SRC-FAMILY TYROSINE KINASES PARTICIPATE IN THE REGULATION OF MAMMALIAN OOCYTE MATURATION AND ZYGOTIC DEVELOPMENT

SRC-FAMILY TYROSINE KINASES PARTICIPATE IN THE REGULATION OF MAMMALIAN OOCYTE MATURATION AND ZYGOTIC DEVELOPMENT

BY

Lynda Kay McGinnis
B.S. Seattle University
M.S. Iowa State University

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_____
David F. Albertini, PhD
Chairperson

_____
Gustavo Blanco, PhD

_____
Lane K. Christenson, PhD

_____
William H. Kinsey, PhD

_____
T. Raj Kumar, PhD

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The Dissertation Committee for Lynda K. McGinnis certifies
that this is the approved version of the following dissertation:

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Committee:

_____
David F. Albertini, PhD
Chairperson*

_____

Date approved: _____

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Abstract

Mammalian oocytes engage in a remarkable series of cytoskeletal and cell cycle modifications that prepare the oocyte for the initiation and continuance of development. Multiple signaling pathways appear to operate during the process of oocyte maturation to ensure that the quality of the cytoplasm and genome will meet the standards required to initiate and complete development. In this thesis we have taken a systematic approach to understand the role of Src-family kinases (SFKs) during oocyte maturation, fertilization and early cleavage in the mouse. We first demonstrate that the SFK that controls the progression of meiosis at the first metaphase anaphase transition is most likely FYN (Chapter 2). This proposal is then supported by the demonstration that tyrosine kinases act upon discrete subcellular compartments that include the oocyte cortex and spindle poles in a way that is spatially and temporally distinguishable from the targets of ser/thr kinases (Chapter 3). Moreover, this work reinforces the specific role of FYN at these sites within mouse oocytes using mice null for this SFK. Finally, in Chapter 4 we show that the functions of SFKs that drive completion of the meiotic cell cycle extend to and through the first embryonic cell cycle after fertilization. Thus, previously unanticipated functions for SFKs have been identified for the first time that mediate the spatial and temporal remodeling of cytoskeleton and cell cycle during oocyte maturation and early development. These findings will have an immediate impact on the field of human assisted reproductive technologies (ARTs) as this pathway has been completely overlooked up to now.

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Chapter One

Introduction

Assisted reproductive technologies (ART) including in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are commonly used to treat infertility. By the year 2008 over 4 million children had been conceived by ARTs [2]. In vitro maturation (IVM) of immature oocytes is now being incorporated into clinical IVF as an additional option for treatment. This procedure is indicated for women who present under the following conditions: (1) patients with polycystic ovarian syndrome who are highly susceptible to ovarian hyper stimulation syndrome; (2) women who have failed to ovulate or had poor quality oocytes from previous ART attempts; (3) young women undergoing treatment for cancer whose ovarian tissues would require cryopreservation to protect immature oocytes from chemical and radiation induced damage.

The first child born from IVF, Louis Brown is now 30 years old [3, 4]. The vast majority of children conceived by ARTs appear to be completely healthy. However, research conducted with animal models as well as numerous clinical studies of IVF and ICSI children have shown a significant rise in developmental abnormalities associated with ARTs (see editorial and discussion from Nature [2, 3]). Animal models of IVM have demonstrated changes in the expression patterns of mRNA & proteins in oocytes and embryos [5, 6]. In vitro matured oocytes exhibit decreased fertilization [7] and reduced embryonic developmental competency [8-11]. In a recent study examining the health and longevity of adult mice produced by IVM, while the majority of parameters measured were normal, adult mice from IVM oocytes had significantly reduced pulse rate and cardiac output as compared to mice produce by IVF from *in vivo* matured oocytes [12]. These studies suggest a need for a better understanding of the intricacies of oocyte maturation and how local environmental factors affects the developmental competence of oocytes and the long-term health of adults derived from this procedures.

Signaling at ovulation

Mature mammalian females experience rhythmic changes in the secretion of endocrine and ovarian factors that lead to the cyclic production of large antral follicles within the ovary. In each estrous cycle, one or a few follicles (depending on species) are selected by unknown mechanisms to undergo growth and differentiation into a mature Graafian follicle [13, 14]. Follicle stimulating hormone (FSH) produced by the anterior pituitary induces ovarian granulosa cell proliferation and subsequent antral follicle development. Each Graafian follicle consists of an outer layer of theca cells lying on a basement membrane that separates the theca from the granulosa cells. As the follicle matures, granulosa cells separate into two distinct populations: 1) the mural granulosa that line the inner follicular wall and 2) the cumulus complex that consists of specialized granulosa cells enclosing a fully grown and developmentally competent oocyte (for early reviews of follicular structure see [15, 16]). Ovulation and the induction of oocyte maturation have long been seen as downstream events following the surge of luteinizing hormone (LH).

Early studies on the effects of LH on the antral follicle leading to ovulation took little notice of the oocyte. Review articles describing the mechanics of ovulation often ignored the presence of the oocyte as it was not considered an active participant within the ovulatory system (for example [17, 18]). Although it was recognized that the oocyte may have some inductive influences on granulosa cells during follicular development [19, 20] and that oocyte growth occurs in concert with the proliferation of the surrounding granulosa cells [21, 22].

Prior to the 1990s, the complexities of intracellular signaling pathways and gene expression patterns were not yet fully appreciated. Autoradiographic studies demonstrated that LH and FSH each bound to specific subsets of cells within the Graafian follicle; LH bound to theca and interstitial cells and occasionally to granulosa, while FSH bound to granulosa cells exclusively (reviewed in [20]). In large antral follicles, FSH could induce the expression of LH receptors on granulosa cells [23]. It was also recognized that FSH and LH activities were linked to the

production of estrogen within the ovary [24] and that the levels of nuclear estrogen receptor seemed to correlate with follicle size [25] but the specific links in these pathways were unknown. By the 1980s, the regulation of follicle development took on a more complex character with the recognition that cAMP levels increased within follicular cells in response to FSH, LH and prostaglandins (reviewed in [17, 20, 26]).

Receptors of this family are transmembrane proteins known as G protein-coupled receptors. Ligand binding the receptor N-terminus on the outer cell surface activates the G-protein at the inner leaflet of the cell membrane. This activation of the G protein in turn activates adenylate cyclase to produce cAMP [27]. Thus, binding of FSH or LH to their specific receptors induces an intracellular rise in cAMP. It was long known that cAMP added to the *in vitro* culture media could prevent gonadotropin induced maturation of mouse oocytes [28, 29]. When Hubbard and Terranova (1982) cultured hamster COC in media with cAMP or cGMP then stimulated cumulus cells with LH, they demonstrated that cumulus cells may play a vital role in suppressing oocyte maturation [30]. Indeed this was confirmed the following year in simultaneous publications from the Eppig and Schultz laboratories with mouse COC [31-33] followed by other mammalian species [34]. The one-directional model arose whereby FSH stimulation of cumulus cells lead to the rise of cAMP in the oocyte and subsequent maturation arrest [35]. However, the model would gain in complexity with the discovery of oocyte produced factors that regulated the companion cumulus cells.

In 1987, Findlay and Risbridger argued that two-way communication between germ cells and gonadal cells was necessary for proper germ cell function [36]. Discovery of the oocyte specific proteins, bone morphogenic protein-15 (BMP15) and growth differentiation factor-9 (GDF9) has proven this to be the case. GDF9 and BMP15 are members of the large TGFβ superfamily of proteins [37]. Oocyte expression and secretion of GDF9 [38-40] and BMP15 [41, 42] are key regulators of ovarian follicular development and subsequent oocyte survival (for review see [43, 44]).

Transmission of molecules between oocytes and their cumulus cells occurs by both secretory and gap junctional communication and is essential for oocyte survival [45]. Oocytes secrete growth factors such as GDF9, BMP15 and FGF8 into the extracellular milieu for paracrine regulation of cumulus cells [46] (for further discussion of interactions during follicle development see [47]). These proteins bind cell surface receptors to initiate intracellular signaling cascades within the cumulus. However, small molecules can pass directly from cell-to-cell through gap junctions.

Cumulus cells develop long trans-zonal projections (TZPs) that reach-out through the zona pellucida to contact the microvilli on the surface of the oocyte. Gap junctions form between the oocyte and these TZPs [48]. The gap junctional complex consists of two units, each comprised of six connexin proteins formed into a characteristic symmetrical unit (connexon) within the cell membrane. Intracellular channels are formed when connexons from neighboring cells are joined. The resulting pore metabolically couples neighboring cumulus cells with each other and the oocyte allowing transfer of low molecular weight (<1 kDa) molecules such as ions, nucleotides, amino acids and other metabolites (for reviews see [38, 39]. The connexin family includes at least 20 genes with three produced in cumulus cells: connexin 32, 43 and 45 [49, 50]. Connexin 43 (Cx43) is the predominant form found in cumulus cells while oocytes express almost exclusively Cx37 [51, 52]. Gap junctions between cumulus cells are mostly homologous connections of Cx43 while gap junctions between oocyte and cumulus are heterologous formed from Cx43 (cumulus cell) and Cx37 (oocyte). Deletion of either Cx43 or Cx37 severely disrupts communication between oocyte and cumulus cells and causes the failure of both oocyte and follicle development [51-54]. Interestingly, while the oocyte produces paracrine growth factors that stimulate cumulus cells, several metabolites required by the oocyte are produced by the cumulus cells and fed into the oocyte through gap junctions [55] including histidine, alanine, pyruvate [56, 57] and possibly ATP [58]. Even intracellular acidity of oocytes is controlled by their companion cumulus cells

via gap junctional passage of pH_i [59] further demonstrating the importance of this cell-to-cell connection.

Communication via gap junctions also returns us to the subject of cAMP and the regulation of oocyte maturation. As mentioned previously, early studies demonstrated that decreased intracellular cAMP content of oocytes lead to a resumption of meiosis both *in vivo* and *in vitro*. FSH binding to its receptor produces increased cAMP in cumulus cells with transient meiotic arrest [33, 60]). However, LH and prostaglandins (produced by granulosa cells in response to maturation [61, 62]) also increase granulosa cell cAMP but subsequent decreases in cAMP in oocytes result in resumption of meiosis (for more details on cAMP signaling see sections Integrating Kinase Signaling and Signaling in Oocyte Maturation) [63]. These differential effects of gonadotropins on oocyte maturation demonstrate a complicated interplay of signaling cascades [48, 50, 52] as well as intact gap junctional communication between the oocyte and cumulus [64, 65]. Further research is needed to fully understand the mechanisms involved but this system is a good example of what was once considered a simple model but is now recognized as extremely complex.

Recent studies have turned toward molecular expression analysis of cell communication and gene regulation within oocytes and cumulus cells [66]. FSH receptor activation initiates multiple signaling cascades including PKA, PKC, MAPK, meiosis activating sterol (MAS) and epidermal growth factor (EGF) in addition to cAMP, cGMP, gonadal steroid hormones and prostaglandins all of which play roles in subsequent follicular development and oocyte maturation [67] (see sections on Integrating Kinase Signaling and Signaling in Oocyte Maturation). Activation of the LH receptors on mural granulosa cells also initiates the production and release of EGF with subsequent activation of EGF receptors on the cumulus cells leading to meiotic maturation [68].

Can the story be any more complicated? The answer seems to be YES. With the advent of microarray technology many studies are now reporting vast changes in gene expression as results of apparently simple receptor activation. As mentioned previously, FSH and LH receptor activation leads to increases in cAMP. We now know that cAMP mediates changes in gene expression by activating PKA which in turn phosphorylates and activates CREB (cAMP-regulatory element binding protein). Once activated, this transcription factor binds to the cAMP regulatory elements present within the promoter region of specific genes such as aromatase and inhibin-A to initiate gene transcription [69]. While cAMP itself can activate entire pathways and regulate gene expression, recent studies have shown that cAMP-independent activities are also initiated following FSH receptor activation including phosphorylation and activation of PKB/Akt and p42/44 MAPK [57, 58].

Wayne et al (2007) published an elegant set of experiments attempting to work-out some of the missing pieces in the FSH signaling pathway that leads to granulosa cell maturation and ultimately oocyte maturation [69]. Using multiple molecular tools, they have pieced apart the signaling pathways of FSH versus EGF and discovered an unrecognized player in follicular regulation: the Src-family kinases (SFKs: For descriptions, see section on Src-Family Kinases in Oocyte Maturation). They concluded that "FSH orchestrates the coordinated activation of three diverse membrane-associated signaling cascades (adenylyl cyclase, RAS, and SFKs) that converge downstream to activate specific kinases (PKA, ERK1/2, and PKB/FOXO1a) that control granulosa cell function and differentiation" [69]. As the story of cumulus cell signaling continues to develop, so too does the web of signaling cascades in the oocyte.

Signaling of oocyte maturation

Oogonia commit to meiosis at the time of birth or shortly thereafter in mouse oocytes. They enter meiosis but arrest at prophase of meiosis-I where they will remain in stasis until signaled to resume in adulthood (for a review of ovarian

development see [47, 70]). During the initial phases of oogenesis in the mouse, meiosis is arrested under the negative control of cell cycle regulatory proteins at the translational and post-translational levels [71, 72]. In the adult ovary, when the follicle begins to grow and an antral cavity begins to form, oocytes acquire the ability to resume meiosis. However within the follicle, resumption of meiosis is prevented by regulatory factors within the follicular environment until a cue is triggered by the LH surge which leads to the resumption of meiosis-I (see Signaling at Ovulation).

The specific activating signals for the resumption of meiosis-I are not known, but several interacting pathways that participate in this process have been identified (see also the sections on Signaling at Ovulation and Integrating Kinase Signaling for additional discussion of this activation process by LH, PKA and other pathways). Immature oocytes arrested at prophase of meiosis-I (GV stage) contain low levels of maturation promoting factor (MPF; Cdk1 and Cyclin B together form MPF; see section on Integrating Kinase Signaling for detailed description). The LH surge initiates the resumption of meiosis and a rise in MPF activity leading to germinal vesicle breakdown (GVBD), condensation of chromatin, formation of the first meiotic spindle and entry into metaphase-I. Once activated, MPF promotes dramatic structural reorganization that drives formation of the metaphase spindle (see section on Integrating Kinase Signaling). In addition to MPF, a rise in MAPK is also essential to the progression of normal maturation. The rise in MAPK and continuous activation of MPF requires the translation of maternal mRNA for MOS (Moloney murine sarcoma oncogene) and Cyclin B. This translation is triggered with the initiation of maturation, although the specifics of this activation are unknown. MOS codes for a serine/threonine kinase that phosphorylates and activates another kinase, MEK (MAP-ERK kinase) which in turn activates MAPK. MOS and cyclin B are maternally derived mRNAs that accumulate during meiosis and are stored at high concentrations in the cytoplasm of the prophase arrested oocyte [73-76]. Resumption of meiosis induces the rapid translation of numerous mature mRNAs including MOS and cyclin B which are essential in the processes of maturation (see also the

activation of CPEB by Aurora kinases in the section on Integrating Kinase Signaling and the discussion of cytoplasmic maturation below) [77]. Activated MAPK in early MI prevents the ubiquitination and degredation of cyclin B by the anaphase promoting complex (APC/C), thus contributing to both the entry into and maintenance of meiosis-I.

Pro-metaphase of meiosis-I is a prolonged cycle with high levels of MPF and MAPK. However, after 7-9h the chromosomes align on the metaphase plate followed by a transient decline in cyclin B allowing for an increase in APC/C ubiquitin-ligase activity [78]. This slight decline in MPF activity promotes the anaphase-telophase transition and extrusion of the first polar body. At this point, rather than enter interphase, cyclin B levels raise again thus activating MPF and deactivating APC/C and the oocyte enters metaphase-II. As with so many other stages of oocyte maturation, this stage of the meiotic cell cycle is tightly controlled and relies on the interplay of multiple signaling systems [78]. With the maintenance of active MPF and MAPK, the oocyte remains arrested in metaphase-II until activated by a fertilizing sperm. The coordination of MPF, MAPK and APC/C are regulated by multiple intersecting pathways and are the subjects of many recent studies (for more details see [78-86]).

Completion of meiosis and entry into the first mitosis

Metaphase-II arrest is released by fertilization which initiates a pulsating release of intracellular calcium. These cytoplasmic calcium waves trigger egg activation. One of the first events following fertilization is the extrusion of cortical granules through the oocyte cortex and into the perivitellin space, thus setting-up the membrane block to polyspermy [87-89]. The full mechanisms involved in this blockade to supernumerary sperm entry require both calcium-dependant and independent mechanisms and it is essential for the prevention of polyploidy and subsequent embryonic death [87, 90, 91]. Calcium waves also induce the full

activation of the APC/C which degrades cyclin B thus inactivating MPF and initiates the exit from M-phase [92].

Proper oocyte maturation involves both nuclear and cytoplasmic maturation which prepare the oocyte to support fertilization and subsequent embryonic development. A critical part of the cytoplasmic maturation is the acquisition of machinery capable of epigenetic remodeling of chromatin after fertilization. Sperm DNA is wrapped tightly around protamines and packaged into teroid coils to form one of the densest tissues found in nature [93, 94] (for discussion of sperm DNA see also [95]). Once inside of the oocyte, egg cytoplasmic machinery must unwind this DNA and remove the protamines in a process known as chromatin remodeling. Protamines are replaced by histones and DNA binding proteins as the nuclear envelope forms to produce the male pronucleus [96]. Coincident with the reprogramming of the male DNA, the female undergoes anaphase-telophase and polar body extrusion to yield a haploid egg. Male and female pronuclear formation accompanies an increase in egg metabolism and chromatin duplication with progression through S-phase [97].

The fertilized egg next enters a lengthy prophase allowing for the synthesis of cyclin B which must rise above a threshold level before entry into the first mitosis can begin. The timing of MPF activation is also determined by the phosphorylation state of the complex. Dephosphorylation of Cdk1 is essential for MPF activity, but the kinase is kept inactive during interphase by dominant inhibitory phosphorylation at T14 & Y15. The final activation step following fertilization is the removal of these inhibitory phosphates by the dual-specificity protein phosphatase Cdc25. This phosphatase is activated downstream of the calcium waves by Ca^{2+}/Calmodium-dependent Kinase-II [98].

Once activated, MPF drives the zygote into the first mitotic cell cycle. This first mitotic cell cycle is almost twice as long as that of subsequent embryonic mitosis due to a prolonged M-phase which is reminiscent of MII arrest. This transient metaphase arrest is likely caused by the presence of maternal factors left-over from

the meiotic milieu, although the precise mechanisms involved are unknown [99]. Regardless of this delay, most of the primary signaling pathways that drive the cell cycle in meiosis are also required for completion of the first and subsequent embryonic mitosis [100].

In addition to cell cycle initiation, fertilization involves changes in protein synthesis and the translational silencing and degradation of maternal mRNA [101, 102]. In mammals, a low level of gene transcription begins in both the male and female pronuclei within hours after fertilization [103] however, the primary zygotic gene activation occurs after several rounds of DNA replication and cell cleavage (2-4 cells in rodents; 8-16 cells in large domestic animals and primates) [104]. Once thought of as a global gene activation, microarray analysis have proven that zygotic gene activation is highly regulated with genes being turned-on or off in a developmentally coordinated pattern [105]. Throughout development from the immature oocyte until full zygotic gene activation, the egg depends on the maternally stored mRNA. Some maternal mRNAs are required even as late as the blastocyst stage of development. For example, maternally derived JY-1 (an oocyte-expressed gene shown to regulate the function of both ovarian granulosa cells and bovine embryogenesis) [106] and β-catenin [107] both of which compose maternal stores of mRNA that are required for the development of blastocysts. Other genes that are oocyte specific are lost after zygotic gene activation and not apparently turned-on again until formation of primordial germ cells in the next generation fetus (for example, GDF9, BMP15, MATER, ZAR1 [108] and FILIA-MATER [109]). Many recent reviews have been published regarding gene expression profiling in mammalian oocytes and is beyond the scope of this thesis (for example see [105]). As more maternal effect genes are discovered, it is becoming evident that proper development and maturation of the oocyte ultimately regulates the fate of the developing embryo and the health of the adult [110].

