster egg after treatment with a mixed-micelle detergent containing 0.2% deoxycholate and 1.0% NP-40 in the extraction medium prior to fixation shows that these sheets, like those in the mouse egg, are composed of distinct fibers (arrows). Each fiber has a diameter of 10 or 11 nm. Figure 5A is reproduced from Gallicano *et al*. (30) with permission of Wiley and Sons, Inc. Figure 5C is reproduced from Capco *et al*. (86) with permission of Wiley, Inc.
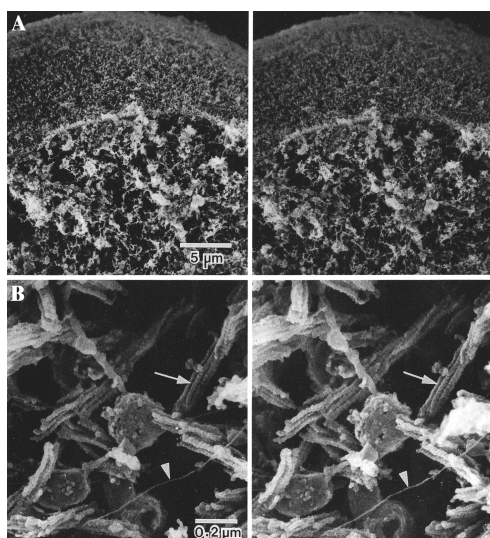
Figure 8. Low and high magnification scanning electron stereo-pair micrographs of a mouse egg, routinely fixed, but damaged during microscopial preparation. (**A**) Low magnification view shows exposed cytoplasmic contents within the egg. (**B**) Higher magnification of cytoplasm of egg illustrated in (**A**). Note the bundles of corrugated filaments (arrow). Microfilaments are also present (arrowhead). Reproduced from Holy *et al*. (87) with permission of Wiley and Sons, Inc.

distinct layer of proteins that coat the sheets removed revealing the underlying 10-11nm filaments lying side-by-side (Figure 7C) (86).

High-resolution images of detergent extracted eggs and embryos using intermediate voltage electron microscopy (IVEM) revealed in more depth the filamentous nature of both the fibrous and planar sheets (78, 86). IVEM was used because it allowed the use of plastic-embedded sections, which were rather thick (~500nm), and thus provided a different approach for analyzing the sheets with increased spatial information. The plastic also prevented any potential collapse of sheets onto the supporting grid material as can happen when using EFEM.

Although EFEM showed fibrous sheets (mouse) containing intermediate filaments lying side-by-side, IVEM revealed more information demonstrating distinct crossbridges spaced 23-25nm apart holding each filament in register (78, 86). Vesicles were also found in close contact with the sheets; however, their composition and function was not determined. Direct cross sectional views of sheets revealed electron-dense dots with each dot being 10-11nm in diameter and linked by crossbridges. The most striking characteristic of these sheets was that they were arranged into cylindrical bundles, a morphology later confirmed by quick-freeze, deep-etch (QFDE) electron microscopy and by scanning electron microscopy (78, 86, 87). Stereoscopic view of a scanning electron micrograph revealed an obvious filamentous nature to the fibrous types of sheets as well as the ease at which the individual filaments can be stripped away from a sheet under the correct conditions (Figure 8) (87).

Comparing the fibrous sheets in mouse eggs to the planar sheets of hamster eggs revealed that only the proteins covering the sheets and the secondary structure of planar sheets were slightly different. The underlying components (i.e., the 10-11nm filaments) of the two types of sheets appeared to be similar. Once again, using EFEM, IVEM, QFDE, and scanning electron microscopy, the planar sheets were analyzed. Cross sectional views using IVEM showed that they were composed of two layers of 10-11nm filaments held in register by vertical cross bridges spaced 23-25nm apart. *En face* views of these sheets extracted with the correct detergent regime revealed the existence of 10-11nm filaments lying side-by-side connected by lateral crossbridges (86). The planar appearance of these sheets, instead of a more fibrous appearance, most likely results from the extra layer of filaments, extra addition of crossbridges that hold the layers together, and the protein coat covering the sheet complex (see below).