In the next section, we will examine some of the known mammalian oocyte signaling pathways and their involvement in oocyte maturation with emphasis on the organization of the cytoskeleton and chromosomes leading to proper chromosome segregation and embryo development. Errors in spindle formation and microtubule dynamics cause deregulation of chromosome organization and aneuploidy. These gross abnormalities are likely a consequence of aberrant molecular signaling pathways and a serious concern in the field of human development and clinical ARTs (advanced reproductive technologies). (See also the section on Src-Family Kinase Signaling in Oocyte Maturation for a discussion on the importance of proper oocyte maturation and concerns of aneuploidy in clinical ART). This coordination of intracellular signaling and cytoskeletal dynamics is essential for the production of a healthy oocyte and subsequent normal offspring.

Integrating signaling with chromatin and cytoskeletal organization

Oocyte signaling is an amazingly complex cellular system with intricate and intersecting molecular pathways. One of the primary components of meiotic maturation is the reorganization of the microtubule cytoskeleton and the formation of the meiotic spindle. Abnormal spindle and chromosome dynamics produce aneuploidy, the primary cause of developmental failure in clinical ART and the oocytes/embryos of older women [111-114]. Because of the critical importance of proper spindle formation and subsequent chromosome segregation, we have focused our studies on the molecular signaling cascades that influence these cytoskeletal and chromosome dynamics.

Prophase-I arrested (GV) oocytes contain long, stable microtubules that radiate throughout the cytoplasm and surround the oocyte cortex. As oocyte maturation progresses through GVBD, cytoplasmic microtubules shorten and become less stable [115, 116] while microtubule organizing centers (MTOCs) coalesce to the region of condensing chromosomes and nucleate dynamic microtubules forming the MI spindle [117]. Chromatin-associated Ran-GTP coordinated microtubules also

play a role in production of the fully functional meiotic spindle [4-8]. Phosphorylation of MTOC proteins increases their microtubule nucleating capabilities during mitosis [9-11]. In meiotic maturation of mouse oocytes, a subset of MTOCs are constitutively phosphorylated [118] although, increased phosphorylation of MTOCs is associated with meiotic competence [119]. Cell cycle dependant phosphorylation of microtubule associated proteins contributes to the regulation of dynamic microtubules [120].

MAPK and spindle microtubules

Mitogen activated protein kinases 42/44 (MAPK, also called ERK1/2) and protein phosphatase 2A (PP2A) are two partners involved in these phosphorylation events [7-11]. Active phospho-MAPK (pMAPK) and PP2A localize to the region of the meiotic spindle in mouse oocytes and both are required for spindle formation [121] although their direct functions on spindle microtubules and centrosomes are unknown.

The Mos protein activates the MAPK pathway [21-24] binds to tubulin in somatic cells [122] and localizes to microtubules of xenopus oocytes [123]. Knock-out mice lacking the MOS gene produce MII oocytes with abnormally diffuse spindles and loose chromosomes [124]. Many of these oocytes fail to arrest at MII, undergoing parthenogenic activation and pronuclear formation. The MOS effector MEK also localizes to microtubules but primarily to the spindle poles [125]. Thus several key regulators of the MAPK pathway are closely association with microtubules and the meiotic spindle [25, 27]. Normal mouse oocytes matured *in vitro* will progress to metaphase-II and extrude the polar body within 14h of culture. When MEK/MAPK was inhibited with 20 μM U0126 beginning at the GV stage, oocytes underwent GVBD but failed to form normal MI spindles. Various abnormalities were seen including oocytes that blocked at the pre-MI stage with central aster of microtubules, monopolar MI spindles and bi-polar spindles with misaligned chromosomes [126]. The majority of oocytes failed to mature beyond MI.

When oocytes were matured for 4 h (allowing for GVBD and entry into pro-MI) before inhibition of MEK/MAPK, oocytes matured to MII and extrude a polar body. However, MII spindle abnormalities were produced including failure to sort chromosomes and formation of irregular spindles. Two proteins that normally associate with the spindle poles in MII oocytes were either absent (NuMA) or dislocated (γ-tubulin) [126]. These abnormities caused by inhibiting the MAPK pathway are reminiscent of our results with SFK inhibition; including a block at MI when Src was inhibited from the GV stage and formation of abnormal MII spindles [127]. This suggests the possibility of an interaction between the Src and MAPK signaling pathways in the progression of meiotic maturation, chromosome sorting and spindle dynamics.

Src kinases are known inducers of MAPK activation through the Src/Raf/MAPK pathway. [For further discussion of this pathway, see the section on Src-Family Kinases in Oocyte Maturation]

Src-family tyrosine kinases and microtubule dynamics

Tyrosine kinases also associate with centrosomes and microtubules. Src family members including Src, Fyn and Lyn and the closely related PTKnamed Fes can bind to microtubules and phosphorylate alpha and beta tubulin in somatic cells [17-19] while Fyn also phosphorylates tubulin in mammalian oocytes [128, 129]. (For a detailed description of SFKs, see the section on Src-Family Kinases in Oocyte Maturation). The centrosomes are another site of tyrosine kinase activity during the somatic cell cycle. γ-Tubulin and ring complex proteins, members of the centrosomal protein milieu are tyrosine phosphorylated by the kinases Fyn and Syk (spleen tyrosine kinase) in budding yeast [130] as well as activated mast cells [131] and differentiating P19 embryonal carcinoma cells [132]. In addition, Fyn PTK and PI3-kinase together interact with γ-tubulin in acentrosomal MTOCs, whereby it appears they regulate microtubule nucleation by membrane bound γ-tubulin in differentiated P19 cells [133]. Interestingly, we have found phosphotyrosyl proteins localized

specifically to the spindle poles in the region of the centrosomes in MII mouse oocytes [134]. Phosphorylated γ-tubulin is likely present at this stage of mouse oocyte also (Barrett and Albertini unpublished observations). This suggests the possibility that the phosphotyrosyl proteins at the spindle poles maybe γ-tubulin and ring complex proteins which comprise the centrosome of the mammalian oocyte [26-29].

Src-family kinase activity is greatly increased during mitosis (reviewed in [135, 136]). This activity is due to the dephosphorylation of the inhibitory Y527 carboxy-terminal regulatory tyrosine by phosphatases (PTP) and an activating phosphorylation by Cdk1. Two of the phosphatases responsible for the dephosphorylation of Y527 include PTP alpha (PTPα) [137] and epsilon (PTPε) [138]. PTP activity is also increased during mitosis [137, 139]. Interestingly, EGF receptor activation causes translocation of PTPε to microtubules [138]. Although this binding causes a decreased PTPε activity, the binding is transient and would provide a mechanism for co-localization of SFKs and their activating PTPs at the site of polymerized microtubules. Interestingly, Wu and Kinsey [140] found Fyn kinase bound directly to PTPα via the SH2 domain in Zebrafish eggs while McGinnis et al [134, 141] and others [142] have found both activated and inactive SFKs associated with microtubules in both oocytes and cumulus cells. Co-localization of Fyn and activating PTPα and/or PTPε at the spindle microtubules may also be involved in meiosis and/or regulation of cellular dynamics. Depolymerization of microtubules with nocodazole causes increased PTPε activity [138]. Therefore, active PTPε maybe in constant flux near the microtubule cytoskeleton where it could activate the Fyn PTK which binds to and phosphorylates tubulin [30, 33, 35, 36, 50]. The functional significance of tubulin tyrosine phosphorylation is unknown.

Tyrosine modifications to microtubules

Microtubules are composed of alternating alpha and beta tubulin heterodimeric subunits that polymerized to form microtubules. Lafanechere and Job

(2000) described that "tubulin is subject to several post-translational modifications, include acetylation at a specific lysine close to the N-terminus of the α-subunit [143], poly-glutamylation near the C-terminus of both the α and β subunits [52, 53] and a cycle of tyrosine removal and addition at the C-terminus of the α-subunit [54-58]" [144]. The reactive tyrosine can be removed by a tubulin carboxypeptidase (TCP) [40, 41] that acts specifically on polymerized microtubules [42-44]. Tubulin tyrosine-ligase (TTL) catalyses the incorporation of tyrosine back into detyrosinated tubulin and acts primarily on free alpha tubulin subunits [62-65]. The interplay of TTL and TCP results in a cycling of tyrosination and detyrosination of α-tubulin.

The significance of tyrosination cycling on α-tubulin is unknown. There appears to be no difference in the assembly or dynamics between microtubules of tyrosinated or detyrosinated tubulin [145]. Although interphase cells contain primarily detyrosinated tubulin [46, 47], detyrosination of tubulin does not directly alter the stability of polymerized microtubules [146, 147]. Inhibition of TTL in cultured cells prevents tyrosination but does not affect the cytoskeletal structure or cell function, at least not in short-term studies [71, 72].

In a study of Xenopus tadpole heart, tyrosinated (Tyr) microtubules were found in the spindle at all stages of the mitotic cycle. The localization of non-tyrosinated (Glu) microtubules changed according to the stage of the cell cycle. The Glu tubules were "mainly restricted to the peripheral regions of the half spindles where the MTs have to sustain a bending stress". Glu tubulin was enriched in the centrosome in prophase and "from metaphase on, exclusively also in the centrioles". During anaphase-telophase transition and remaining during telophase, detyrosinated microtubules were enriched at the interzonal spindle region. Treatment of cells with millimolar vanadate caused additional assembly of tyr-MTs and a "drastic disarrangement of the Tyr-staining spindle fiber component became evident". "At the onset of anaphase, an extreme spindle lengthening presumably due to the separation of the Tyr- and Glu-MTs occurred. Obviously, the Glu-spindle fibers were less affected and remained largely in their original spindle position. Redistribution of anti-

dynein staining following vanadate incubation suggests a causal relationship between inhibition of dynein motor proteins and disarrangement of different microtubular spindle components. These results suggest that the changes in the spindle framework are at least partly due to misregulation of centrosomal phosphorylation events, respectively to inactivation of special cross-bridging proteins interacting between distinct MT-subsets by a phosphate mimicking effect of vanadate and finally, by a vanadate-induced displacement of polar asters." [148]

Other kinases associated with spindle and chromosome dynamics

Cyclin-dependant kinases

One of the primary control mechanisms of the cell cycle, both mitosis and meiosis is a family of serine/threonine kinases termed "cyclin-dependant kinases" (Cdk). The Cdks are tightly regulated by their close association with protein sub-units; the cyclins. Together, the choreographed increases and decreases of Cdks and their regulatory cyclins drive the cell cycle [149]. In mitosis, cellular levels of Cdks tends to remain stable throughout the cell cycle, however their activity changes with the availability of the regulatory cyclins which increase or decrease according to the stage of cell cycle [149]. This regulation is slightly different during meiotic maturation in the mouse where levels of Cdk1 as well as cyclin B proteins increase during maturation [39, 40]. While the levels of cyclins oscillate largely by changes in transcription or degradation, cyclin availability is also regulated by changes in subcellular localization. Maturation promoting factor (MPF) was first discovered by Masui and Makert for its ability to initiate and maintain metaphase in xenopus oocytes and early embryos [150] and has since been identified as active Cdk1/cyclin B [151]. MPF activity is regionalized within the oocyte [152, 153]. Individual Cdks bind to and are activated by specific cyclins. For instance, Cdk1 and its regulatory subunit cyclin B1 make-up the maturation promoting factor [150] and are key regulators of meiotic maturation in many species (see section on meiosis). Cdk1 can

also bind cyclins A and B2 while, Cdk2 is commonly regulated by either cyclin E or A, each producing a cyclin-specific function for paired Cdks. In addition to regulation by cyclins, inhibitory phosphorylation also influences Cdk activity. Cdk1 in particular is inhibited during prophase arrest by dual phosphorylation at T14 and Y15. Each site is phosphorylated by a specific kinase; Myt1:T14 and Wee1:Y15. Dephosphorylation by the dual phosphates Cdc25 (A, B and C) leads to activation of the Cdk1/cyclinB enzyme and meiotic resumption begins. Interestingly, Cdk1 and cyclin B localize to the centrosomes during late prophase, spindle poles during metaphase and the mid-body at anaphase [154, 155] in a cyclic pattern similar to Aur-A (see below). Cdk1/cyclinB are involved in microtubule dynamics stabilizing long microtubules during interphase and controlling formation of bipolar spindle in metaphase [46, 47]. During meiosis, the Cdk1 inhibitor Wee1 has also been identified on the meiotic spindle and disruption of Wee1 causes maturation failure and abnormal spindles [156-158]. Knockdown of Cdc25 also causes an MI block and abnormal congression of chromosomes [159]. Thus, various members of the Cdk1 regulatory system localize to the spindle and centrosomes and participate in metaphase spindle and chromosome dynamics.

Cdk2 and its partner cyclins A and E drive the G1/S phases of the cell cycle [152]. It is also responsible for the function of the centrosome cycle where in mitotic centrosomes are duplicated during the G1/S phase and reach final maturity at G2/M [160]. Relatively less research has been done on Cdk2 as compared to Cdk1 with respect to oocyte maturation, possibly because the majority of mammalian oocytes progress through the G1/S phase early in ovarian development. The final stages of meiotic maturation skip the G1/S portion of the cell cycle progressing directly from G2/MI to arrest again at MII.

Given the importance of Cdk2 in both the centrosome and cytoplasmic cell cycles, the results of CDK2 gene deletion in mice was a surprise. CDK2 (-/-) mice survive through adulthood, but are infertile [161]. Ovaries of Cdk2 (-/-) females contain apparently normal oocytes at E17.5 however, oocytes fail to complete

prophase, undergo apoptosis around P1 and are absent from the ovary by P21 [161]. This emphasizes an unexpected importance for Cdk2 in germ cell development [160] and vital roles for Cdk2 have been discovered in later stages of meiosis as well. For instance, work with xenopus egg extracts suggests that Cdk2/cyclinE is complementary to MAPK for the maintenance of metaphase-II arrest by inhibiting the anaphase-promoting factor [162]. Interestingly, Cdk2 phosphorylation of Cdc25 is requisite for full activation of Cdc25 and its subsequent activation of Cdk1 and entry into metaphase using xenopus cell extracts [54, 55]. Many of the proteins essential for this pathway have also been identified in meiotic maturation of xenopus and mouse oocytes [93-95] suggesting a key role for Cdk2 in the resumption of maturation and metaphase-II arrest. Further studies will be needed to determine if Cdk2 truly plays a vital role in the regulation of mammalian oocyte maturation.

It is important to note, the extensive use of the inhibitor drug roscovitine for studies of Cdk1 function in mammalian oocytes. Numerous studies, too many to list here, have relied strongly on roscovitine with the claim that this drug is Cdk1-specific. However, roscovitine is an equally potent inhibitor of Cdk2 (IC50 for Cdk1/cyclinB, Cdk2/cyclinA and Cdk2/cyclinE = 0.7 μM each) and is considered by cell cycle biochemists to be a "pan-cdk inhibitor" [96, 97]. This must be considered when examining past studies which relied on the Cdk1 inhibitory effects of roscovitine and may suggest the results in these results were combined effects of inhibiting both Cdk1 and Cdk2.

Aurora Kinases regulate meiotic centrosomes and spindle microtubules

Aurora proteins constitute another family of kinases closely associated with centrosome and spindle function. Aurora (Aur) was first identified as a kinase in drosophila [163]. In embryos from Aur mutant mothers, centrosomes duplicate but fail to separate. This produces monopolar spindles with large circular microtubule arrays emanating from a central pole with chromosomes dispersed around the microtubule array resulting in asynchronous divisions and polyploidy. The Auroras

are an evolutionarily conserved family of serine/threonine kinases found in diverse species, including Ipl1 (increase in ploidy-1) of budding yeast, Eg2 (protein identified from xenopus egg extracts), IAK proteins (Ilp1 and aurora-related kinases from human cells), AIE1 (Aur-C) and others [164-166]. Across species, the general function and localization of these proteins is consistent although slight variations exist. Three forms of aurora kinases have been identified in mammals such as humans and mice (A, B and C). Aurora A localizes with centrosomes while Aurora B and C associate with the centromeres and kinetochores of chromosomes. All three are found at the spindle mid-body during cytokinesis and are involved in centrosome and chromosome separation during mitosis and meiosis (for a review of Aurora kinases see [167]). Aurora C is expressed in the testis and ovary and associates with germ cells [75-77]. In sperm, Aur-C co-localizes with Aur-B on chromosomes and spindle mid-bodies [168]. Over-expression of kinase defective Aur-C in HeLa cells demonstrated its involvement in the localization of Aur-B and other passenger complex proteins such as INCENP and Bub1 onto the centromeres and mid-bodies [101], but little is known about specific activities in oocytes.

While Aur-B and C interact with CENP and other chromosome passenger complex proteins and participate more specifically in the organization and separation of chromosomes, Aur-A participates in the regulation of centrosome and spindle dynamics. The primary aurora found in mammalian oocytes is Aur-A [81, 83]. Inhibition of Aurora kinases with chemical inhibitors prevents oocyte maturation. During *in vitro* maturation, bovine oocytes exposed to the Aurora inhibitor VX680 had significantly reduce maturation to metaphase-II with many oocytes failing to extrude a polar body or to arrest at metaphase-II, resulting in the formation of multi-nucleate eggs [169]. Likewise, the drug ZM447439 (Aur inhibitor) disrupted maturation of mouse oocytes resulting in abnormal spindle formation and failed chromosome condensation [170]. These results might not be surprising considering the reduced levels of c-mos and low MAPK activity following inhibition of Aur-A in these bovine oocytes and the fact that knock-out of MOS in mice results in a very

similar egg phenotype [171]. Interestingly, Uzbekova et al (2008) did not find any specific localization of Aur-A to the spindle poles of bovine oocytes or embryos until after activation of the maternal genome at the 8-cell stage. After this, Aur-A was found at spindle poles and mid-bodies of mitotic embryonic cells [169] similar to what has been reported in somatic cells [167]. Interestingly, a recent study has reported that Aur-A kinase is activated prior to GVBD in mouse oocytes and is independent of both PI3K-PKB and CDK1 activities. Over-expression of Aur-A caused the formation of abnormal MI spindles while siRNA knock-down disrupted resumption of meiosis and spindle assembly [172]. Taken together, these studies implicate Aur-A in many essential roles during meiotic maturation.

In addition to its role in cytoskeletal dynamics, Aur-A (Eg2) has been implicated in the activation of c-mos transcription in xenopus eggs by phosphorylation and activation of CPEB leading to increased poly-A adenylation of stored messages, a necessary step leading to the translation of the dormant maternal mRNAs [173]. Subsequent studies have supported this role for Aur-A (IAK1/Eg2) in the phosphorylation of CPEB in mouse oocytes and its requirement for up regulation of maternal mRNA [174]. Similarly in bovine oocytes under going *in vitro* maturation, Aur-A was found at high levels in oocytes and possibly involved in the regulation of CPEB and c-mos [169]. However, these findings have recently been contradicted by another xenopus study which found that perturbation of Aur-A or Aur-B had no effect on CPEB phosphorylation nor subsequent poly-adenylation of maternal mRNAs [175]. Therefore this role for Aur-A in oocytes awaits further investigation.