Since the substructural composition of both types of sheets was undoubtedly that of intermediate filaments, the next question was to determine which type? At first it appeared that the intermediate filaments within sheets were not of the keratin types. This observation stemmed from two facts: 1) that antibodies to K8 and K18 (the first keratin genes expressed in embryos) did not bind to sheets, and 2)
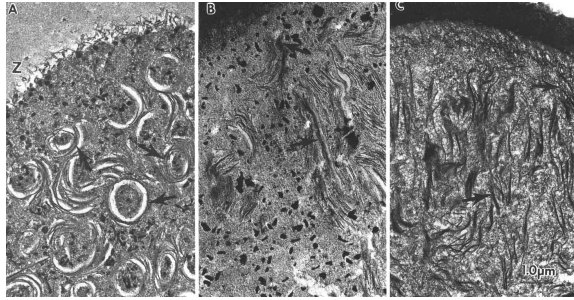
Figure 9. Cytoskeletal sheets in detergent-extracted, fertilization-competent hamster eggs and detergent-extracted, fertilized eggs. (**A**) Unfertilized hamster egg shows sheets (arrows) in a whorl-like conformation. Sheets are excluded from the egg periphery. The egg is surrounded by its zona pellucida (Z). (**B**) Sheets (arrows) in the fertilized egg are linear and also appear to extend into the zygote periphery. (**C**) Sheets in hamster eggs are activated by PMA are linear and appear to extend into the egg periphery. Similar results are obtained when eggs are incubated in DiC8 or calcium ionophore (data not shown). All eggs were judged fertilized or activated from the existence of a second polar body viewed prior to extraction and fixation. Reproduced from Gallicano *et al*. (11) with permission from Academic Press, Inc.

antibodies to other keratin types also did not bind to sheets in eggs that were not detergent extracted (Gallicano unpublished observations). However, based on the fact that different detergent regimes could systematically remove material from the sheets and uncover their underlying intermediate filament substructure, this approach was used in conjunction with embedment-free electron microscopy and broad-spectrum keratin antibodies to analyze the biochemical nature of the sheets. While antibodies to keratins 5, 6,, 16, and an unknown type termed "Z" (24) bound to sheets after localization with gold-labeled secondary antibodies, antibodies to K8 and 18 as well as tubulin, and vimentin did not (78). Antibodies to actin weakly bound to sheets, but the relevance of this staining has not been determined. Verification of keratin labeling was seen in one- and two-dimensional western blots made from 250-300 detergent extracted cytoskeletal enriched mouse eggs (78). In two dimensional western blots, antibodies directed against five keratin types revealed K5, K6, its pair K16, K8 (at very low levels), and a 61 kD keratin similar to type Z found by Jackson *et al*. (88). As added proof, an antibody directed against K15 recently stained the cytoskeletal fraction of 200 mouse eggs in a one-dimensional western (Gallicano unpublished data). Finding K15 in eggs was important because K5, which usually partners with K14 to make a filament in the basal layer of the epidermis (89), can also pair with K15 in the absence of K14 (Lloyd *et al*., 1995). K14 was not found in one or two-dimensional westerns of mouse eggs. As a result, K5 may pair with K15 in the sheets.

Why would K5, 15, 6, and 16 reside in eggs and early mammalian embryos? These keratins are interesting in that they are found primarily in stem cells or cells undergoing marked proliferation (89, 90). The ultimate stem cell is the fertilized egg and while K5 and K15 are not needed (at least structurally as in a typical keratin filament network) until the morula or blastula stage when they would function by binding to desmosomes, they may be stored in the egg so the embryo does not have to waste energy synthesizing the intermediate filaments necessary for survival. K6 and K16 on the other hand are special keratins. Except for some low level expression in palmer and planter epidermis, tongue, and outer root sheath of the hair follicle, they are usually only highly expressed in hyperproliferative epithelia that have been wounded (90, 91, 92). Recently, a novel function for K16 was introduced after transgenic mice were constructed in which either a full-length K16 gene or a K16 head-rod/K14 tail chimeric gene was targeted to the epidermis. While the full length K16 gene drastically altered the cytoarchitecture of epithelial cells, the K16/K14 hybrid did not suggesting that K16 may function by altering the cytoarchitecture of certain cell types. Furthermore, the results also implied that it was the COOH-terminal tail domain of K16 that was responsible for its function (93). The fact that the egg and early embryo must undergo dramatic cytoarchitectural changes in conjunction with the stem cell function of each blastomere may explain the presence of K5, 15, 6 and 16.

To date a direct function for the sheets in the fertilization process has not been determined. Their function appears to be storage sites for intermediate filaments, which are used later in development (see below). However, to say the sheets are without function before, during, and immediately after fertilization may be a bit premature. Both the fibrous and planar types of sheets have been found to be very dynamic structures in that they undergo drastic spatiotemporal changes soon after fertilization (11, 29, 30, 78). Before fertilization sheets are excluded from the cortical region of the egg. Approximately 45-50 minutes after egg activation using a calcium ionophore or by *in vitro* fertilization, the sheets enter the cortical region of the egg. They do not directly contact the plasma membrane of the cell, but electron micrographs show them enmeshed in the cortical cytoskeleton. Planar sheets have an added feature. Prior to fertilization 80-85% of these large sheets are distinctly arranged in a whorled configuration (Figure 9A). Again, 45-50 minutes after egg activation (fertilization) 80-85% of the planar sheets are completely linearized (Figure 9B,C) (11). Why sheets undergo such drastic changes upon fertilization is not known; however, it appears that specific signal transduction events are responsible for regulating the spatiotemporal changes the sheets undergo.

While investigating the role signal transduction events played in remodeling the egg into the zygote, a serendipitous observation resulted in the first evidence of an active form of protein kinase M, the catalytic subunit of PKC-alpha, in mammalian eggs (11). PKC-alpha is activated as a dual result of the increase in intracellular free calcium and the increase in diacylglycerol in the plasma membrane (see above). Once PKC performs its function phosphorylating components at or near the plasma membrane it must be down regulated. PKC has been shown to be down regulated by a number of ways, one being cleavage between its membrane-binding domain and the

catalytic subunit of the kinase (94-99). The catalytic subunit is then free to diffuse away from the membrane where it becomes both membrane and calcium independent. The catalytic subunit can be tracked in the cytoplasm by its reduced molecular weight (upon western blotting), by antibodies or dyes specific for the catalytic subunit of PKC-alpha, and its ability to phosphorylate cytoplasmic targets *in vivo* and synthetic peptides *in vitro* (11). PKM was identified in the literature as early as 1977 by Inoue *et al*. (100) and Takai *et al*. (101). The kinase associated with the sheets after fertilization was the correct molecular weight to be PKM (no kinase with a molecular weight appropriate for PKC-alpha was detected associated with the sheets), it was recognized by an antibody directed against the catalytic subunit of PKC-alpha, and it was able to phosphorylate an exogenous substrate for PKC/PKM under conditions in which only PKM, and *not* PKC-alpha, would be active. In addition, inhibitors to protein kinase A, tyrosine kinase, and myosin light chain kinase, added to the reaction mixture, did not block the phosphorylation of the exogenous substrate; however, inhibitors to PKC/PKM added to the reaction mixture inhibited phosphorylation (11). Phosphorylation experiments showed two substrates for PKM, K16 and one of the coat proteins that cover the sheets, p69 (11).

Further evidence for the role of PKM and sheet restructuring was observed in an *in vitro* set of experiments in which hamster eggs (containing planar sheets) were permeabilized, followed by perfusion with ATP, and ATP regenerating system, and PKM. Under these conditions, the planar, whorled sheets linearized and entered the cortical region of the egg, a result virtually identical to that seen *in vivo* upon fertilization or parthenogenic activation. It must be noted that when PKM was excluded from the perfusion mixture, the sheets did not alter their whorled configuration nor did they enter the egg cortex (11).

Evolution of a PKC/PKM pathway seems reasonable primarily because of its efficiency. PKC acts first at the cell periphery triggering cellular events such as cortical granule exocytosis and second polar body initiation, followed by its cleavage to deactivate its role in the periphery. The titer of PKM slowly builds resulting in its association with the sheets. This progression follows the logic that the egg periphery is remodeled, followed by the interior upon fertilization.