Numerous other centrosome and cytoskeletal proteins are also targets of Aurora kinases in mitosis including the kinesin motor protein Eg5, mitotic kinesin-like protein 1 (MKLP1) and survivin (for review of mitotic kinases, see [176]). (For a discussion of SFK interactions with Aurora kinases, see the section on Src-family Kinases in Oocyte Maturation)

Polo-like Kinase

The polo-like kinases (Plk) are another family of the so called "mitotic kinases" [176] that share important roles during meiotic maturation in diverse species. Polo was first discovered in a screen of "maternal effect mutations" in drosophila embryos [177]. Embryos derived from polo mutant mothers exhibited abnormal spindles and aberrant mitosis as well as multi-polar and tetrapoid male meiotic germ cells. The Polo gene sequence shares high homology to Cdc5p in *Saccharomyces cerevisiae*, Plx1 in xenopus and Plk1 in mammals [177]. In mouse oocytes, Plk1 protein levels do not change during maturation, however an activating phosphorylation on Plk1 occurs 30 min before GVBD and remains high throughout maturation. Activity declines following egg activation and progression from metaphase-II to pronuclei [177]. This phosphorylation was inhibited by treatment with Cdk inhibitors roscovitine and butyrolactone. Since Plk1 is a known activation target for Cdk1 and activation of Plk1 was prevented by Cdk inhibitors, it was suggested that Plk1 participates in the Cdk1 self-activating cycle during meiosis as it does in mitosis [177]. This pathway maybe related to one which occurs in xenopus egg extracts and starfish oocytes where in Plk1 activates the dual phosphatase Cdc25 which in turn increases the activity of Cdk1 [92, 93]. Plk1 localizes to MTOCs and spindle poles during meiotic maturation in mouse oocytes, then shifts to the spindle mid-body during telophase in a pattern similar to the Aurora kinases (see above) [177]. Interestingly, Plk1 and Cdk1 also function sequentially in the phosphorylation and subsequent degradation of CPEB which is required for full maturation to MII in xenopus oocytes [178]. It will be interesting to see if this is also true in mammalian oocyte maturation. It is also interesting to note that although a recent study suggests an interaction of Plk1 and MAPK during mouse oocyte maturation, no correlation between SFK signaling and the Plk kinases have been reported.

Protein Kinase C

Protein kinase C (PKC) encompasses yet another family of serine/threonine kinases important in many aspects of cell cycle dynamics. Similar to other regulatory kinases, PKCs are co-factor dependant with isotype specific activities. The family is divided into three categories which include: "1) conventional isotypes which are activated by calcium, diacylglycerol (DAG) and phospholipids, 2) novel isotypes which are activated by DAG and phospholipids and, 3) atypical isotypes which are activated by phospholipids" [179]. Four types of PKC have been identified in mouse oocytes: PKCα, γ, δ and ζ [96-98].

Studies in somatic cells have proven an interesting pathway relating to metaphase spindle localization and cell polarity involving PKC and recent studies indicate a role for this pathway in mouse oocyte maturation. An intracellular complex composed of PAR3/PAR6/Cdc42 and PKCζ causes phosphorylation of Glycogen Synthase Kinase 3β (GSK3β) which in turn leads to stabilization of the metaphase spindle and polarization of the cell [99-103]. Phosphorylated (active) forms of PKCζ, PKCδ and GSK3β localize to the spindle poles and centromeres in metaphase mouse oocytes [95, 104]. Interestingly disruption of Cdc42 causes a loss of PKCζ at the spindle poles and prevents the cortical localization of the MII spindle [180]. For the oocyte to undergo proper asymmetric division and polar body extrusion, the meiotic spindle must move to the cortex [181]. Disruption of PKCζ causes elongated astral centrally located meiotic spindles [179]. Without this polarity induced by a cortically localized spindle, the oocyte is not able to undergo asymmetric meiotic division and meiosis arrests prior to polar body extrusion [180].

Knock-down of PKCδ in mouse oocytes induces a different phenotype signified by abnormally shaped spindles and misaligned chromosomes [182], not unlike those seen in our studies with Src kinase inhibitors (for discussion of PKC and SFK interactions, see section on Src-Family Kinases in Oocyte Maturation). Downs et al. (2001) examined PKC activities in both cumulus-enclosed and denuded oocytes [183]. When oocytes and COC were held arrested in hypoxanthine-containing media,

it was “concluded that direct activation of PKC in (denuded) oocytes suppresses maturation, while stimulation within cumulus cells generates a positive trigger that leads to meiotic resumption” and cumulus expansion [183]. On the other hand, Viveiros and colleges [83, 184] found PKCδ essential for the initiation of anaphase-I and regulation of the MI-MII transition. More recent studies of cumulus-enclosed oocytes demonstrate a requirement for PKC activation in cumulus cells combined with gap junctional communication to stimulate resumption of meiosis in the companion oocytes [60]. This suggests a message produced by cumulus cells involving a PKC pathway and subsequent transport of this message through gap junctions to trigger meiotic maturation in the oocyte. These studies demonstrate that PKC isoforms are involved in oocyte maturation and cumulus-oocyte communication, however identifying the specifics of these pathways will require further investigation.

Protein Kinase A

One of the points that we hope stand-out from this review of cell cycle and meiotic kinases is that no one kinase pathway stands alone. The intracellular milieu is exceedingly complex; all signaling pathways seem to include multiple crossovers into other pathways. The cAMP-dependant protein kinase A (PKA) is a prime example of this interrelationship. Long known as an integral player in the maintenance of meiotic arrest, new roles and intersecting pathways are still appearing.

The PKA holoenzyme consists of two catalytic (C) and two regulatory (R) subunits and is activated by increased levels of intracellular cAMP. When combined into a tetramer, the kinase is inactive. However, binding of cAMP to the R subunits causes the C subunits to be release thus activating PKA. Small changes in the levels of cAMP can result in strong changes in PKA activity [27]. In addition to regulation of its catalytic activity, PKA signaling is tightly controlled by PKA-anchoring proteins (AKAPs). The AKAPs are a diverse family of proteins, specific to cell-type and intracellular locations which, as the name suggests, anchor PKA to specific

subcellular regions. This provides a mechanism to maintain PKA in close proximity to other regulatory molecules for quickly and efficiently activating and deactivating of the kinase thus limiting its activity within both space and time. (For more on PKA and cAMP see the chapters on Signaling at Ovulation and Oocyte Maturation).

Studies on the localization of PKA have shown movements of the various PKA subunits to discrete subcellular locations at different stages of oocyte maturation and the presence of multiple PKA binding partners including the anchoring protein, AKAP1 (AKAP140) [185-187]. Disruption of PKA/AKAP1 association can induce meiotic maturation, even in the presence of high levels of cAMP. Translocation of PKA-RII to the mitochondrial membrane where is it anchored by AKAP1 is essential for the continuation of maturation [188]. Therefore, the specific subcellular localization of PKA is essential for proper regulation of oocyte maturation.

In addition to the AKAPs which regulate the localized activation of PKA signaling, PKA exists in two distinctly different forms (RI and RII) based on the type of R subunits. Studies that examined the roles of the two different types of PKA found them to have opposite effects on oocyte maturation. Varying formulations of cAMP can be incorporated into *in vitro* culture experiments to provide specific stimulation of one or both of the R subunits. Using this technique, it was found that stimulation of PKA-RI in cumulus enclosed and denuded oocytes prevented GVBD as expected. However similar activation of PKA-RII allowed GVBD in oocytes and stimulated cumulus expansion. Previous studies have indicated that cAMP levels in cumulus cells rise following FSH and LH receptor activation. Interestingly, when intact COC were cultured in 300 μM dbcAMP to maintain high intracellular levels in the cumulus cells and their companion oocytes, an additional pulse of PKA-RII induced meiotic maturation. Similar experiments with denuded oocytes failed to initiate GVBD. These studies demonstrate a mechanism where by activation of PKA in cumulus cells can have an opposite effect to that in the companion oocyte [145]. (For interactions of Src kinases with PKA, see section on Src-Family Kinases in Oocyte Maturation.)

Other Kinases

Other signaling pathways that participate in the resumption and progression of oocyte maturation include AMP-activated protein kinase [189], myosin light-chain kinase [190], Src-family kinases [134, 141, 191], protein kinase B/AKT [172, 192], phospholipase C [193], phosphoinositide 3-kinase (PI3K) [172, 194], myristoylated alanine-rich C kinase (MARCKS) [195], β-catenin and the Wnt pathway [196] and of course, the antagonists of the kinase pathways: the phosphatases [151, 197-204]. As research continues and our understanding of the complexity of cellular communications expands, additional pathways and intersecting ties are likely to be found.

Src-family kinases in the regulation of mammalian oocyte maturation

Recent evidence has implicated SFKs in the maturation of mouse oocytes [134, 141, 191] and development of the early preimplantation embryo [134, 142]. These studies suggest a role for SFKs in at least two stages: 1) maintenance of meiotic arrest and progression to metaphase-I; 2) chromosome spindle dynamics through meiotic metaphase-I and II and the first mitosis. Although the details of the Src signaling partners in the mammalian oocyte are not yet known, in our next section, we will examine the molecular interactions of SFKs with other cell cycle regulated signaling pathways and explore the few details of known SFK activities during meiotic maturation.

Src-Family Kinases in Oocyte Maturation

The Src-family of protein tyrosine kinases (SFKs) includes 9 members: Src, Fyn, Yes, Lyn, Lck, Blk, Fgr, Hck and Yrk. Three of these, Src, Fyn and Yes are ubiquitously expressed although their kinase activities are selectively regulated by phosphorylation, conformation changes and interactions with other proteins [205]. Because of similarities in SFK structure, there is some level of redundancy and over-lap in the activities of various family members thus allowing loss of one SFK to be

compensated for by the presence of another. The SFKs are generally regulated through phosphorylation on two tyrosine residues [206]. Autophosphorylation in the activation loop (Y416 in Src) increases kinase activity, while phosphorylation of Y527 in the C-terminus induces a conformational change inactivating the kinase. Phosphorylation of Y527 is catalyzed by the C-terminal Src kinase (Csk). Therefore, the most prominent sites of SFK activation are the dephosphorylation of Y527 and autophosphorylation of Y416.

Src-family kinase activity is greatly increased during mitosis (reviewed in [135, 136]). This activity is due to the dephosphorylation of the inhibitory Y527 by phosphatases (PTP) and an indirect activating phosphorylation of Y416 induced by an activated cdk1. The phosphatases responsible for the activating dephosphorylation of Y527 include PTP alpha (PTPα) [137] and epsilon (PTPε) [138]. PTP activity is increased during mitosis [137, 139]. Interestingly, EGF receptor activation causes translocation of PTPε to bind to microtubules [138]. Although this binding causes a decreased PTPε activity, the binding is transient and would provide a mechanism for co-localization of SFKs and their activating PTPs at the site of polymerized microtubules. Depolymerization of microtubules with nocodazole causes increased PTPε activity [138]. Therefore, active PTPε maybe in constant flux close to the microtubule cytoskeleton where it could activate the Fyn PTK which binds to and phosphorylates tubulin [10-14].

Src and Cdk1

As mentioned previously, Cdk1 is required for both the G1-S and the G2-M cell cycle transitions in mitotic cells. Eukaryotic Cdk1 is required for entry into mitosis and its kinase activity is essential for cell cycle function [207]. At least three substrates are known to be phosphorylated by Cdk1 during mitosis: Histone H1, Src and Abl, another nonreceptor tyrosine kinase that is closely related to Src. Mitotic phosphorylation of histone H1 is thought to cause chromatin condensation. Increased tyrosine kinase activity of Src has been observed during mitosis which is partially

regulated by Cdk1 phosphorylation of Src kinase [16, 17]. The specific mechanism of action is unknown, but this ser/thr dual phosphorylation of Src by Cdk1 during mitosis results in either an increase in Y527 phosphatase activity or the decrease in a Y527 kinase thus increasing Src kinase activity [208]. Src also phosphorylates lamin-B and may be involved in nuclear envelope breakdown [207].

In somatic cells, SFK activity is required for the G2/M transition, cleavage furrow progression and for full abscission during cytokinesis [10-13]. Treatment of MDA-MB-468 breast cancer cells with the Src/Abl inhibitor PD173955 or the specific disruption of Fyn, Yes or Src in fibroblast cells caused a block of mitosis at the G2/M phase [209]. Similarly, prolonged treatment of HELA cells with the SFK inhibitor PP2 prevents transition from G2/M phase in most cells. HELA cells that escaped inhibition with PP2, were found to arrest during cytokinesis with an elongated cytoplasmic bridge containing the mid-body [210]. This data demonstrates that SFK activity is required for entry into mitosis. During M-phase, Src activity is necessary for later cytokinesis events. Our recent results with the SFK inhibitor SKI606 or specific knock-down of Fyn kinase in GV or MII stage oocytes suggest that SFKs may not be required for the initiation of the meiotic cell cycle at GVBD or following fertilization although, a total knock-down of all three oocyte SFKs (Src, Yes and Fyn) would be necessary to truly prove this point. Our research does however, indicate that SFKs and specifically Fyn kinase play a critical role in chromosome and spindle dynamics through metaphase of meiosis I and II and the first mitotic cell cycle [134, 141].

Src and MAPK

MAPK is another critical player in meiotic and mitotic cell cycles (see section on Integrating Kinase Signaling). In oocytes, MAPK is primarily activated downstream of Mos in the MOS/MEK/MAPK pathway and has been studied mostly for its role in cell cycle regulation of maturation. Interestingly, there are alternative pathways that activate MAPK in somatic cells and which may play a role in the

oocyte; pathways that integrate SFKs and MAPK signaling downstream of growth factor and steroid hormone receptors.

Numerous receptors are known to activate SFKs. Examples found in mammalian oocytes include EGF and a-typical progesterone receptors. Activation of receptor-associated SFKs in somatic cells induces the activation of Ras GTPase which in turn activates Raf-1. Similar to Mos, Raf-1 is a serine/threonine dual specific MAP kinase kinase kinase that activates MEK which in turn activates p42/44 MAPK [211, 212]. Thus Raf-1 links Src and MAPK signaling pathways in somatic cells.

Raf-1 kinase is also present in mouse oocytes [171, 213]. Study of Raf-1 protein during spontaneous *in vitro* maturation of mouse oocytes in a basal M2 media found Raf-1 present throughout maturation. However, Raf-1 phosphorylation/activation was not detectable by western blot analysis until the MII stage [171]. The authors of this study felt this supported their theory that Raf-1 kinase had no activity in maturation of the mouse oocyte. However, Raf-1 kinase is activated downstream of cues such as growth factors, integrins and steroid receptors and the simple *in vitro* culture system used in this study contained no factors that would activate Raf-1. Since they did not examine *in vivo* matured oocytes nor attempt to directly stimulate the Raf-1 signaling pathway, the question of Raf-1 activity during maturation is still unanswered. Since all of the players are present in the mammalian oocyte, the possibility still exists for activation of the Src/Raf-1/MAPK pathway during mammalian oocyte maturation. What would be its specific role and what might trigger activation of the SFKs in meiotic maturation are also not known.

In xenopus, progesterone receptor activation through non-genomic pathways is considered the initiator of maturation [214-216]. Interestingly, membrane associated SFKs are activated within 3 minutes following progesterone exposure; one of the earliest measurable changes in xenopus oocytes and in direct line with the increase in MAPK activity [217]. The involvement of steroid hormones in

mammalian oocyte maturation is controversial. Granulosa cells of the antral follicle produce estrogens in response to FSH and increasing amounts of progesterone following the LH surge. Since steroid hormones pass freely through the lipid by-layers of cell membranes, it is likely the maturing oocyte is exposed to these hormones. Unfortunately, *in vitro* maturation studies have shown both activation [218] and failure of activation [219, 220] following exposure of mammalian oocytes to steroid hormones. While this suggests that steroid hormones are not "obligatory activators" of mammalian oocyte maturation *in vitro* [220], the question on whether they participate in the proper maturation and subsequent development *in vivo* remains unanswered.

In addition to the traditional steroid hormones, other triggers of steroid receptors exist within the antral follicle. Studies on the meiosis activating effects of follicular fluid lead to the isolation of FF-MAS (follicular fluid - meiosis activating sterol) [221]. Interestingly, FF-MAS was able to activate resumption of meiosis in denuded oocytes even in the presence of phosphodiesterase inhibitors which maintain a high level of cAMP and meiotic arrest. However, inhibitors of Src, Ras or MAPK block the effects of FF-MAS suggesting that this naturally occurring sterol uses the Src/Ras/Raf/MAPK pathway in oocytes [221]. It must be noted, however, that like other steroids and growth factors, the activities of FF-MAS directly on oocytes remain controversial [71].

Similar to the conflicting evidence for steroid receptor activation, *in vitro* studies have been unable to find direct roles for EGF receptors in mammalian oocyte maturation although the receptors are present and EGF-like ligands are produced by maturing cumulus cells [8, 222, 223]. Discrepancies amongst *in vitro* culture experiments maybe due at least in part to the large variations in the *in vitro* culture conditions used for oocyte maturation studies. However, the presence of growth factor and steroid receptors and downstream mechanisms within the oocyte suggest the possibility for their activation of the Src/Raf-1/MAPK pathway in the mammalian oocyte.

Src and Aurora B

Interestingly, recent studies have also implicated the Src/Ras/Raf/MAPK signaling pathway as the link between estrogen steroid receptor activation and the regulation of Aur-B and the spindle assembly check-point during mitosis [224, 225]. Since Aur-B is also present in mammalian oocytes, this leads to another possible pathway linking SFK signaling and regulation of chromatin and spindle assembly (see section on Integrating Kinase Signaling).

Src and PKC

As mentioned in our section on Integrating Kinase Signaling, PKC seems to play an integral part in oocyte maturation. Knock-down of PKCδ in mouse oocytes induces a aberrant phenotype signified by abnormally shaped spindles and misaligned chromosomes [182], not unlike those seen in our studies with SFK inhibitors. Interestingly, PKCδ can activate SFKs, but does this indirectly. In smooth muscle cells, PKCδ activates PTPα which in turn dephosphorylates and activates SFKs (see previous discussion of SFKs and PTPα [226]). PKCδ can also be phosphorylated by Src increasing PKCδ activity [227] and suggesting the possibility of an amplification loop between SFKs and PKCδ. In xenopus oocytes, injection of active PKCδ leads to Src activation and initiates GVBD at levels comparable to progesterone stimulation [228]. Since mammalian oocytes also express PKCδ [183] and the apparent importance of PKC in oocyte maturation (see section on Integrated Signaling) one wonders if PKCδ activity may play a role in SFK signaling during oocyte maturation.

Src and PKA

Src-family kinases can also be activated by PKA. cAMP stimulation can result in PKA phosphorylation of Src on S17 (pS17) [229]. Depending on cell type, pS17 may or may not increase the activity of SFKs [229, 230]. In cultured CHO (Chinese Hamster Ovarian) cells and hyper-activated sperm cells, PKA/cAMP stimulation leads to the pS17 and increased SFK activities [231-233]. A second

pathway by which PKA increases Src activity has also been identified. PKA serine phosphorylates Csk protein causing a decrease in kinase activity of Csk in somatic cells [234] and sperm [233]. This kinase is responsible for the regulatory phosphorylation of Y527 within the C-terminal tail region of SFKs. pY527 renders the SFK inactive, therefore, PKA can both stimulate Src activation by direct phosphorylation of pS17 and indirectly by phosphorylating and inhibiting Csk for a double amplification of Src activity. Since PKA is an important regulator of oocyte maturation (see sections on Integrated Signaling and Meiotic Maturation), it seems likely that PKA plays a role in the control of SFKs in the oocyte.

SFKs and meiotic maturation

Recent studies have demonstrated an essential role for SFKs in granulosa cell maturation and ovulation [69, 235]. As we have seen above, several pathways also exist for the activation of SFKs within the mammalian oocyte. Our studies detailed in the following chapters demonstrate the importance of SFKs within the oocyte during maturation and their effects in the dynamics of chromosomes and meiotic spindles. Due to the complexity of these signaling pathways and the many interacting players involved, we have not yet identified the specific triggers or downstream mechanisms of SFK signaling in the oocyte. Further extensive research will be required to determine the activators and specific interacting pathways involved.

Summary

With this background the stage has been set for the experiments that comprise this thesis. Given the overall complexity of interactive signaling pathways during oocyte maturation and fertilization, it was the singular purpose of this work to begin to unravel the role of SFKs and their relationship to the regulation of the cell cycle and cytoskeleton in the mouse oocyte.