3.2. The cytoskeleton during compaction

Embryonic compaction occurs at the 8-16 cell stage and is marked by two distinct changes in the embryo. The first is a change in blastomere shape from spherical to flattened, while the second is a change in shape of the entire embryo as a result of blastomere flattening. As a result of flattening, the embryo forms a smooth ball of cells known as a morula. The primary cytoskeletal cues that regulate the formation of the morula are those associated with cell-cell junctions, which at compaction quickly form at the boundaries of the flattening juxtaposed blastomeres. Adherens junctions, desmosomes, and tight junctions all form at this stage, and each is involved in a different aspect of compaction (5, 21, 22, 25, 102, 103)

One primary function of the actin network during compaction is its association with adherens junctions. The basic components of an adherens junction in embryos begin with the transmembrane cadherins, of which E-cadherin is the embryonic prototype. The extracellular domain of E-cadherin functions by forming homotypic, calcium-dependent interactions with E-cadherins on the surface of juxtaposed blastomeres. Its cytosolic domain functions by binding cytosolic components that ultimately cluster the cadherins to form the junction. The remaining components that comprise a basic adherens junction include beta-catenin, which bridges E-cadherin and alpha-catenin, and finally the actin cytoskeletal network, which binds to alpha-catenin. Most of the work uncovering the components comprising adherens junction has been worked out on cell lines and tissues from adult organisms. An insightful review on cell adhesion via the actin network recently described many of the novel components comprising adherens junctions (104). Each set of components (e.g., vinculin, p120, alpha-actinin, vasp, etc.) appears to lead to a different configuration of a typical adherens junction (104). Whether all of these novel components comprising the different actin-based cell adhesion configurations found in somatic cells are found in embryos during compaction is not known. However, it is clear that the basic adherens junction (i.e., e-cadherin/beta-catenin/alpha-catenin/actin) is present and is very active in the compaction process; an observation highly supported when adherens junction proteins such as E-cadherins are missing due to gene targeting. In these embryos, the blastomeres fail to compact leading to embryonic lethality at the 8-16 cell stage (105). Interestingly, this phenotype appears to be relatively similar to other components of adherens junctions (i.e., alpha-catenin and beta-catenin) if they are ablated (106, 107).

Although typical adherens junctions exist in embryos, it must be noted that their formation and their direct association with the cytoskeleton is unique during embryogenesis primarily because all of the components necessary for assembling these junctions are present by the two-cell stage (103, 108). Consequently, an intriguing question has persisted. If all of the components are present then why doesn't compaction occur earlier than the 8-16 cell stage? Actually, compaction will take place earlier if embryos are induced to do so *in vitro* using activators of PKC (103, 109, 110, 111). Evidence is accumulating suggesting that PKC must interact, most likely by phosphorylation, with beta-catenin to promote its binding to E-cadherin, alpha-catenin, and actin to form the junction (103, 111). *In vivo*, however, compaction takes place with acute timing under strict regulation. This regulation appears to be due to tyrosine phosphorylation of beta-catenin, which in its tyrosine phosphorylated form functions to keep E-cadherin/beta-catenin adhesion complexes nonfunctional until needed for adhesion during compaction and blastocyst formation (108).

Independent of cell adhesion, the actin network also functions during compaction by enabling blastomeres to undergo cytocortical microfilament polarization (112). The morphology of the polarization process in mammalian embryos has been known and described for decades; however, the regulatory process of polarization in embryos

is only recently becoming evident. Granted, polarization is promoted by the formation of adhesive junctions (i.e., adherens and tight junctions), a process, which can be inhibited by incubating embryos in cytochlasin D. However, a novel investigation by Clayton *et al.* (112) showed that cytocortical microfilament polarization and adhesion was prevented in embryos incubated in media containing Clostridium botulinum C3-transferase, an inhibitor of the small Rho GTPase protein. Cytochalasin D incubation only inhibited adhesion suggesting the two actin-based processes are controlled by different mechanisms. Further evidence suggesting that Rho GTPase controls polarization was seen in experiments where constitutively active recombinant Rho protein was injected into four cell embryos. In these embryos, the cortical microfilament network was disrupted and apical displacement of nuclei occurred along with a typical polarization of the microtubule filament network (112).