Chapter Two

Functions of Fyn kinase in the completion of meiosis in mouse oocytes

ABSTRACT

Oocyte maturation invokes complex signaling pathways to achieve cytoplasmic and nuclear competencies for fertilization and development. The Src-family kinases FYN, YES and SRC are expressed in mammalian oocytes but their function during oocyte maturation remains an open question. Using chemical inhibitor, siRNA knockdown, and gene deletion strategies the function of SFK was evaluated in mouse oocytes during maturation under *in vivo* and *in vitro* conditions. Suppression of SFKs as a group with SKI606 greatly reduced meiotic cell cycle progression to metaphase-II. Knockdown of FYN kinase expression after injection of FYN siRNA resulted in an approximately 50% reduction in progression to metaphase-II similar to what was observed in oocytes isolated from FYN (-/-) mice matured *in vitro*. Meiotic cell cycle impairment due to a Fyn kinase deficiency was also evident during oocyte maturation *in vivo* since ovulated cumulus oocyte complexes collected from FYN (-/-) mice included immature metaphase-I oocytes (18%). Commonalities in meiotic spindle and chromosome alignment defects under these experimental conditions demonstrate a significant role for Fyn kinase activity in meiotic maturation. *[McGinnis LK, Kinsey WH and Albertini DF. Functions of Fyn kinase in the completion of meiosis in mouse oocytes. Dev Biol (2009) 327:280-287}*

INTRODUCTION

Mammalian oocytes acquire essential properties for fertilization and early development as they mature coincident with the process of ovulation. Meiotic maturation is elicited *in vivo* by the periovulatory LH surge and encompasses a series of events that occur over a time span of hours in rodents to days in the case of humans and other mammalian species [236]. While it has long been recognized that mammalian oocytes proceed from prophase-I arrest (GV) through to metaphase-II (MII) when removed from the inhibitory environment of the ovarian follicle [29], there is an emerging notion that a complex signaling dialogue between companion granulosa cells and oocytes modulates the reinitiation, progression, and arrest of meiosis at metaphase-II. Thus, original studies invoking cAMP and protein kinase A (PKA) signaling and communication between the oocyte and cumulus granulosa cells [29, 31, 32, 48, 237] appear as oversimplifications since recent studies have identified a complex multifactorial sequence of signaling effectors to be operative [67]. Specifically, reception of LH in mural granulosa cells, at least in rodents, is followed by de novo production of EGF family ligands that drive the reinitiation of meiosis. At the level of the oocyte, mechanisms involving selective inactivation of oocyte phosphodiesterases leading to decreased cAMP and PKA activities within the oocyte appear linked to a rise in Cdk1/cyclinB and p44/42 MAPK supporting meiotic maturation from the GV to metaphase-II stages [31, 238-240].

Of the pathways recently implicated in oocyte maturation and egg activation in mammals are those involving SFKs [134, 142, 191, 241, 242]. Src-family protein tyrosine kinases are a family of nine closely related protein tyrosine kinases of which three (FYN, YES and sometimes SRC) have been identified in mammalian oocytes [142, 241-243]. The involvement of SFK signaling pathways in mammalian oocyte maturation was shown by *in vitro* maturation studies in mouse oocytes where the SFK inhibitor PP2 blocked germinal vesicle breakdown (GVBD) [191]. Src-family kinases localize to region of the meiotic metaphase-II spindle and Fyn kinase co-precipitates with tubulin from egg cytoplasm [129, 134, 142, 243] further implicating

participation of SFKs in meiotic spindle function. An idea supported by the fact that aged rat oocytes undergo meiotic spindle disruption in the presence of SFK inhibitors (PP2 or SU6656) [129]. Moreover, while chemical inhibition of SFKs during fertilization of mouse oocytes permitted resumption of meiosis, the completion of meiosis-II and the first mitotic cell cycle were inhibited in association with severe abnormalities in spindle microtubule organization and chromosome alignment [134]. Since injection of a constitutively active form of Fyn into MII rat oocytes caused egg activation, metaphase-anaphase transition and extrusion of the second polar body [129] these studies collectively implicate SFKs s in microtubule dynamics and cell cycle progression in the mature mammalian oocyte and fertilized egg. The rather limited evidence implicating SFKs in earlier stages of meiotic maturation of mammalian oocytes prompted the present investigation into the role of SFKs in meiotic cell cycle progression in mouse oocytes with reference to meiotic spindle morphogenesis and functionality.

MATERIALS AND METHODS

Oocyte collection

Cumulus-Oocyte-Complexes (COC) were collected from 6-7 week old female mice. Most experiments used CF1 female mice (Harlan Sprague-Dawley, Indianapolis IN or Charles River Laboratories, Wilmington MA). FYN knock-out mice (B6/129S7-Fyn^{tm1Sor}/J; FYN (-/-) [244]) and the recommended control (B6/129SF2/J) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and a homozygous knock-out colony was maintained at the University of Kansas Medical Center. Mice were housed in a temperature and light-controlled room on a 14L:10D light cycle and experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Academy of Sciences 1996). Mice were euthanized by isofluorothane inhalation anesthesia followed by cervical dislocation. Females were stimulated with 5 IU equine chorionic gonadotropin (eCG; Calbiochem, San Diego CA). Ovaries were collected at 42-46 hours (h) post-eCG.

COC were released from large antral follicles into HEPES-buffered KSOM (FHM, Chemicon-Millipore, Billerica MA) with 4 mg/ml BSA (mFHM). For experiments in which ovulated oocytes were used, female mice were stimulated with 5 IU eCG followed 48h later with 5 IU human chorionic gondadotropin (hCG) and ovulated oocytes were collected from the oviducts 15-16 h post-hCG. For experiments examining the effects of chemical inhibitors of SFKs, COC were released from the ovary directly into mFHM containing the final concentration of the inhibitor treatment, then transferred directly into maturation medium supplemented with the same concentration of the chemical inhibitors. Prior to release of COCs, each ovary was cut into 2 pieces and the 4 ovarian pieces from each donor female were allocated to different treatments. In other experiments, COC were released into media without SFK inhibitor, with or without 300 μM cAMP according to the individual experimental protocol (see below). While most experiments were conducted with KSOM_MAT (see below) as a semi-defined culture medium, *in vitro* maturation media for the FYN -/- and their control COC were supplemented with 5% fetal bovine serum to provide optimum conditions for cumulus expansion. In experiments that used oocytes without cumulus cells, COC were collected as stated then cumulus cells manually removed by repeated pipetting with a pulled glass pipette.

Pharmacological treatment of oocytes

To test the effects of SFK chemical inhibitor on meiosis, oocytes with or without their companion cumulus cells were cultured for 16-17 h in KSOM-MAT ($KSOM^{AA}$ (Chemicon-Millipore, Billerica MA [245]) supplemented with 5 mM glucose [246], 1 mM glycyl-glutamine, 0.23 mM pyruvate, 4 mg/ml BSA, 0.6 mM L-cysteine, 0.5 mg/ml D-glucosamine, 0.02 μM ascorbate, 1% insulin-transferrin-selenium (ITS), 0.2 IU/ml recombinant human FSH (Serono Reproductive Biological Institute) and 10 ng/ml EGF Calbiochem, San Diego CA). SKI606 (Calbiochem, San Diego CA) was prepared as 10 mM stock solution in DMSO and stored -20˚C. Media were prepared fresh on the day of oocyte collection and pre-equilibrated in a

humidified (6%CO_2, 5%O_2 and 89%N_2) incubator (Sanyo) for at least 2 h before oocyte culture. Denuded oocytes were matured in groups of <10 per well in 60-well NUNC Terasaki plates with 10.5 µl medium over layered with 3 µl oil (0.22 µm sterile filtered Sigma Embryo Tested Mineral Oil stored in the dark; cat. M8410). Intact COC were cultured in 30-50 µl drops covered with oil in NUNC 4-well plates. After 16-17 h *in vitro* maturation, oocytes were fixed for labeling and confocal image analysis.

Fixation and immunohistochemical staining

Methods for fixation and immunohistochemistry were similar to those previously reported (McGinnis et al 2007). Briefly, oocytes and COC were fixed for 10 min at room temperature in FHM medium with 3% paraformaldehyde followed by 30 min at 35˚C in 2% formaldehyde microtubule stabilization buffer (MTSB-XF [247]). After fixation, COC were transferred into wash solution (McGinnis et al 2007) and held overnight at 4˚C. All fixatives and wash solutions were supplemented with 40 µM phenylarsine oxide, 100 µM sodium orthovanadate and 10 µM okadaic acid to inhibit phosphatase activity. Antibodies used included Clone 28 antibody (Biosource International, Camarillo CA USA) to localize activated forms of SFKs, anti-phosphotyrosine antibody (clone 4G10, Upstate, Lake Placid NY, USA), α and β tubulin (Sigma). All of these were mouse monoclonal antibodies. YOL 1/34 rat monoclonal α tubulin (Abcam, Cambridge MA). Secondary antibodies were Alexa 488 or Alexa 568 (goat anti-mouse or goat anti-rat depending on the source of the primary antibody; Molecular Probes, Eugene OR). Oocytes were labeled with primary antibodies at 35ºC for 1h or overnight at 4˚C followed by secondary antibody for 1h. After secondary labeling, oocytes were transferred to a wash solution containing 1 µg/ml Hoechst 33258 with or without 1:100 Alexa 568-phalloidin and stored in the dark overnight at 4˚C. Oocytes and COC were mounted the following morning and imaged (mounting medium consisted of 1:1 glycerol: PBS supplemented with 5 mg/ml sodium azide and 1 µg/ml Hoechst 33258). All chemicals, hormones

and reagents were purchased from Sigma Chemical Company, St. Louis, MO unless otherwise stated.

siRNA Knock-down of FYN PTK

COC were collected in mFHM supplemented with 300 μM cAMP to prevent GVBD. Most of the cumulus cells were removed by brief exposure to 0.3 mg/ml hyaluronidase and gentle pipetting with a fine glass pipet. FYN siRNA (Santa Cruz #sc-35425) and a 20-25 nt non-targeting scrambled control siRNA (Santa Cruz #sc-37007 & sc-36869) were prepared with supplied diluent at a concentration of 100 μM. Immediately before injections, siRNA was thawed and centrifuged at 16,000g for 10 min at 4˚C then back loaded into 0.3 μm Egg-Jek needles (MicroJek , Kansas City, KS). Injections were performed on an inverted Nikon Eclipse TE2000-S with an Eppendorf FemtoJet injection system. Preliminary studies were conducted to determine the optimum siRNA concentration. Three concentrations were tested including, 0.7, 1.40 and 2.8 μM. At 2.8 μM, control siRNA caused a reduction in the percentage of oocytes maturing to MII therefore this concentration was considered too high. The lower two concentrations of the control siRNA produced no measurable inhibition of maturation therefore 1.4 μM was selected for use in these studies. Following completion of siRNA microinjections, oocytes were transferred to Terasaki plates and culture for 4-5 h in KSOM-MAT supplemented with 300 μM cAMP to maintain GV arrest and to allow for siRNA inhibition of endogenous mRNA. Oocytes were examined at the beginning and end of this culture and graded for presence of a visible GV. Following this culture, oocytes were washed without cAMP and matured 17h in KSOM-MAT. The selective knock-down of Fyn was examined by semi-quantitative RT-PCR for both Fyn and Yes kinases.

Semi-quantitative RT-PCR

Groups of 10 oocytes (Fyn siRNA, control siRNA and non-injected) were dissolved into 500 μl of TRIzol-Reagent (Invitrogen) and stored at -80C. A known

concentration of Rabbit αGlobin mRNA was added to each tube of oocytes (1.0 pg/oocyte) in TriReagent. The methods for cDNA preparation and RT-PCR were as previously published [248]. Primers for Fyn and Yes kinases were generated using Primer Express 2.0 from Applied Biosystems (Fyn5'AGT GCC ATA CCC AGG CAT GA; Fyn3'GTG GGC AGG GCA TCC TAT AG; Yes5'GCT TCC ACA GCT GGT TGA TAT G; Yes3'AGA TCT CGG TGA ATA TAG TTC ATT CTT TC; Integrated DNA Technologies, Coralville IA). Relative levels of mRNA as determined by RT-PCR were statistically compared by t-test. P-value of less than 0.05 was considered significant.

Imaging and data analysis

Oocytes were imaged by serial z-sections (1-2 μm depth) on a Zeiss LSM510 confocal microscope. Serial z-sections were used to establish 3-dimensional relationships between the oolema, chromatin, meiotic spindle, first polar body and companion cumulus cells. Statistical analysis of meiotic maturation was performed using SPSS software (SPSS Inc, Chicago IL). Data were analyzed by ANOVA followed by Bonferonni post-hoc comparisons for experiments with 3 or more treatments. P-value of less than 0.05 was considered significant.

RESULTS

Activated Src-family kinases localize to microtubules in mouse oocytes throughout meiotic maturation.

Previous work had demonstrated that activated SFKs were concentrated in close association with microtubules of meiotic spindles in MII oocytes [134]. While Fyn kinase has been shown to localize in the region of premetaphase spindle [129], the specific association of activated SFKs to microtubules at the germinal vesicle stage oocyte has not been demonstrated [191]. Therefore, as an initial step we sought to determine the localization of activated SFKs during the early stages of meiotic maturation. To accomplish this, we used a phosphorylation site-specific antibody

(clone 28) that recognizes the activated (dephosphorylated) Y527 in the carboxyl tail region of SFK proteins [249]. Epitope distribution was determined by confocal fluorescence microscopy in oocytes fixed following 0, 8 and 16 hours of *in vitro* maturation as seen in fig. 1. In germinal vesicle stage immature oocytes, clone 28 staining was observed in a pattern that has been associated with cytoplasmic microtubules that surround the GV [247] as well as those emanating from cortical microtubule organizing centers (* MTOCs; fig. 1 GV). Clone 28 also labeled cumulus cells associated with the zona pellucida with staining being disposed along the cumulus cell surface and throughout the cytoplasm. Mitotic figures are commonly seen in the cumulus and in dividing cells, a prominent spindle labeling was readily apparent (fig.1, insert). In oocytes at metaphase of meiosis-I or II (fig. 1 MI and MII), activated SFKs were distributed throughout the meiotic spindles in a pattern not unlike that seen in our previous study of MII oocytes [134]. These findings clearly indicate that the clone 28 epitope is specifically expressed in a microtubule-associated pattern throughout all stages of oocyte maturation in mouse oocytes.

Chemical inhibition of Src-family kinases during meiotic maturation causes a dose dependant failure of progression through metaphase of MI.

To determine whether meiotic progression requires SFK activity, cumulus enclosed or denuded oocytes were matured in the presence of the inhibitor, SKI606. COC collected from ovaries of eCG primed mature CF1 female mice were released directly into media containing 1, 5 or 10 μM SKI606 or the solvent DMSO as a control. SKI606 is a new and highly selective inhibitor of SFKs and the closely related Abl kinases [250, 251] and has been used previously in studies of SFK activities in mouse zygotes [134]. The three concentrations of SKI606 were selected on the basis of preliminary dose response experiments (not shown) and our previously published studies with pronuclear stage zygotes [134]. SKI606 had no effect on the ability of oocytes to resume meiosis (GVBD 75-100% in all groups), however, maturation through metaphase-I was significantly reduced as seen in fig. 2A.

Exposure of either denuded oocytes (Egg) or cumulus enclosed oocytes (COC) to concentrations of SKI606 at 5μM or greater caused approximately 50% maturation failure. Oocytes that were enclosed by cumulus exhibited a similar response to SKI606. Oocytes matured in the continuous presence of the SFK inhibitor and progressed to metaphase-II exhibited marked defects in spindle and chromatin organization (fig. 2B). Notably at 5 and 10 μM concentrations, all of the oocytes that matured to MII in the presence of SKI606 were distinctly abnormal as detailed below. The effect of SKI606 was reversible (table 1).Oocytes exposed to the inhibitor for 5 h followed by wash-out and 17h of culture progressed to MII at a frequency that was 87% of that observed in controls and was not statistically significant (P>0.05).

Table 1. The short-term exposure inhibitory effects of SKI606 are reversible

Exposure	Time	N	%GVBD	%MII
5h	DMSO	12	100_a	100_c
5h	SKI	35	80_a	87_c

Treated oocytes matured through GVBD to metaphase-II at rates similar to controls (P>0.05).

Inhibition of Src-family kinases during meiosis results in spindle and chromosomal abnormalities.

To ascertain how SFK inhibition was influencing meiotic progression, confocal microscopy was performed on control and treated oocytes matured under the conditions described above. While phalloidin (*f*-actin) staining failed to reveal major changes in actin organization within oocytes, tubulin and DNA labeling revealed a range of defects in chromatin and microtubule disposition (fig. 3). Control oocytes matured to metaphase-II exhibited extruded polar bodies and chromosomes aligned on a bipolar meiotic spindle (fig. 3A). SKI606 induced abnormalities that appeared to reflect disruption of the interactions between chromosomes and spindle microtubules. For example, displaced chromosomes were found on MI (fig. 3B, 3G) or MII spindles (fig. 3H**) and in many cases bivalent segregation failed to occur at

anaphase or telophase of MI (fig. 3B, 3E-F). Thus, MI stage oocytes entered anaphase without disengaging homologues as evidenced by the presence of elongated anaphase spindles contained unresolved bivalents along nearly the entire length of the spindle (fig. 3E, 3F). Telophase oocytes also exhibited lagging chromosomes trapped within the contractile ring of forming polar bodies (fig. 3D, 3H*). In addition to the positioning of chromosomes, SKI606 induced a variety of severe spindle aberrations during meiosis. Many spindles were loosely organized and irregular in shaped, extending microtubule bundles towards displaced chromosomes (fig. 3G). Some oocytes exhibited segregated chromosomes in anaphase, but failed to extrude a polar body (fig. 3C). In such cases, oocytes with two spindles were formed, one of which was monopolar and the other being bipolar. In the example shown here (fig. 3C), the monopolar spindle was attached to a single pole of the bipolar spindle. However, this characteristic was not consistent for all oocytes that failed to complete cytokinesis. In most cases, bipolar and monopolar spindles were completely separate entities. Monopolar spindles displayed microtubule bundles that extended up to and along the oolema (not shown). Cortical actin localization appeared normal: As has commonly been seen, chromatin subjacent to the oolema was associated with cortical *f*- actin caps (fig. 3E-H) and *f*-actin density was increased in cortical regions involved in cytokinesis and the polar body extrusion (fig. 3D). The abnormalities observed after continuous exposure to SKI606 indicate that while spindle morphogenesis following

Src-family kinases are required for maintenance of meiotic arrest.

In the above inhibitor study, it was noted that oocytes exposed to SKI606 tended to undergo GVBD earlier than controls suggesting that meiotic resumption might be accelerated by this treatment. To further test this with better meiotic synchrony, we cultured immature oocytes with 300 μM dbcAMP which has been shown to prevent GVBD for up to 6 hours even in the presence of the maturation stimulators FSH and EGF [60]. Thus, to establish whether SFKs participate in maintenance of meiotic arrest, we cultured oocytes in KSOM-MAT (FSH and EGF

supplemented media) with 300 μM dbcAMP with or without 10 μM SKI606 for 5 hrs, washed samples in drug free medium, and allowed maturation to proceed for17h (in absence of dbcAMP). Following wash-out and IVM, both control and SKI606-treated oocytes matured to normal metaphase-II (93% with SKI606 vs 100% controls, table 2) confirming the reversibility of SKI606. However, inhibition of SFKs in the presence of dbcAMP either permitted or induced the resumption of meiotic maturation within 5 hr of treatment (60% GVBD with SKI606 vs 0% controls) suggesting that SKI606 sensitive kinases may participate in GV arrest. GVBD appeared to occur on schedule relative to controls, treated oocytes are impaired in their ability to support chromosome segregation during the metaphase-anaphase transition of meiosis-I.

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Table 2. Precocious meiotic resumption of oocytes cultured with Src inhibitor

Exposure	Time	N	%GVBD	%MII
5h	cAMP	10	0_a	100_c
5h	SKI+cAMP	43	60_b	93_c

Oocytes cultured with the Src inhibitor and dbcAMP resumed meiosis and initiated GVBD while control oocytes remained arrested at GV (a,b) $P>0.05$. Following wash-out from the dbcAMP both control and SKI606 treated oocytes matured to MII(c) $P>0.05$.

Injection of FYN siRNA into GV stage oocytes demonstrates a specific requirement for Fyn kinase during meiotic maturation.

One drawback of the pharmacological approach described above is that SKI606 inhibits the activity of all members of the Src-family and Abl-family kinases. Since FYN kinase is the most highly expressed of the Src-family members in mouse oocytes (Novartis Mouse Gene Atlas; https://biogps.gnf.org), we sought to more specifically test the role of Fyn kinase in oocyte maturation. Mouse oocytes arrested at the GV stage using dbcAMP were injected with either FYN siRNA or a 20-25 nt non-targeting control siRNA.

Following injections, oocytes were cultured for 4-5h in complete maturation medium supplemented with 300 μM dbcAMP to prevent resumption of meiosis and to allow time for depletion of FYN mRNA. GV stage oocytes were then washed free of dbcAMP and matured *in vitro* for 17h. After 17h of culture, oocytes were fixed and labeled for tubulin, actin and DNA for confocal microscopy as above. To confirm the knock-down and specificity of the Fyn siRNA, sets of 10 oocytes were pooled after 17h of culture and tested for mRNA levels of Fyn and Yes kinases. Yes kinase is a Src-family member closely related to Fyn and is the second highest expressed of the SFKs in mouse oocytes (Novartis Mouse Gene Atlas; https://biogps.gnf.org). Kinase mRNA levels were normalized against a known concentration of rabbit alpha-globin mRNA. The Fyn siRNA caused a significant (~80%, $P<0.05$) decrease in Fyn mRNA as compared to scrambled control siRNA

injected or non-injected oocytes (n=4, 2 and 4 replicates, respectively; fig. 4A). There was no difference in the level of Fyn mRNA between control siRNA injected versus non-injected oocytes and no effect of Fyn siRNA on the levels of Yes kinase demonstrating the specificity of the Fyn knock-down (fig. 4B).

Oocytes injected with FYN siRNA undergo GVBD but exhibited a reduced capacity to progress to metaphase-I (83% vs 100% of controls; $P<0.05$, fig. 5). Few oocytes were able to mature to metaphase-II (39% vs 86%; $P<0.05$). Injection of FYN siRNA did not induce premature resumption of meiosis in the presence of dbcAMP nor were overt chromatin or spindle organizational defects observed (not shown). The selectivity of FYN siRNA relative to controls implies a direct role for FYN in meiotic progression of mouse oocytes. To further test this, we next examined the phenotypes of oocytes collected from FYN knockout animals.

FYN (-/-) mice ovulate oocytes exhibiting defects in meiotic progression and spindle organization.

To further examine the role of Fyn kinase during oocyte meiotic progression, we compared oocyte maturation of normal and FYN (-/-) mice both *in vivo* and in vitro. Mature females were superovulated and oocytes were collected from oviducts 16 h post-hCG. The total number of oocytes ovulated by FYN (-/-) females were not different from control females (17 and 22, respectively; table 3). While all ovulated oocytes isolated from both FYN (-/-) and control animals had undergone GVBD (68 and 111 oocytes, respectively), only 85% (58/68) of oocytes from FYN (-/-) animals had matured to metaphase-II compared to 99% (110/111) of controls ($P<0.05\%$). Moreover, ovulated FYN (-/-) oocytes exhibited a slightly higher percentage of spindle and chromosome alignment abnormalities relative to controls (19% (11/58) versus 4% (4/110), respectively) although this difference was not statistically significant. The types of abnormalities detected in oocytes from Fyn (-/-) animals were associated with chromosome alignment and meiotic spindles (fig. 6). Since ovulated FYN (-/-) oocytes exhibited reduced maturation, we next tested maturation

using our in vitro culture system. This provided a more consistent controlled environment under which the maturation competence of FYN (-/-) oocytes could be ascertained without in vivo variability due to endocrine and/or ovarian factors.

Table 3. FYN (-/-) mice ovulate oocytes with meiotic progression defects

Strain	# Donors	# Oocytes	%GVBD	%MII	% abnormal MII
B6/129	5	111	100	99a	4c
Fyn KO	4	68	100	85b	19c

All ovulated oocytes initiated GVBD. A significant percentage of ovulated FYN (-/-) oocytes failed to reach metaphase-II and were blocked at metaphase-I (a,b) $P<0.05$. There was a tendency for FYN (-/-) oocytes to exhibit abnormal spindle and chromatin. alignment however this difference was not statistically significant (c) $P>0.05$.

FYN knock-out mice exhibit a meiosis I defect.

FYN (-/-) oocytes exhibited a slightly higher percentage of spindle and chromosome alignment abnormalities relative to controls (19% (11/58) versus 4% (4/110), respectively) although this difference was not statistically significant. The types of abnormalities detected in oocytes from Fyn (-/-) animals were associated with chromosome alignment and meiotic spindles (fig. 6). Since ovulated FYN (-/-) oocytes exhibited reduced maturation, we next tested maturation using our *in vitro* culture system. This provided a more consistent controlled environment under which the maturation competence of FYN (-/-) oocytes could be ascertained without *in vivo* variability due to endocrine and/or ovarian factors.

Fully grown oocytes (with or without cumulus cells) were collected from the ovaries of mature females at 44 h post-eCG similar to previous experiments. More oocytes from antral follicles were obtained from FYN (-/-) mice (n=3) as compared to normal control females (n=4; 53 oocytes versus 26 oocytes per donor, respectively). COC from FYN (-/-) mice matured *in vitro* for 16 h exhibited cumulus expansion similar to wildtype controls indicating that cumulus cell expansion is not influenced by the loss of Fyn.

The ability of FYN (-/-) oocytes to mature *in vitro* was significantly reduced compared to controls. FYN (-/-) oocytes progressed through GVBD but arrested at various stages between GVBD and metaphase-I. Only 20% (32/158) of FYN (-/-) oocytes matured to metaphase-II as compared to 49% (51/105) of controls (fig. 7). Of those oocytes from FYN (-/-) females that matured to metaphase-II, 22% of these (7/32) exhibited abnormal spindle formation and/or chromosome misalignment compared to only 4% (2/51) of controls although this difference was not statistically significant. FYN (-/-) oocytes that blocked at metaphase-I also demonstrated spindle and chromosome alignment errors but at a low incidence (5/78, 6%). No cytoskeletal defects were observed in metaphase-I arrested oocytes from control animals (0/31). The errors observed in FYN (-/-) oocytes resembled those identified in our earlier chemical inhibitor studies with misaligned chromosomes and malformed spindles. Thus, three distinct experimental strategies including biochemical inhibition of Src-related kinases , as well as siRNA knock-down, and gene knockout of Fyn kinase have yielded results consistent with a role for SFKs in meiotic progression, chromosome segregation and spindle function in mouse oocytes.

DISCUSSION

Oocytes prepare for fertilization and later development by undergoing a programmed series of maturational events in response to an ovulation-inducing surge of LH. At the level of the ovarian follicle, prominent changes in several signaling pathways occur during ovulation that ensure the coordinate induction of ovum release, cumulus expansion and oocyte maturation. Amongst these signaling pathways, SRC, RAS, and EGF have all been implicated in the terminal differentiation of granulosa cells [69, 252, 253] and preliminary reports have invoked a role for Src within the oocyte itself with reference to meiotic maturation and egg activation [134, 142, 191, 241, 242].

The present studies aimed to better define the activities of SFKs during meiotic maturation in mouse oocytes by taking advantage of the experimental

tractability of this model system. A series of *in vitro* maturation experiments showed that SFKs function in meiotic progression through the metaphase-anaphase transition of meiosis-I with the most prominent defects being a failure to segregate homologous chromosomes. From pharmacological inhibition, FYN siRNA knockdown, and the use of FYN knockout mice, resolution of bivalents and progress to metaphase-II appears to require the activity of SFKs, and in particular FYN kinase. The fact that genetic depletion of FYN kinase activity caused a similar series of meiosis defects both *in vivo* and *in vitro*, involving failure of chromosome disjunction during anaphase and abnormal organization of spindle microtubules, suggests that FYN assists in the coordination of karyokinesis and cytokinesis during meiotic maturation and that additional effectors such as other SFKs are likely to integrate meiotic cell cycle progression in this system. Fyn (-/-) mice are viable but females produce only 2-3 litters after which reproduction fails (Kinsey lab unpublished data). Interestingly, histological examination of ovaries following superovulation found antral follicles in Fyn (-/-) females that failed to ovulate (not shown). This may explain the disparity in the number of oocytes obtained between our *in vitro* maturation studies where oocytes are manually extracted from all antral follicles as compared to ovulated oocytes retrieved from the oviducts. Fyn (-/-) ovaries yielded far more oocytes than wildtype ovaries and a major proportion of the Fyn (-/-) oocytes were incapable of normal oocyte maturation, while the superovulated oocytes retrieved from the oviducts were more similar between the Fyn (-/-) and control animals. Studies are currently ongoing in our laboratory to better define these issues in the knock-out mouse.

The mechanism of Fyn action in chromosome segregation and spindle function may involve its close association with spindle microtubules. Previous studies on rat oocytes demonstrated localization of Fyn kinase to microtubule-containing structures [129, 243]. The fact that FYN is the most highly expressed SFK in mouse oocytes (Genomics Institute of the Novartis Research Foundation ("GNF"), https://biogps.gnf.org [254]), together with our earlier finding that activated SFKs

were associated with spindle structures after fertilization [134] led us to further define the functional significance of this association during oocyte maturation. The results presented here indicated that this spindle-associated Fyn is highly active since it was detected with the Clone 28 antibody. This association with microtubules continued during spindle morphogenesis following GVBD and persisted within spindles throughout meiotic maturation. Activated SFK localization was also maintained with MTOCs at all stages consistent with previous studies using pericentrin and MPM2 which demonstrated Ser/Thr phospho-proteins associated with MTOCs throughout meiotic maturation [247]. Talmor, Kinsey and Shalgi (1998) first demonstrated the co-localization of Fyn kinase to the meiotic spindle in mammalian oocytes and later proved the association of Fyn with tubulin in co- immunoprecipitation assays [129]. We have now demonstrated activated SFKs associate with meiotic spindles and cytoplasmic microtubules at all stages of meiosis.

SFKs in general and Fyn in particular are known to be involved in somatic cell mitotic cell cycle progression [255, 256]. Direct interactions of Fyn with dynein [257] and γ-tubulin [131, 132] have been proposed as responsible for mitotic arrest and microtubule stability in a variety of mammalian cell types [209]. In the mammalian oocytes, γTubulin is involved in spindle positioning and size [248]. Together with our current results this suggests a possible relationship between Fyn kinase signaling and γ-tubulin in the regulation of the meiotic spindle. Moreover, the presence of monopolar spindles and displaced chromosomes further implies that as yet unresolved forces exerted during bivalent attachment to the spindle may be under the local control Src-related kinases integrated into the meiotic spindle.

We have also shown that inhibition of Src-related kinases with the chemical inhibitor SKI606 and siRNA of Fyn kinase prevented the progression of meiosis from MI to metaphase-II. This finding contrasts those reported by [191] in which mouse oocytes matured in the presence of the SFK inhibitor (PP2) were unable to undergo GVBD. Our studies using the inhibitor SKI606 did not reveal a significant inhibitory effect on GVBD. In fact, we found that under meiosis arresting conditions, this drug

induced the resumption of meiosis (Table 2). The variance in the results of Zheng et al. (2007) and this work may be due in part to differences in the specificity or action of the inhibitors. For example, SKI606 represents a new generation of quinolinecarbonitrile derivatives with high specificity for SFKs [250]. This compound inhibits Src *in vitro* with an IC50 of 1.2 nM, while having low affinity for other kinases such as receptor protein tyrosine kinase ErbB-2 (IC50 of 2.6 μM) and Ser/Thr kinase Cdk4 (IC50 of 19 μM) [251]. The affinity of SKI606 is similar between Src and other family members such as Fyn [251]. The SFK inhibitor PP2 has an *in vitro* IC50 of 5 nM for Fyn kinase [258]. Like PP2 [259], SKI606 also inhibits the Src-related Abl kinase with an *in vitro* IC50 of 1.0 nM. While the general activities of SKI606 and PP2 are similar, slight differences in affinity and selectivity may account for the varied effects reported here and elsewhere. More likely, variations in chemical inhibitor results may take their origins in IVM culture protocols and/or differences in mouse strains used since mouse strain variations have been reported for both *in vivo* and *in vitro* matured oocytes [260, 261]. Additionally, Zheng and colleagues (2007) cultured naked oocytes in basal M2 medium without supplementation. While basal salt media allow for spontaneous meiotic resumption of cultured oocytes from many mammalian species [236], these conditions are inadequate for production of oocytes of high developmental potential [45]. In our studies we used a modified $KSOM^{AA}$ medium supplemented with factors designed to induce meiotic maturation and cumulus expansion (FSH, EGF, cysteine, glucosamine and ascorbic acid; see methods section). FSH and EGF are known activators of SFKs in somatic and granulosa cells [69, 262], therefore media containing these hormones may activate signaling pathways during meiotic maturation that are not operative in a basal salt medium.

In summary, the suppression of Fyn activity caused defects in spindle organization and chromosome segregation that in some cases brought meiotic maturation to a halt whereas in other cases permitted advancement of the cell cycle to metaphase-II albeit with significant distortions in the spindle. The lack of complete

penetrance as seen in oocytes from FYN (-/-) animals, implies some level of redundancy in function for ovarian SFKs. Taken together, these results suggests that the primary active SFKs associated with the spindle of the mammalian oocyte is Fyn and that disruption of Fyn kinase leads to failure of meiotic maturation by disrupting the cell cycle dynamics of spindle and chromosome organization.

ACKNOWLEDGMENTS

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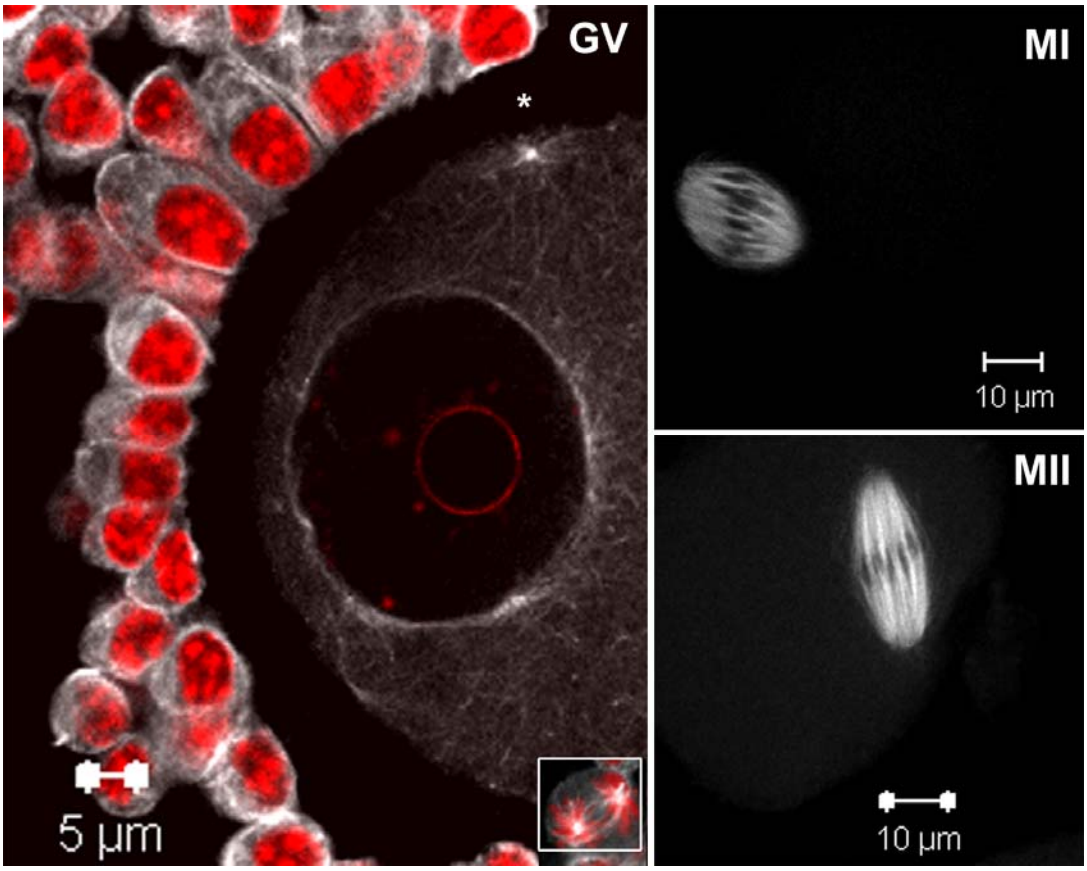

Figure 1

Fig. 1. Activated SFKs distribute in microtubule-like patterns at the GV, MI and MII stages of meiosis. Germinal vesicle stage oocytes were collected from PMSG primed mouse ovaries and matured in vitro for 0, 8 or 16 hours followed by fixation. Oocytes were labeled with a monoclonal antibody against activated Src-family PTKs (clone 28) and detected with Alexa-488-goat anti-mouse IgG (white) and co-labeled with the DNA dye Hoechst 33258 (red). Active SFKs are distributed in microtubule-like patterns consistent within oocytes and companion cumulus cells; see mitotic spindle microtubule labeling in cumulus cell (GV inset). Within GV stage oocytes, SFKs localized to cytoplasmic microtubule arrays surrounding the nuclear envelope as well as cortical microtubule organizing centers (*). At MI and MII, SFKs localize primarily to microtubules of the metaphase spindle.