The fact that Rho GTPases may regulate aspects of the polarization process in mammalian embryos makes sense since these proteins have been repeatedly shown to be involved in polarity generation, cytoskeletal organization, and control of intercellular adhesion in other cell types (104, 113, 114, 115). Interestingly, both the Rac1 and Cdc42 (rho GTPase family members) knockout mice can form blastocysts and implant normally (116, 117) suggesting that the polarization phenotype found by Clayton *et al.*, (112) may be Rho specific.

The role of the microtubule cytoskeletal network during compaction is still not fully understood. At compaction, microtubules do undergo dramatic changes in their posttranslational modification as well as a change in their intra- and intercellular distribution (118). In compacted blastomeres, two types of posttranslationally modified tubulins are detected, a typical tyrosinated alpha-tubulin and to a lesser extent, an acetylated alpha-tubulin. It was shown that while tyrosinated alpha-tubulin residing in typical microtubules redistributed towards the apical domain of blastomeres, acetylated alpha-tubulin in mictrotubules relocated to the basal portion of the cell cortex (118). Moreover, microtubules containing acetylated alpha-tubulin were found primarily in cells that would typically form on the inside of the compacted embryo suggesting a role for microtubules in the polarization process of compaction. In support of this notion, it was recently shown that the outward migration of nuclei at the eight-cell stage may be microtubule-dependent since the process of microtubule redistribution closely precedes nuclear relocation (119).

Although, a number of investigations have shown that microtubule redistribution is accompanied by mitochondrial and lipid droplet redistribution, it has not been established if the redistribution of microtubules is a cause or an effect of compaction. Based on present data, it appears that microtubules function in compaction by controlling normal cellular trafficking.

Ultrastructural and biochemical evidence shows that the cytoskeletal sheets are a major cytoskeletal component in eggs and early embryos especially in rodents

(29, 30, 83). Although the sheets undergo changes at fertilization (see above), the most significant change in the sheet network (both fibrous and planar sheets; see above) occurs at compaction (29, 30, 83, 78, 86). It is at this stage that sheets begin to splay apart into clearly visible 10-11nm filaments. Electron microscopic analysis of embryos clearly showed filaments emanating from sheets and associating with junctional complexes that resembled desmosomes (30, 86). It is well known that one role intermediate filaments fulfill in cells is to bind to desmosomes, while another well known role is their responsibility in maintaining structural integrity of a cell. Both functions have been well tested in keratin homologous recombination experiments, which repeatedly show increased fragility in cells where keratin expression has been ablated (90, 120, 121).

To date ablation of cell-specific keratins has resulted in similar cell-fragile phenotypes. Removal of K14 in the epidermis leads to severe skin blistering due to epidermal cell fragility, while complete removal of the K18, K19 (both of which pair with K8 to make an intact filament) keratin network in trophectoderm cells results in cytolysis and embryonic lethality at about embryonic day 9.5 (121). With this in mind, then, how does the early preimplantation embryo survive prior to E9.5 without a keratin network? One answer may be that a keratin network is not needed; however, this answer is difficult to accept based on the stresses the embryo undergoes (e.g., hatching, implantation, etc.). An alternative answer to this perplexing question may be the intermediate filaments stored in the cytoskeletal sheets. It is highly possible that they function early on to provide structural integrity for embryonic cells until the embryonic genome can take over and express enough keratins for cell and embryonic survival.

The fact that the filaments emanating from the sheets clearly associate with desmosome-like junctions suggest that these filaments maintain the structural integrity of the embryo at these early stages. In addition, some of the individual intermediate filaments, which splay out from the sheets also associate with the nucleus of each blastomere (78). This association of intermediate filaments with the nuclear envelope has been reported to occur in somatic cells, and is thought to be involved in the positioning of the nucleus within the somatic cells (85, 122-124). As a result, the intermediate filaments may also be positioning the nucleus within the blastomeres beginning at the time of embryonic compaction.

3.3. The cytoskeleton of the blastocyst ... and beyond

The blastocyst stage of embryogenesis is the first stage where distinct cellular differentiation is identified. The outer layer of cells that surround the entire embryo differentiate into a primary epithelium, while the inner cell mass (ICM) cells remain pluripotent. The cytoskeleton within these two populations of cells differs significantly primarily because the outer cells are very polarized and have a distinct function (i.e., initiating and regulating implantation).