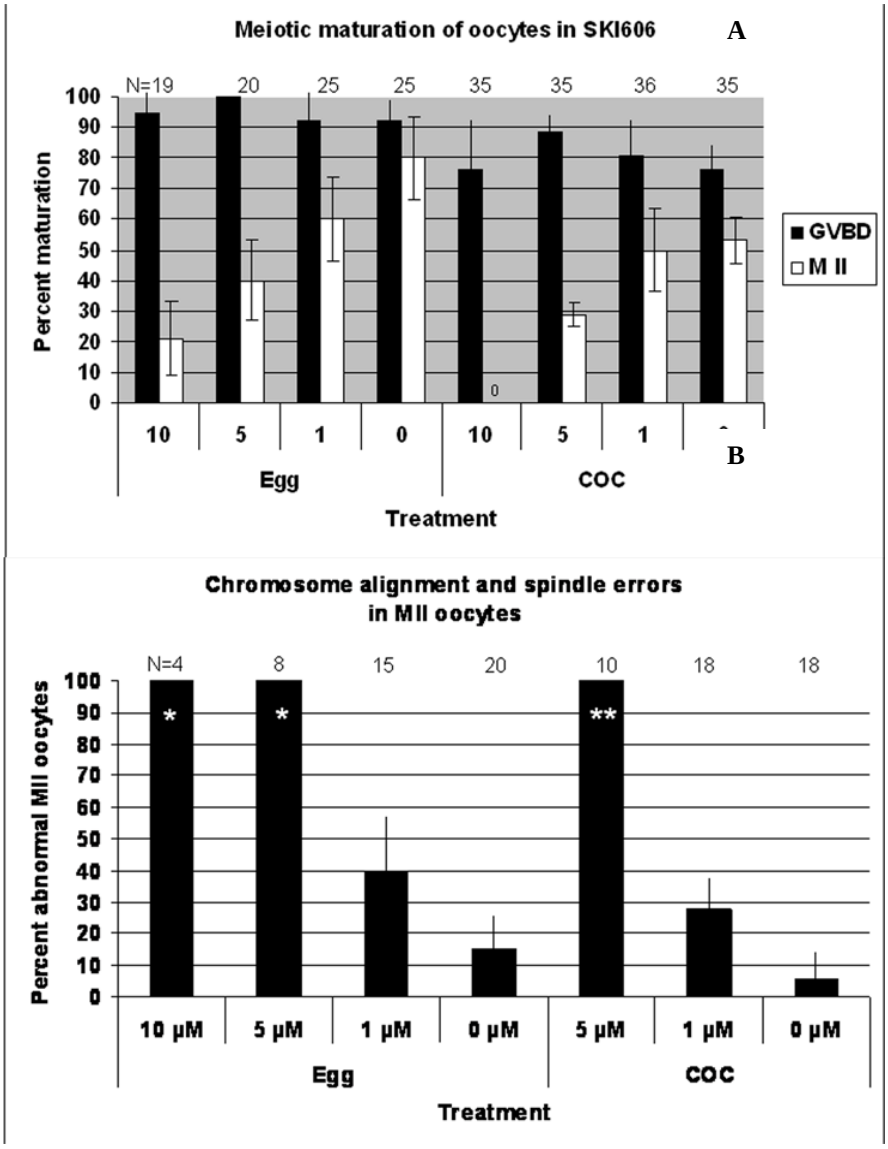
Meiotic maturation of oocytes in SKI606
A
N=19
20
25
25
35
35
36
35
Percent maturation
GVBD
M II
10
5
1
0
10
5
1
B
Egg
COC
Treatment
Chromosome alignment and spindle errors in MII oocytes
N=4
8
15
20
10
18
18
Percent abnormal MII oocytes
*
*
**
10 µM
5 µM
1 µM
0 µM
5 µM
1 µM
0 µM
Egg
COC
Treatment

Figure 2

Fig. 2. **Chemical inhibition of SFKs during maturation reduces meiotic potential and induces metaphase chromosome alignment and spindle errors.** COC were released from the ovaries of PMSG primed female CF1 mice at 40h post-hCG then cultured in KSOM-MAT medium supplemented with SFK inhibitor SKI606 for 17h. The concentration range of 0-10 μM chosen was based on previous studies [134]. (A) Inhibition of SFKs blocked meiosis at metaphase I in a dose dependant manner in both cumulus intact (COC) and denuded oocytes (Egg). No significant effect on the number of oocytes that underwent GVBD (black bars) was observed. However, maturation to metaphase-II (white bars) was inhibited. Cumulus-enclosed oocytes were more sensitive to inhibitor, with none maturing to MII in the 10μM dose and maturation in 5 μM being significantly lower than 1 μM or 0 μM ($P<0.05$). The ability for denuded oocytes to mature was also dose dependant with significantly fewer oocytes maturing to meiosis II in medium with 10 μM as compared to 0 or 1 μM. (B) SKI606 treated oocytes that progressed to metaphase II exhibited chromosome and spindle abnormalities. Of those oocytes that progressed to metaphase II in 5 or 10 μM (*) SKI606, 100% were abnormal as compared to the 1 and 0 μM concentrations.

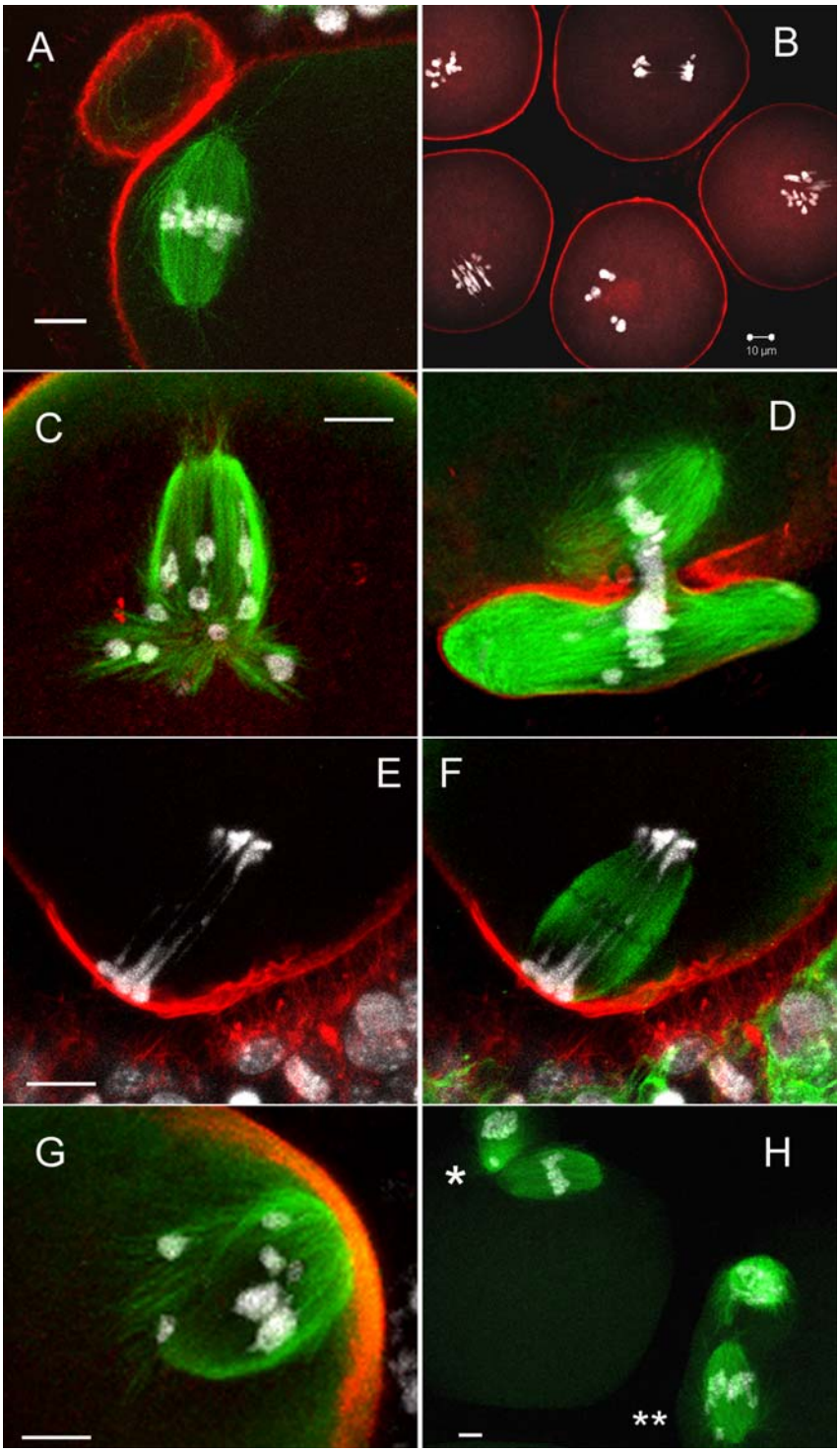

Figure 3

Fig. 3. Inhibition of Src-related kinases with SKI606 induced abnormal spindles and misalignment of chromosomes during meiosis. Denuded oocytes (eggs) and intact COC matured 17h in 0, 1, 5 or 10 μM SKI606 (fig. 2) were fixed and co-labeled with monoclonal mouse anti-αβ tubulin (microtubules; green) and goat anti-mouse Alexa-488, phalloidin-Alexa-568 (*f*-actin; red) and Hoechst 33258 (DNA; white) and oocytes were confocal imaged and examined as described previously. Three-dimensional reconstructions were used to examine oocyte meiotic status and the associations of tubulin, actin and DNA. The majority of control oocytes (A = 0 μM SKI606) matured and arrested at metaphase of meiosis II with normal spindle morphology, chromatin aligned on the metaphase plate and an extruded polar body. Oocytes exposed to SKI606 arrested at various stages of meiosis, many with displaced chromosomes and spindle microtubules that branch abnormally in seeming attempts to enclose these aberrant chromosomes (B-H). Fewer abnormal oocytes occurred in the lower doses of SKI606 as compared to the higher doses, however the general types of abnormalities were the same across all concentrations of the inhibitor (malformed spindles and wayward chromosomes: B, C, G = 10 μM; D, E, F = 5 μM; H = 1 μM SKI606, respectively). Panels E (tubulin label turned-off) and F (with tubulin (green)) are the same oocyte. Both images are shown to demonstrate the stretched chromosomes that failed to separate (E) even though the spindle microtubules have attempted to elongate and undergo anaphase (F). Panel H demonstrates two oocytes cultured in low (1 μM) SKI606 which extruded polar bodies but have displaced chromosomes. One oocyte has a chromosome that failed to segregate and was trapped in the constriction site between the polar body and the oocyte (*) while the second oocyte has multiple chromosomes displaced along the spindle (**). [scale bar = 10 μm]

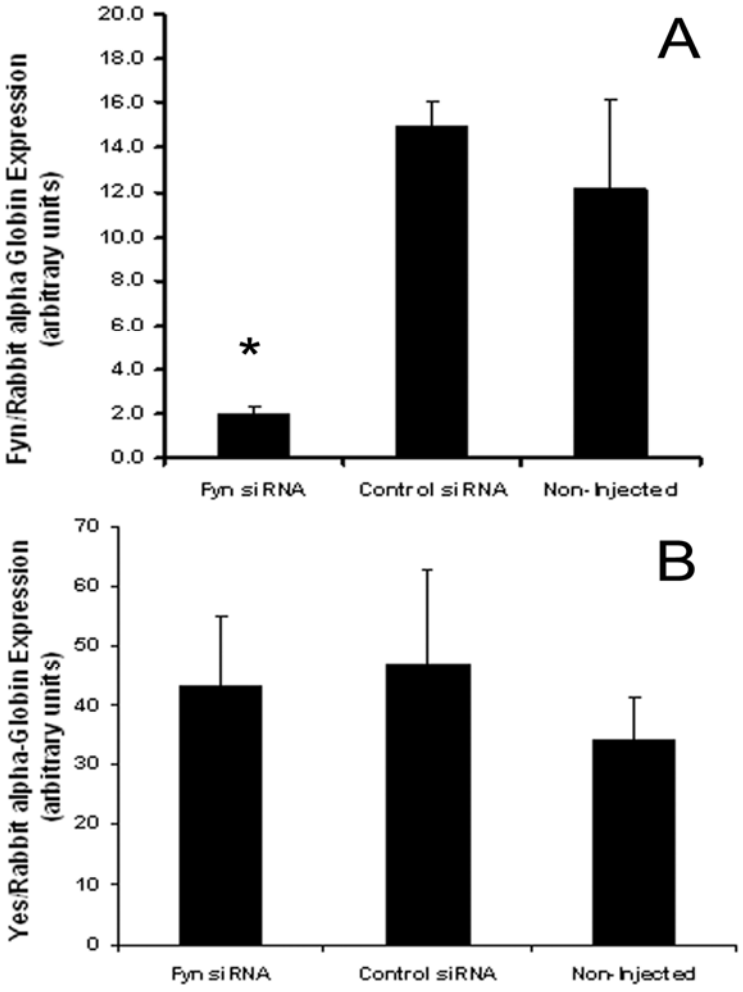
A
Fyn/Rabbit alpha Globin Expression (arbitrary units)
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
*
Fyn siRNA
Control siRNA
Non-Injected
B
Yes/Rabbit alpha-Globin Expression (arbitrary units)
70
60
50
40
30
20
10
0
Fyn siRNA
Control siRNA
Non-Injected

Figure 4

Fig. 4. Microinjection of Fyn siRNA significantly reduced levels of Fyn mRNA. Oocytes injected with Fyn siRNA were matured 17h then pooled in sets of 10 and tested by semi-quanititative RT-PCR for levels of Fyn mRNA and the closely related Yes mRNA. Injection of Fyn siRNA resulted in a significant (~80%; *$P<0.05$) knock-down of Fyn mRNA as compared to scrambled control siRNA injected and non-injected oocytes (A). Fyn siRNA had no effect on the levels of Yes kinase mRNA (B). (n = 4, 2, 4 replicates, respectively)

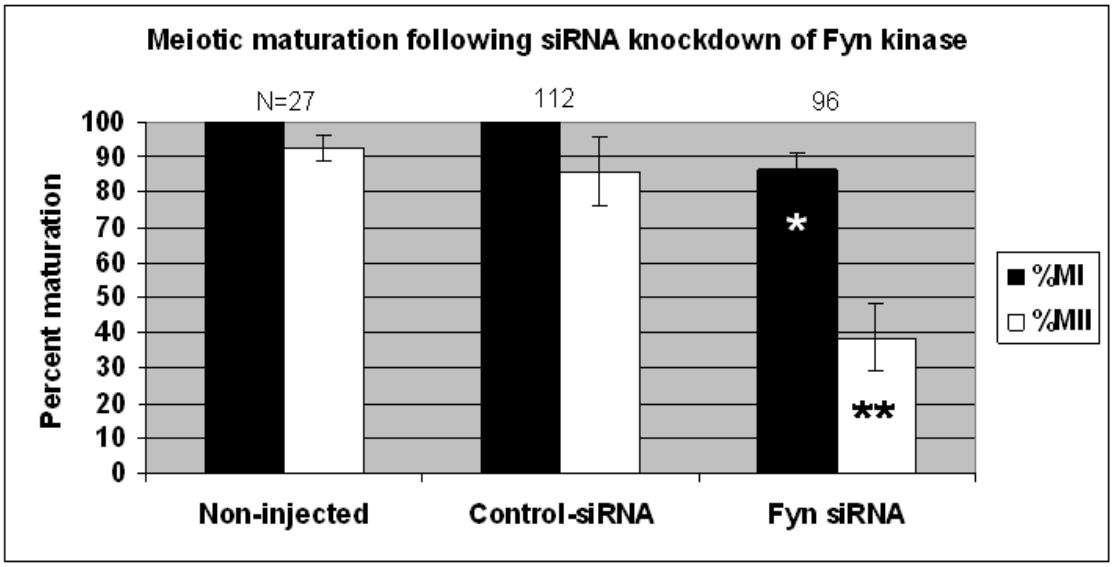
Meiotic maturation following siRNA knockdown of Fyn kinase
N=27
112
96
Percent maturation
100
90
80
70
60
50
40
30
20
10
0
*
**
%MI
%MII
Non-injected
Control-siRNA
Fyn siRNA

Figure 5

Fig. 5. **Fyn PTK activity is required for meiotic maturation of mouse oocytes.** Oocytes injected with FYN siRNA were held arrested at the GV stage in 300 μM cAMP for 4-5 h, washed in cAMP-free media, and cultured in maturation media for 16-17 h. Controls included injection with an equal concentration of scrambled control siRNA or processing in the above solutions without injection. Seven replicates with siRNA were conducted using both types of controls. Oocytes were labeled for microtubules and DNA as described above (fig. 3) and scored to determine meiotic stage. Injection of FYN siRNA into GV stage oocytes significantly decreased meiotic maturation since fewer FYN siRNA oocytes reached metaphase of MI compared to control siRNA injected or non-injected oocytes (83% versus 100% and 100%, respectively; *$P<0.05$). Maturation to MII was also impaired with only 39% of FYN siRNA injected oocytes reaching metaphase-II as compared to control siRNA and non-injected oocytes (86% and 93%, respectively; **$P<0.05$).

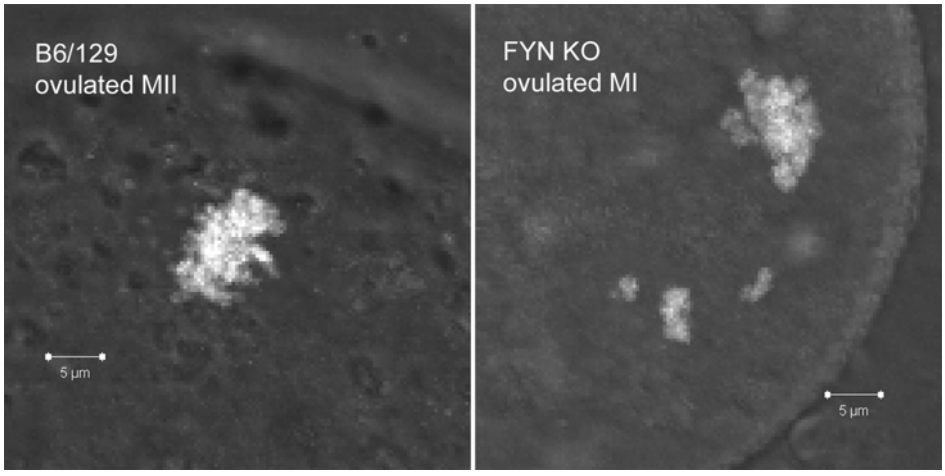

Figure 6

Fig. 6. Superovulated FYN (-/-) oocytes exhibit misaligned chromosomes. Oocytes were collected from superovulated FYN (-/-) and wildtype control mice 16h post-hCG. Control mice ovulated metaphase-II stage oocytes, 110/111, 99%, whereas only 85% (58/68) of FYN (-/-) had reached metaphase-II. Analysis of cytoskeleton and chromatin organization showed normal chromosome alignment in 99% of control MII oocytes (B6/129), however 19% of the MII and 6% of MI stage FYN (-/-) oocytes exhibited misaligned chromosomes. Differential interference contrast (DIC) images of ovulated oocytes B6/129 (MII) and FYN KO (MI) labeled with DNA dye Hoechst 33258 (white).

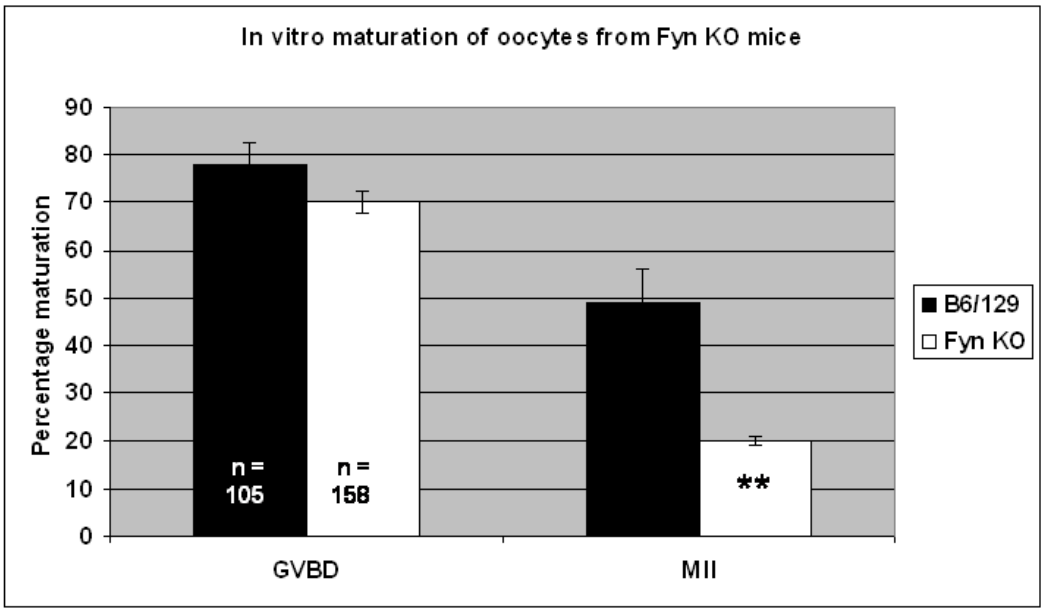
In vitro maturation of oocytes from Fyn KO mice
Percentage maturation
90
80
70
60
50
40
30
20
10
0
n = 105
n = 158
**
GVBD
MII
B6/129
Fyn KO

Figure 7

Fig. 7. Oocytes from FYN (-/-) mice exhibit reduced meiotic potential.
Oocytes and COC from FYN (-/-) and wildtype controls collected at the GV stage were matured in vitro for 16hrs and subjected to fluorescence analysis as before. FYN (-/-) oocytes exhibited reduced meiotic potential as indicated by the fact that while FYN (-/-) oocytes underwent GVBD to comparable levels as wildtype controls (70 % versus 76%, respectively), significantly fewer FYN (-/-) oocytes achieved MII (20% versus 49%; **$P<0.05$).

Chapter Three

Dynamics of protein phosphorylation and nuclear actin during meiotic maturation

ABSTRACT

Kinases and their targets require specific regional distributions to effect changes in cell function. Here, the protein phosphorylation patterns were mapped topographically over the course of meiotic cell cycle progression in *in vitro* matured mouse oocytes. Oocytes collected at the GV stage were matured *in vitro* in either a basal embryo medium (IVMb) or a medium designed to improve oocyte developmental quality (IVMh). After meiotic reinitiation, oocytes matured for 0, 2, 4, 8 and 16h were fixed and probed for phosphotyrosine (pTyr) or mitosis associated phosphoserine/threonine (MPM2) epitopes. Quantitative and qualitative analyses were performed on oocytes at progressive stages of maturation using confocal microscopy and image analysis. pTyr epitope was evident throughout the cytoplasm at all stages with intense staining at the cortex. Cytoplasmic and cortical labeling increased significantly between MI and MII. Supplemented media significantly increased cortical levels of pTyr proteins at MII when compared to oocytes matured under IVMb conditions. In contrast, MPM2 epitope was distributed in patterns distinctly different from those bearing pTyr. Cytoplasmic MPM2 epitope localized to the meiotic spindle, spindle poles and cytoplasmic MTOCs but was not detectable in the cortex. Cytoplasmic MPM2 localized in a subcortical region. In meiotic spindles, pTyr epitope was concentrated at spindle poles whereas MPM2 was distributed throughout the spindle in a pattern distinct from pTyr. Both cortical and spindle pole pTyr epitopes were absent in oocytes obtained from Fyn (-/-) oocytes suggesting a role for the Fyn tyrosine kinase in meiotic spindle integrity and cortical remodeling. These findings demonstrate that tyrosine kinases impose spatially discrete patterns of protein phosphorylation that may underlie the distinction between nuclear and cytoplasmic maturation in mammalian oocytes.

INTRODUCTION

Interacting cascades of signaling pathways dependent upon protein phosphorylation drive many aspects of cellular metabolism. These pathways are essential in the life cycle of an oocyte and may be used discriminately during the growth and maturation stages of oogenesis [263]. In the case of oocyte maturation, regional modifications in protein phosphorylation contemporaneously support the maturation of both the nucleus and cytoplasm during meiotic cell cycle progression and are required for developmental competence following fertilization [196, 264-268].

Alterations in the timing or topographical distribution of protein phosphorylation during meiotic maturation can impose detrimental effects on embryonic, fetal or offspring health. For instance, *in vitro* culture of oocytes and embryos contributes to fetal wastage due to aneuploidy or abnormalities in imprinting and gene expression [269]. Disruption of kinase signaling pathways in oocytes results in defects in chromosome condensation and segregation that have been linked to embryonic aneuploidy [263]. Moreover, failure to correctly localize and activate kinases cause disruptions in fertilization and embryo development [266]. While ser/thr kinases are well known to drive the meiotic cell cycle, only recently has it become apparent that tyrosine kinases are also operative during the process of oocyte maturation and early development in mammals. For example, SFKs appear to mediate meiotic spindle and chromatin modifications in rodent oocytes [129, 134, 142, 191]. Until the interplay between discrete protein kinase signaling pathways can be resolved in mammalian oocytes, the role of these factors in determining the quality of oocyte nuclear and cytoplasmic maturation will remain unclear. Knowledge of the regional patterns of phosphorylated proteins during maturation may help in the diagnosis of egg quality. Indicators of oocyte quality would be useful in the development of *in vitro* systems for ART and may provide insight into the regulatory mechanisms that are required to obtain and express developmental competence.

In somatic cells it is well accepted that global changes in protein phosphorylation are triggered in response to extracellular cues that activate growth factor and integrin receptors and stimulate entry into the cell cycle. Ligand receptor interactions and the ensuing signaling events are often regionalized to achieve tighter temporal control. Oocytes present a unique set of problems with respect to signaling and cell cycle regulation. For example, a strict localization of protein kinase-A (PKA) to the cytoplasm is essential for the maintenance of G2/M meiotic arrest. To reinitiate the meiotic cell cycle PKA migrates from the cytoplasm to the mitochondrial membrane where it is sequestered by one of the many forms of A-Kinase anchoring proteins (AKAP1) [188]. And as mentioned above, tyrosine kinases have recently been shown to increase significantly following fertilization [270] but the origins of these changes during meiotic maturation have not been rigorously studied. Thus, the importance of kinase regionalization and the lack of information on the relative contributions of tyr or ser/thr kinases has prompted this investigation [188].

MATERIALS AND METHODS

Oocyte collection

Cumulus-Oocyte-Complexes (COC) were collected from 6-7 week old female mice. Most experiments used CF1 female mice (Harlan Sprague-Dawley, Indianapolis IN USA or Charles River Laboratories, Wilmington MA). Fyn knock-out mice (B6/129S7-Fyn^{tm1Sor}/J) and the recommended control (B6/129SF2/J) mice were purchased from Jackson Laboratories (Bar Harbor, ME USA) and a homozygous knock-out colony was maintained at KUMC. Mice were housed in a temperature and light-controlled room on a 14L:10D light cycle and experiments were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Academy of Sciences 1996). Mice were euthanized by isofluorothane inhalation anesthesia followed by cervical dislocation. Females were stimulated with 5 IU equine chorionic gonadotropin (eCG; Calbiochem, San Diego CA USA). Ovaries were collected at 42-46 hours (h) post-eCG. COC were released

from large antral follicles into HEPES-buffered KSOM (FHM, Chemicon) and 4 mg/ml BSA (mFHM) with 300 μM dbcAMP to prevent meiosis resumption during the collections. COC were cultured in either $KSOM^{AA}$ (basal IVM media; IVMb; (Chemicon-Millipore, Billerica MA) or $KSOM^{AA}$ medium supplemented with components previously shown to improve cumulus expansion and oocyte maturation (1 mM glycyl-glutamine, 0.23 mM pyruvate, 4 mg/ml BSA, 0.6 mM L-cysteine, 0.5 mg/ml D-glucosamine, 0.02 μM ascorbate, 1% insulin-transferrin-selenium (ITS; Sigma Corp, St Louis MO), 0.2 IU/ml recombinant human FSH (Serono Reproductive Biological Institute, Rockland, MA) and 10 ng/ml EGF (Calbiochem, San Diego CA); IVMh).

Fixation and immunohistochemical labeling

Methods for fixation and immunohistochemistry were similar to those previously reported [134]. Briefly, oocytes and COC were fixed for 10-20 min at room temperature in FHM medium with 3% paraformaldehyde followed by 30 min at 35°C in microtubule stabilization buffer with 2% formalin and 0.5% triton-X100 (MTSB-XF [118]). After fixation, eggs and embryos were transferred into wash solution (McGinnis et al 2007) and held overnight at 4°C. All fixatives and wash solutions were supplemented with 40 μM phenylarsine oxide, 100 μM sodium orthovanadate and 10 μM calyculin-A to inhibit phosphatase activity. Antibodies used included mouse monoclonal anti-phosphotyrosine antibody (clone 4G10, Upstate, Temecula CA) and MPM2 (Upstate, Temecula CA). Secondary antibodies were Alexa 488 goat anti-mouse (Molecular Probes, Eugene OR). Oocytes were labeled with primary antibodies overnight at 4C followed by secondary antibody for 2h at 35C. After secondary labeling, oocytes were transferred to a wash solution containing 10 ug/ml Hoechst 33258 with 1:100 phalloidin-Alexa 568 and stored in the dark overnight at 4°C. Oocytes that were not part of the fluorescence intensity pools were co-labeled with rat monoclonal tubulin antibody (YOL1/34; Abcam, Cambridge MA) followed by secondary antibody goat anti-rat or goat anti-rabbit

Alexa-568; primary antibodies labeled for 1h at 35C followed by secondary for 1h at 35C). Oocytes and COC were mounted the following morning and imaged (mounting medium consisted of 1:1 glycerol: PBS supplemented with 5 mg/ml sodium azide and 10 μg/ml Hoechst 33258). All chemicals, hormones and reagents were purchased from Sigma Chemical Company, St. Louis, MO unless otherwise stated.

Oocyte processing

Oocytes were matured *in vitro* for 0, 2, 4, 8 or 16h in IVMb or IVMh media. At specific times, a random pool of COC were removed from culture, cumulus cells were stripped manually by pipetting with a fine tipped glass pipet followed by brief washing in hyaluronidase (0.3 mg/ml) and fixation. Following fixation, oocytes were sorted into groups containing 2 oocytes from each time point selected at random from the pools of fixed oocytes. This permitted the labeling and imaging of oocytes from all time points together in one cohort and eliminated any signal variability due to labeling or imaging conditions; in this way, we were able to determine variations in staining intensity attributable to phosphoepitope abundance between oocytes within each set.

Imaging and data analysis

Serial z-sections (1 μm depth) were obtained for each oocyte using a 40x water immersion lens on a Zeiss LSM510 confocal microscope. These data sets were used to measure 3-dimensional relationships between the oolema, chromatin, meiotic spindle and first polar body. Fluorescence intensity levels at the central plain of each oocyte (6 oocytes/ time/ treatment) were considered to represent the overall amount of phospho-protein at each stage of maturation. A single TIFF image from the central plain of each oocyte set was exported for Metamorph image processing. This method allowed us to compare the localized patterns of phospho-proteins at each time point and between the types of maturation conditions. Line-scans were produced for each

oocyte to allow for comparisons of localized fluorescence intensity. Measurements taken for each oocyte included: (1) a line-scan (7 um thick) covering the maximum diameter of the oocyte; (2) linescan around the circumference (7 μm thick) that included the oolema and cortex; and (3) the central cytoplasm that excluded the oolema/cortical regions of the original linescan. Average fluorescence intensity was statistically compared between stages of maturation and culture media treatment using SPSS software (SPSS Inc, Chicago IL). Data were analyzed by ANOVA followed by Sidak post-hoc comparisons. P-value of less than 0.05 was considered significant.

RESULTS

Tyrosine phosphorylation increases during oocyte maturation.

Oocytes were matured *in vitro* for 0, 2, 4, 8 or 16-17h. Two oocytes from each time point were pooled, labeled and imaged together to enable a direct comparison of fluorescence intensity between oocytes during maturation. Images taken from the central plain of each oocyte were compared visually (confocal images, fig 1-2), and used for linescan comparisons of fluorescence intensity across the median and around the cortex of each oocyte (fig 3-4). Intense cortical labeling associated with the oolema and large cytoplasmic patches were seen at all stages (fig 1-2). Figure 1 demonstrates pTyr levels in oocytes at the plane containing the chromatin and shows the diversity of cytoplasmic pTyr labeling. Most cytoplasmic patches were subcortical however, in GV stage oocytes one or two large cytoplasmic patches were localized adjacent to the GV (fig 1A-B). By 2h of maturation (GVBD) pTyr labeling was more diffuse throughout the cytoplasm, but still exhibited cytoplasmic patches (fig 1C). Oocytes at metaphase-II were easily distinguished from immature oocytes by increased pTyr proteins throughout the cytoplasm (fig 1F). Intense label was evident at the spindle poles of metaphase-II (fig 1F arrow) of IVMh and IVMb oocytes (5/5 and 3/6, respectively), which is similar to our previous studies with oocytes matured *in vivo*[134]. Tyrosine phosphorylated proteins were also

evident at the spindle poles of most metaphase-I (fig 1E arrow) oocytes (4/5 IVMh and 4/6 IVMb). Figure 2 demonstrates oocytes from three stages of maturation that were labeled and imaged together in a single set of oocytes. These images were all taken from the same central plain of the oocytes and no changes have been made to the intensity levels in order to demonstrate the original images from which intensity comparisons were made. Cytoplasmic pTyr epitope staining was similar between oocytes at the GV (fig 2A) and metaphase-I (fig 2B) stages. Notably, total cytoplasmic pTyr epitope levels increased between MI-MII (fig 2B, 2C).

Stage specific Tyrosine phosphorylation at the oocyte cortex.

To quantify protein tyrosine phosphorylation, images from oocytes in the previous experiment were analyzed by linescan comparisons and statistical analysis of fluorescence intensity levels. Linescan tracings graphically demonstrate alterations evident in fluorescence images. As seen in photographs (fig 1-2), pTyr epitope is highly localized in the oocyte cortex at all stages of maturation. During maturation, the transition from metaphase-I to metaphase-II was associated with an increase in both cytoplasmic and cortical pTyr epitope (fig 3-4). In addition to showing high levels of pTyr localized at the cortex (Fig 3*), levels were increased as oocytes transitioned from MI to MII. Although present at much lower levels (fig 3*****), cytoplasmic pTyr epitope also increased during the MI to MII transition (fig 3). Statistical analysis of cortical and cytoplasmic pTyr levels confirmed significance at the MII stage (fig 4). Moreover, cortical pTyr levels (fig 4A) were also statistically higher in oocytes matured in IVMh medium (12.3 ±1.49) as compared to IVMb (17.4 ±1.53; $P<0.05$) suggesting that culture conditions influence the magnitude of cortical protein tyrosine phosphorylation. However, no such difference was observed between levels of cytoplasmic pTyr (fig 4B) in either culture treatments (7.12 ±0.87 and 5.47 ±0.87, respectively). Thus stage and culture conditions are associated with differential quantity of pTyr epitopes during meiotic maturation in the mouse. We next examined epitopes detected by the monoclonal antibody MPM2 that is known to mark many relevant substrates for M-phase specific kinases.

Patterns of MPM2

Sets of oocytes from the same cohorts that were labeled for pTyr proteins were labeled for mitosis-associated ser/thr phospho-proteins (MPM2). This antibody has been used by our laboratory in previous studies to identify meiosis-specific phosphorylation patterns associated with the meiotic spindles and MTOCs, but overall cytoplasmic patterns of localization have not been reported. Figure 7 compares oocytes from three stages of maturation. These images were taken at the central focal plane under the same conditions as described above. Little MPM2 epitope is detectable in the cytoplasm of GV stage oocytes (fig 5A) but levels progressively increased from GV to MI (fig 5B) to metaphase-II (fig 5C). A gradient of epitope distribution is apparent in an eccentric pattern characterized by higher levels near the cortex as relative to the center of the oocyte. Unlike pTyr epitope, which aligned with the cortex and/or oolema, MPM2 epitope is concentrated in a subcortical zone (fig 5). Fluorescence intensity measurements were performed as described for pTyr proteins. Because of the intense labeling of MPM2 at the meiotic spindles (see below), the spindle region was excluded from measurement of the cytoplasmic fluorescence intensity. Linescans confirmed a continuous increase in MPM2 epitope both in the sub-cortical region (fig 6*) and cytoplasmic region (fig 6*****) as maturation progressed from GV to MII (fig 6). Increased MPM2 levels were statistically significant in both cytoplasmic (fig 7A) and subcortical zones (fig 7B) at MI and MII when compared to earlier stages (fig 7; a, b and c are statistically different, $P<0.05$). There was an additional significant increase between MI and MII. Interestingly, and unlike the pTyr results presented earlier, culture conditions had no effect on the levels of MPM2 epitope present at all cell cycle stages that were assayed ($P>0.05$).

Fyn knock-out oocytes fail to undergo cortical pTyr epitope expression

Our studies of SFKs during meiotic maturation and fertilization have demonstrated an essential function for SFKs for proper organization of chromosomes and spindles [127, 134, 142]. Of the nine SFKs produced in mammalian cells, FYN kinase is expressed at very high levels in oocytes. YES and SRC kinases are also expressed, but at lower levels (BioGPS.gnf.org, mouse, GeneAtlas GNF1M, gcrma). To determine if Fyn kinase maybe responsible for the patterns of tyrosine phosphorylation demonstrated above, Fyn (-/-) oocytes and B6/129 wildtype control oocytes were *in vitro* cultured for 17h (IVMh), fixed, labeled and imaged as in our earlier pTyr and MPM2 experiments. MPM2 proteins localized to the spindles and spindle poles as seen in previous experiments and no difference could be detected between Fyn (-/-) and wildtype (fig 8A and 8B, respectively). However, a difference was seen in pTyr epitope localization. Most (11/12; 92%) wildtype oocytes exhibited the expected cortical localization of pTyr proteins (fig 8C), however only 4/28 (14%) of the Fyn (-/-) oocytes exhibited cortical expression as seen in wildtype oocytes. The remaining 21/28 (75%) Fyn (-/-) oocytes displayed barely detectable and patchy cortical pTyr while 3/28 (11%) had no detectable pTyr at the cortex (8D). It was also apparent that Fyn (-/-) oocytes had no detectable spindle associated pTyr epitope at either MI or MII spindle poles (0/25) whereas all wildtype control oocytes exhibited prominent spindle pole staining (10/10; not shown). Thus, genetic ablation of Fyn kinase causes reduced cortical and spindle associated tyrosine phosphorylation. Given the discrete spindle patterns shown by pTyr (spindle poles) or MPM2 (entire spindle and poles), we explored the origins of these differences by examining earlier stages of meiotic cell cycle progression.

MPM2 proteins localize near to chromatin and meiotic spindles

At the GV stage, intense labeling of MPM2 was detectable on MTOCs near the cell cortex and adjacent to the GV as previously reported (not shown [247]). In addition, novel localization patterns were detected that we believe are due to the inclusion of phosphatase inhibitors in our fixation cocktail. Thus MPM2 epitope formed dense clusters near chromatin within the GV (fig 9 GV) and occasionally was localized at the surface of nucleoli but was never detected within nucleoli based upon z-section analysis. Aggregates of MPM2 epitope associated with condensing chromosomes and developing spindles (fig 9 GVBD & pre-MI). And, as noted earlier, MPM2 decorates MI and MII spindles as well as spindle pole MTOCs (fig 9 MI & MII). Labeling at MTOCs was clearly spatially separated from the minus ends of spindle microtubules (note gap in fig 8A & 8B) with either a single large focus or characteristic "C" and "O" ring configurations as we have previously reported for pericentrin [271].

Patterns of nuclear *f*-actin

To further examine the relationship between cortical phosphorylation and actin remodeling, a common target for SFKs, oocytes were co-labeled for *f*-actin using Alexa 568-conjugated phalloidin. Changes in cortical actin reorganization in maturing mouse oocytes have been extensively studied. But to our surprise, we noted several striking alterations in *f*-actin patterning that have not been detected in earlier studies. We suspect two factors have resulted in these novel findings. First, we have formulated a fixation method that incorporates multiple phosphatase inhibitors. Second, imaging of all oocytes and COC within 2 days of fixation with an emphasis on imaging within 24h after being mounted onto glass slides. These adaptations in specimen preparation result in preservation of actin cytoskeletal organization that have not been previously reported.

In particular, GV stage oocytes in early diakinesis contained a thick coil of *f*-actin within the GV. To further analyze the timing of formation for this structure,

oocytes were collected into media containing dbcAMP to arrest GVBD. They were then washed and matured (IVMh) for 0, 30, 60, 90 and 120 minutes followed by fixation and labeling for tubulin (green), DNA (red) and *f*-actin (white). At all time points, oocytes were surrounded with a thick actin cortex, hundreds of actin TZPs stretching from the neighboring cumulus and a dense actin cytoskeleton enclosing the nuclear envelope (fig. 10). No intranuclear *f*- actin was seen within arrested (0 min) GV stage oocytes displaying either non-surrounded (fig. 10A, NSN) or surrounded nucleoli (fig. 10B, SN). Remarkably, a thick *f*-actin coiled filament formed within the GV after the initiation of maturation. This structure was apparent during GVBD through diakinesis (fig. 10C-F) a time frame coinciding with the initial 30 and 60 minutes of maturation. These transient filaments appeared to branch between bundles of condensing chromatin (fig 10D) and were no longer detectable by 90-120 minutes of maturation by which time chromosome condensation had occurred.