The actin filament network in trophectoderm resides primarily in the peripheral region of each cell, while actin filaments are much more diffuse in ICM cells (125).

The integrity of the actin filament network appears to rely, in part, on the cell cycle because as the mitotic apparatus forms during normal blastomere divisions, the amount of actin in the cortex of each cell significantly decreases. Once M-phase is over in a trophectoderm cell, actin becomes reconcentrated in the cortex of each daughter blastomeres (23). One role of the cortical actin network is to form microvilli on the apical surface of trophectoderm cells. A recent investigation delving into microvilli characteristics and epithelial differentiation of the trophectoderm described a novel posttranslational modification in the actin-associated protein, ezrin, which has been shown to bind actin filaments to the plasma membrane via proteins such as ICAM 2, CD43, and CD44. Ezrin has been widely studied in somatic cells (126, 127) and has been shown to be an important component of apical microvilli as well as function in determining cell surface morphology (128). It shares high homology to radaxin and moesin, all of which belong to the super family of band 4.1 actin-binding proteins (129). It was shown that in early trophectoderm cells (outer blastomeres of compaction-stage embryos, ezrin was localized to the apical region of each cell, while in ICM cells ezrin was found as a diffuse cytoplasmic pool. Interestingly, ICM cells that were isolated from blastocysts and cultured for increasing time intervals without leukemia inhibitory factor (LIF; culturing ICM cells in the absence LIF causes them to differentiate into a primary epithelia; 33 and references therein) showed a distinct reorganization of ezrin to the cortical region of outside cells (i.e., those that saw an outside and inside environment). Once ezrin had redistributed to the cortical region of cultured ICM cells, microvilli formed (130). Interestingly, the redistribution of ezrin was linked to distinct O-glycosylation of the ezrin moiety and not to its phosphorylation (130). The hypothesis being considered is that this posttranslational modification appears to, in part, be responsible for the redistribution of ezrin to the apical plasma membrane, which in turn, allows for the formation of, as well as stabilization of, the pole of microvilli (130).

Another distinct characteristic of the blastocyst is that it must hatch from its pertinacious, polysaccharide shell known as the zona pellucida. Recently found in trophectoderm cells during hatching was one of the most dramatic changes in the actin filament network seen to date. It had been thought that blastocysts hatch primarily as a result of protease-mediated lyses of the zona pellucida, followed by pressure-induced (from the expanding blastocoel) squeezing through the lyses site. Although these phenomena may play a role, a recent set of experiments showed that blastocyst hatching was completely inhibited at each stage (i.e., expanded, lobe-formed, and hatching blastocyst stages) when embryos were incubated in cytochalasin B, highly suggesting a role for the actin network. Interestingly, in each case, upon removal of cytochalasin B, the embryos resumed development and hatched from their zona pellucida (131). Immunocytochemistry revealed that the concentration of actin filaments had risen dramatically in trophectoderm cells at the site of hatching. However, more intriguing was evidence that showed trophectoderm cells possessing a motile phenotype as they past through the lysed zona (131).

As a result of those experiments, an underlying, novel function for the actin network in trophectoderm cells was revealed. Trophectoderm cells are capable of briefly modifying their actin network to produce cellular locomotion, a function resembling that of many types of somatic cells, which use actin filaments for motility (e.g., fibroblasts). Fascinatingly, this adaptation is short-lived as the actin network redistributes to its pre-hatching state once free of the zona pellucida. Yet to be defined, however, are all of the signaling mechanisms responsible for these actin network modifications during hatching. This is a special case of actin filament reorganization as it happens only once in the entire lifespan of the individual. Consequently, it will be necessary and interesting to see if there are unique as well as known proteins involved in this novel adaptation of actin network reorganization.

A key actin binding protein, myosin, also reorganizes in cooperation with actin filament redistribution. During late compaction, early blastula formation, myosin moves from area of contact between blastomeres to areas that contain actin (i.e., cortex region in trophectoderm cells; 132-134). Other actin binding proteins such as vinculin, fodrin (spectrin), and alpha-actinin, also undergo spatiotemporal reorganizations throughout early development especially at the blastula stage (135-137). However, how each of these proteins function to initiate or regulate compaction and blastula formation has only been speculated. Experiments utilizing inhibitory antibodies to these proteins or possibly ablation of each gene are needed to understand the role of each protein at each stage of development.

Microtubules in the blastocyst have been observed by immunofluorescence in different mammalian species showing similar distributions (135, 138). In trophectoderm cells, microtubules form a network-like cortical layer beneath the actin cortical network (138). In ICM cells the microtubule network is much more diffuse throughout the cytoplasm of each cell. Besides their function separating chromosomes during M-phase, microtubules have not been shown to be directly involved in the cytoarchitectural process of blastocyst formation; however, it appears that they are intricately involved in regulating critical cellular processes, which enable the blastocyst to continue developing. Recently a report demonstrated that microtubule-associated proteins such as cytoplasmic dynein (cDHC) use the microtubule network for transport of golgi, and lysosomes in cells of the blastocyst (139). cDHC -/- blastocysts grown *in vitro* showed a highly vesiculated golgi complex that was distributed throughout the cytoplasm. Endosomes and lysosomes were found attached to microtubules in cDHC-/- blastocysts; however, their distribution also was disrupted, suggesting that DHC is necessary for proper positioning of the golgi, endosomes, and lysosomes in blastomeres. Since ablation of DHC primarily disrupted vesicle positioning it is thought that other microtubule-associated proteins may be necessary for attachment of vesicles to the microtubules (139).

Individual intermediate filaments in the blastula are prominent throughout trophectoderm cells but are less abundant in ICM cells (20). Cytoskeletal sheets on the other hand are virtually absent in trophectoderm cells,
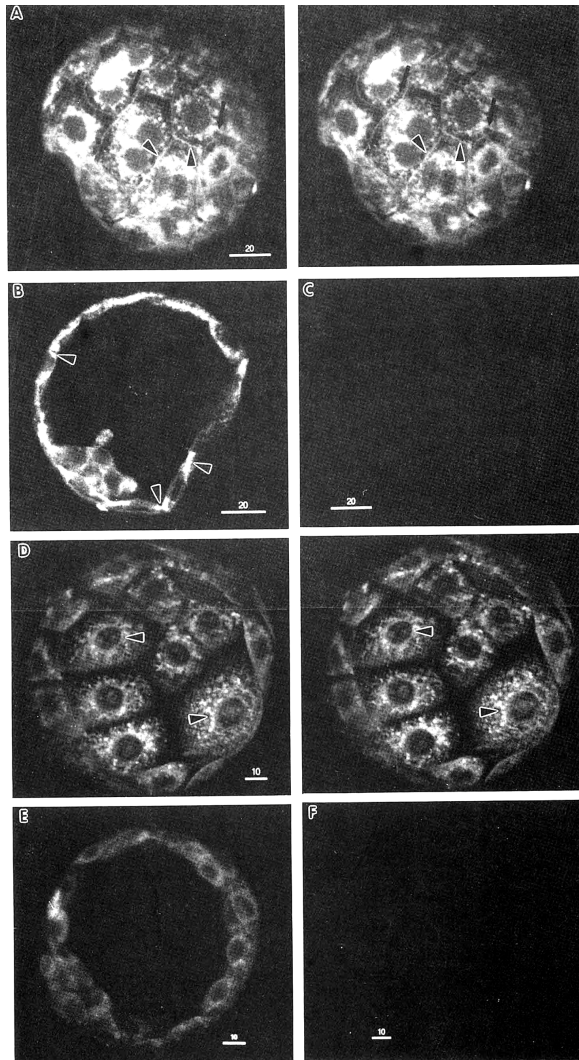
Figure 10. Hatched blastocysts were subjected to antibodies directed against K5/6 (**A** and **B**) or K8 (**D** and **E**) followed by fluorescently tagged second antibodies and analyzed using laser scanning confocal microscopy. (**A**) Steroscopic view of a stack of twenty 1.5 micro-meter optical sections from the trophectoderm cells of a hatched embryo demonstrates staining of K5/6 at intercellular junctions (arrowheads) and in distinct tracts extending between perinuclear regions and the intercellular junctions. (**B**) A stack of ten 1.5 micro-meter sections through the middle of an embryo demonstrates K5/6 in a diffuse pattern in ICM cells and at intercellular junctions (arrowheads) in trophectoderm cells. (**C**) Control embryo stained only with rhodamine-conjugated goat-antimouse antibodies shows no staining. (**D**) Steroscopic view of a stack of 20 1.5 micro-meter optical sections from a hatched embryo demonstrates staining of K8 at perinuclear regions (arrowheads) in trophectoderm cells. No staining was observed at intercellular junctions or on tracts. (**E**) A stack of 10 1.5 micro-meter sections through the middle of an embryo shows K8 surrounding nuclei in both ICM cells and trophectoderm cells. (**F**) Control embryo stained with FITC-conjugated goat-antirat antibodies shows no staining. Reproduced from Schwarz *et al*. (20) with permission from Elsevier Science Ireland Ltd.

while prominent in the ICM cells (20). These correlative results began to uncover a role for the cytoskeletal sheets; they are storage sites for intermediate filaments (20, 78, 86). Further evidence for this role was seen in western blots from at least 6 different studies that revealed at least five to six different keratins residing in blastocysts (20, 26, 27, 88, 125). Although later studies showed K8 and 18 to be responsible for at least two of these keratins in western blots, many investigators dismissed the others (which have different molecular weights than K8 and 18) that had been found by those earlier investigators. Schwarz *et al*., (20) distinctly showed antibodies to K5/6 staining filamentous material in trophectoderm cells as well as staining bands at the correct molecular weight for K5 and K6 on western blots (Figure 10). The immunofluorescent staining disappeared after the blastocyst stage at about the time of implantation where K8 and K18 were found to be the prominent keratins in trophectoderm cells and primitive endoderm (26).

Maintenance of cell integrity appears to be a universal function for intermediate filaments (90, 140). However, this function was challenged not too long ago when Baribault *et al*., (141) knocked out K8. Most of those mice died at embryonic day 12-13 due to hemorrhaging in the liver. However, a following report by Baribault *et al*. (142) showed that on a different genetic background, K8 null mice lived to adulthood. The confusion mounted as K18 was ablated in mice resulting in relatively normal progeny (28). Based on these data, it was thought that K8 and K18 when ablated separately were not necessary for development (28, 142). The confusion was cleared up, however, upon ablation of other keratins such as K19, which is not found in embryonic (trophoblast) tissues until after implantation (121). When both K18 and K19 were ablated, embryonic lethality occurred with 100% penetrance at E9.5 due to trophoblast fragility (121). Magin *et al*., (28) had previously shown that in the absence of K18, K19 can pair with K8 to make a filament, thus circumventing embryonic lethality. Based on these reports, as well as others, which showed that the loss or disruption of the keratin network contributes to cell fragility in all cell types (26, 90, 120), a rather important question remains. How does the early embryo, especially at the blastula stage, survive stressful situations such as hatching, and implantation without the K8, K18 network if, by some investigators interpretations, these are the only intermediate filaments present? Although it is possible that intermediate filaments are not necessary prior to and during implantation, it is more likely, based on the plethora of biochemical and ultrastructural evidence placed forth in past investigations and reviewed here, that the cytoskeletal sheets provide the intermediate filaments necessary for epithelialization of the trophectoderm, cellular integrity for the drastic forces each cell must endure as the blastocyst hatches, and finally cellular integrity for trophoblast cells during invasion into the uterus.

4. PERSPECTIVE

The evidence is clear that all three cytoskeletal systems are necessary for normal development of the egg and embryo. However, from acentriolar centrosomes to the cytoskeletal sheets, investigators have shown that the

cytoskeleton in mammalian embryogenesis has adapted some very unique structures and functions, which enable the egg and embryo to undergo very remarkable changes found at developmental transitions. Understanding how the cytoskeleton is involved in the developmental changes of the early embryo is important for many fields of cell biology including cell adhesion, cell cycle regulation, and control of cell polarization. Our understanding of the cytoskeleton in eggs and embryos, however, is still in its infancy when compared to knowledge about the cytoskeleton in somatic cells. Fortunately, though, with the advent of technologies such as those that allow experimentation on low amount of material found in eggs and embryos are developed or refined, cell signaling mechanisms as well as novel components and functions are slowly being uncovered for each cytoskeletal system during early mammalian development.

5. ACKNOWLEDGMENTS

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