DISCUSSION

The present studies address the question of how the topography of kinases and their targets is regulated during the meiotic cell cycle progression as exhibited by the mouse oocyte. Three main points emerge. The first is methodological. Given that protein phosphatases are instrumental in maintaining phosphorylation states of many kinases and their substrates, we adopted a fixation protocol that includes several phosphatase inhibitors to minimize the loss of phospho epitope integrity that occurs upon fixation. In this way, quantitative measures of both M-phase related (MPM2) and tyrosine kinase targets are stabilized at least at the level of the phospho epitopes being recognized by these immunological probes. Not only has this revealed discrete temporal and spatial patterns of phosphorylation during the rather synchronous cell cycle progression exhibited by *in vitro* matured mouse oocytes, but novel structural features in nuclear and cytoplasmic cytoskeletal elements were uncovered. Most dramatic perhaps is the demonstration of a transient assembly of actin filaments within the GV that appears to tether chromosomes during the active and early process

of chromosome condensation. This finding sheds new light on both older and recent inquiries [272-275] into the role of the actin cytoskeleton in chromosome function.

Intracellular localization and dynamics of protein phosphorylation

The current studies have demonstrated regional changes of protein phosphorylation during oocyte maturation. Clone 4G10 antibody is a well characterized antibody that specifically recognizes a diverse array of tyrosine phosphorylated proteins. We used this reagent to identify changes in pTyr proteins during *in vitro* maturation. Tyrosine phosphorylated proteins increased in both the cell cortex and the cytoplasm as the oocyte matured (fig 1-4). This disagrees with previous studies showing no change in overall levels of pTyr proteins between GV and MII [270]. Interestingly, a recent study examining changes in protein phosphorylation and the kinome of porcine oocytes during maturation found that the number of tyrosine phosphorylated proteins increased was equal to the number that decreased during transitions from GV-MI and MI-MII [276]. This may explain why traditional global protein measurement methods are unable to detect small changes in protein phosphorylation.

Immunohistochemical labeling and confocal microscopy combined with image and statistical analysis has enabled us to further define localized changes at an intracellular level and with high spatial resolution. That pTyr epitopes concentrate in cytoplasmic patches, MI and MII spindle poles and the oocyte cortex are novel observations that will inform future studies into the identity and function of subcellular domains (fig. 1). While pTyr levels rose in all regions of the oocyte during the MI-MII transition, the change in cortical intensity was the most dramatic. Interestingly, culture media had a significant effect on the concentration of pTyr proteins in the cortex. Supplemented IVMh media resulted in higher levels of cortical tyrosine phosphorylation that basal IVMb (fig. 4A). Due to difficulties in timing of IVO versus IVM it is not currently known whether this higher level signifies improved oocyte quality. Since cortical pTyr levels further increase at fertilization,

the levels of pTyr proteins present during MII arrest maybe important to proper sperm incorporation, fertilization success and zygotic development [277]. Previous studies have demonstrated an increase in embryo survival from oocytes matured in supplemented media [45]. Further studies will be necessary to determine whether fertilization and embryo developmental rates between oocytes matured under the current culture conditions are due to effects on the efficiency of tyrosine kinases or phosphatases. As shown here, Fyn kinase is a very likely candidate for such regulation and may be modified under less than optimal culture conditions.

MPM2 antibody was deployed here to track serine and threonine phosphorylated protein epitopes known to be phosphorylated by M-phase kinases during mitosis [278, 279]. This antibody has been used in our laboratory for the identification of M-phase protein phosphorylation in oocytes during *in vitro* maturation [247, 280]. These studies extend that work identifying unreported dynamics of the MPM2 labeled phosphoproteins. The incorporation of phosphatase inhibitors in the fixation solutions and minimization of storage time is likely to be partly responsible for improved phosphoprotein preservation given the notorious stability of phosphatases. As reported previously, MPM2 labeled MTOCs in the cytoplasm and near the GV at all stages of maturation. MPM2 proteins also formed aggregates surrounding the condensing chromosomes as well as the meiotic spindle microtubules and spindle poles (fig. 9). Of particular interest were aggregates of MPM2 proteins found within the GV that were closely associated with chromatin (fig 9A-B). The diversity detected between oocytes in the abundance, location and size of MPM2 aggregates within GV suggests the presence of protein assemblies, such as centrosomes are transient and subject to changes during meiotic arrest of GV intact oocytes. Interestingly, our methodology also demonstrates the cytoplasmic localization of MPM2 proteins. Unlike pTyr proteins which were localized directly at the cell cortex (fig 1-3), MPM2 proteins were concentrated sub-cortically and in a pattern that decreased in intensity towards the center of the oocyte (fig 5). The concentration of MPM2 proteins increased steadily from GV to MII both in the

cortical region and the central cytoplasm (fig 6). This was strikingly different from the changes in pTyr which remained relatively constant from GV to MI then significantly increased by metaphase-II. This suggests a steady increase in MPM2 phosphoproteins as maturation progresses. However, a significant increase in tyrosine phosphorylation is associated specifically with metaphase-II arrest and preparation for fertilization. A division of labor between targets of tyr versus ser/thr kinases would be consistent with these observations although the identity of these components remained ill-defined with one exception as shown here.

Our recent studies have focused on the role of SFKs during oocyte maturation and fertilization [127, 134, 142]. The present data reinforce a role for Fyn kinase in what we suggest is an example of cortical maturation. Fyn kinase is associated with changes in cortical actin dynamics of cultured cells [281-283] and sertoli cells [284]. Since pTyr proteins localize specifically at the oocyte cortex, we examined the localization of pTyr in oocytes from Fyn (-/-) mice to determine whether Fyn kinase participated in this phosphorylation event. Interestingly, oocytes from wildtype control mice exhibited intense cortical pTyr localization as seen in the earlier studies. However, pTyr localization in Fyn (-/-) oocytes was heterogeneous wherein some oocytes showed no cortical staining while others displayed extremely low level and patchy staining at the cortex. Expression of mitotic/meiotic cell cycle specific MPM2 epitopes was not visibly affected by the lack of Fyn kinase (fig. 8A-B). However, Fyn (-/-) oocytes lacked pTyr spindle pole labeling at MI or MII. This distinct spatial change in spindle pole foci helps explain our previous data showing spindle and chromosome dysfunction during meiosis of Fyn (-/-) oocytes [127, 134]. Collectively, these findings implicate a role for Fyn kinase in establishing spindle pole integrity and chromosome segregation at meiosis I and II. Furthermore, the loss of cortical epitope in Fyn null oocytes suggests a role for Fyn kinase in oocyte cortical dynamics and preparation for fertilization. Studies are ongoing in our laboratories to further define the functions of Fyn kinase in mouse oocytes.

Nuclear actin dynamics

During these studies, a remarkable modification in nuclear actin was observed that we believe may be linked to separate roles for actin during the nuclear and cytoplasmic maturation of mouse oocytes. Phosphatase inhibitors were included in the fixation and processing steps as mentioned earlier and may account for these observations. Since phosphatase activities are associated with changes in actin dynamics, inhibiting them may provide for better actin stabilization [226, 252, 285-287]. Oocytes were cultured for short times (0, 30, 60, 90 or 120 minutes) to allow for the initiation of maturation, diakinesis and GVBD. Interestingly, diakinesis was associated with the formation of a thick f-actin filament within the GV (fig 10 C-F). This actin structure was not seen in fully arrested oocytes (time 0 and held in cAMP supplemented media; fig. 10A-B). It was also absent from nuclei with condensed chromatin. While direct contact with chromatin was difficult to establish, we note that many earlier studies proposed a role for actin-based contraction during GVBD [271, 274]. Moreover, nuclear lamina contraction has been reported and together with the appearance of a contractile force during diakinesis [275, 288], these results uncover a very early and potentially relevant role for the cytoskeleton. Further studies are ongoing to evaluate actin involvement in spindle assembly and chromosome alignment.

In conclusion, our evidence suggests a separation of function in kinases that operate through distinct signaling pathways. That there is clear topographical segregation in epitope disposition suggests further that these pathways serve to integrate the progression of nuclear and cytoplasmic maturation in the mouse oocyte.

ACKNOWLEDGEMENTS
Supported by NICHD 42076, The Hall Family Foundation and the ESHE fund.

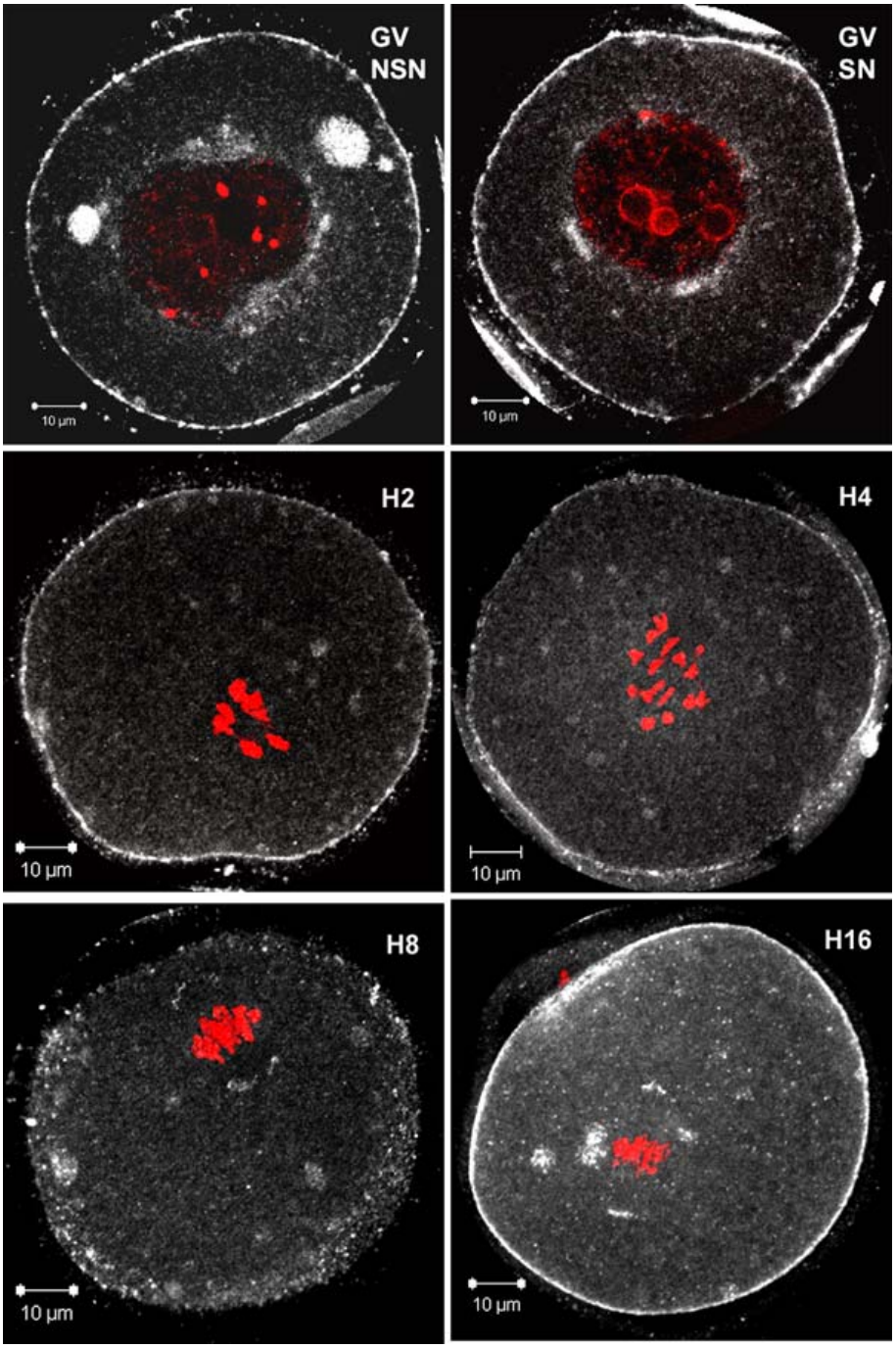

Figure 1

Figure 1. Phospho-tyrosine proteins increase at MII and localize primarily to the oocyte cortex and within large cytoplasmic patches. Oocytes were matured in vitro and fixed at specific times of maturation. Two oocytes from each time were pooled together, labeled and imaged as a set to enable direct comparisons of fluorescence intensity between oocytes at different stages of maturation. Complete serial z-sections of oocytes labeled with pTyr and imaged in sets were examined to identify patterns of pTyr proteins associated with cellular structures at each time point of maturation (H0=GV, H2=GVBD, H4=pre-MI, H8=MI, H16=MII). pTyr labeled oocytes are shown here in the plane where chromatin was found. Two to three sections have been compressed together to enable visualization of entire structures such as both poles of the spindles. Image intensity has been increased in this figure to improve visualization of labeled structures. Dense patches of pTyr proteins were seen in the cytoplasm of all oocytes and near to the nucleus in GV stage oocytes with both non-surrounded (NSN) and surrounded (SN) nucleoli. pTyr proteins were associated with the cortex at all time points and with spindle poles (arrows) at both MI (H8; 4/5 IVMh and 4/6 IVMb) and MII (H16; 5/5 IVMh and 3/6 IVMb). (white=pTyr, red=red).

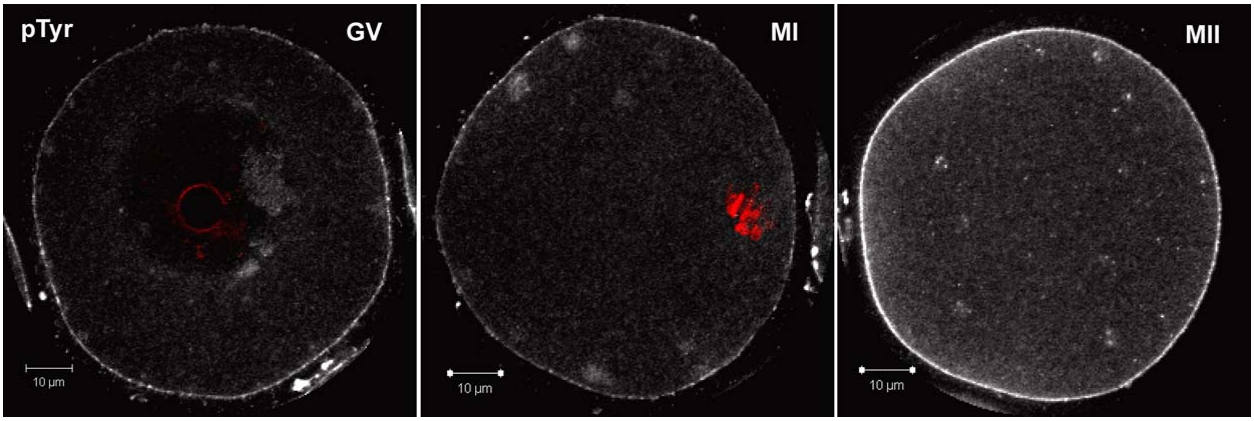

Figure 2

Figure 2. Patterns of tyrosine phospho-proteins change during meiotic maturation. The oocytes in this figure were selected from a single set of oocytes imaged together as a set to demonstrate the actual differences of fluorescence intensity between GV, MI and MII. Each image shown here is a single section (1μm thick) at the central plane. The intensity of these images has not been modified as to enable a demonstration of fluorescence intensity differences between oocytes at different stages of maturation. This is the same plane used for later measurements for linescans and fluorescence intensity comparisons. As seen in fig. 1, pTyr proteins were found throughout the cytoplasm but congregated in cytoplasmic patches and at the cell cortex at all stages. Overall intensity remained the same as oocytes matured from GV through GVBD to M-I. However, pTyr levels in the cytoplasm and at the cortex were greatly increased at MII. (white=pTyr, red=DNA).

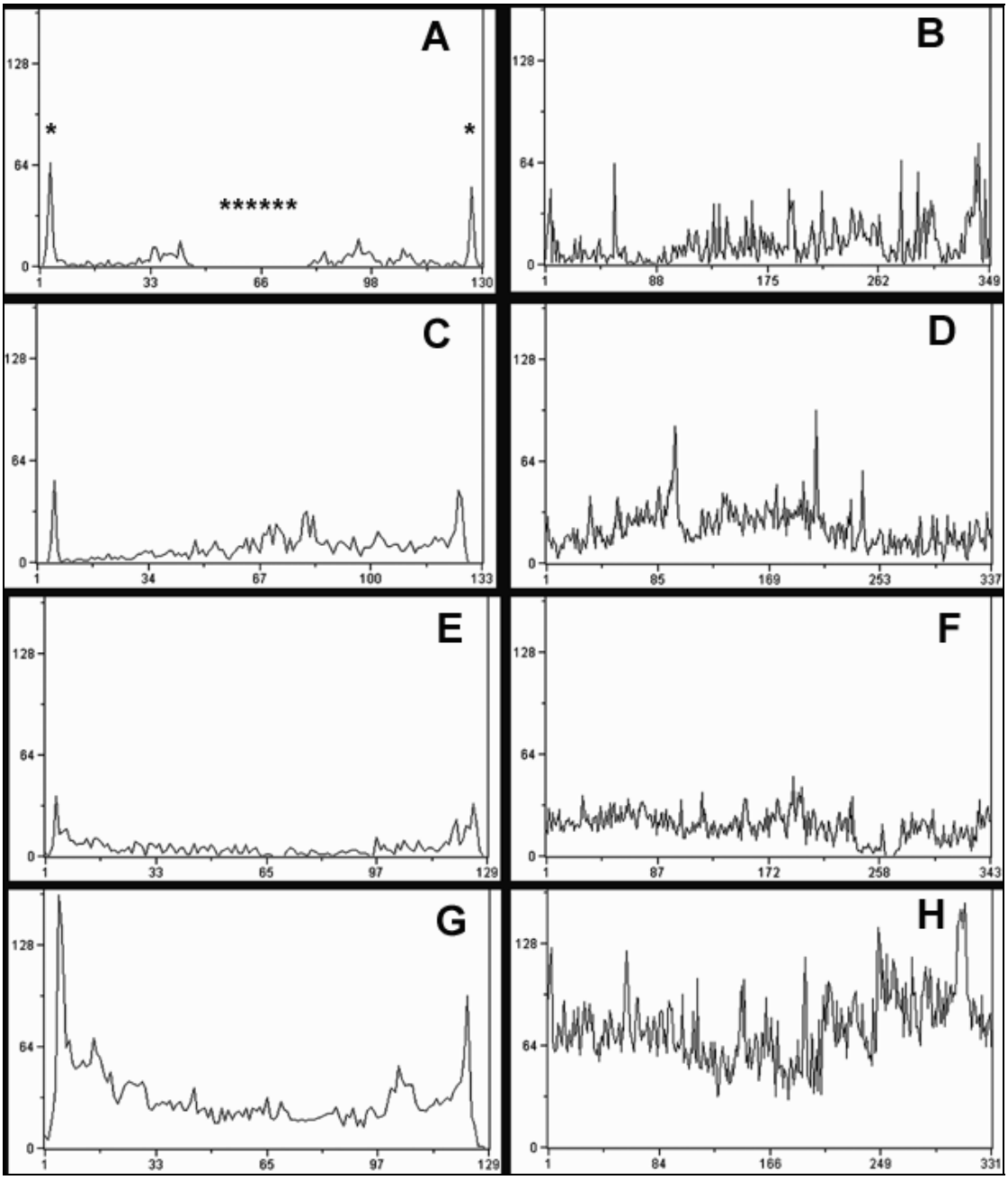
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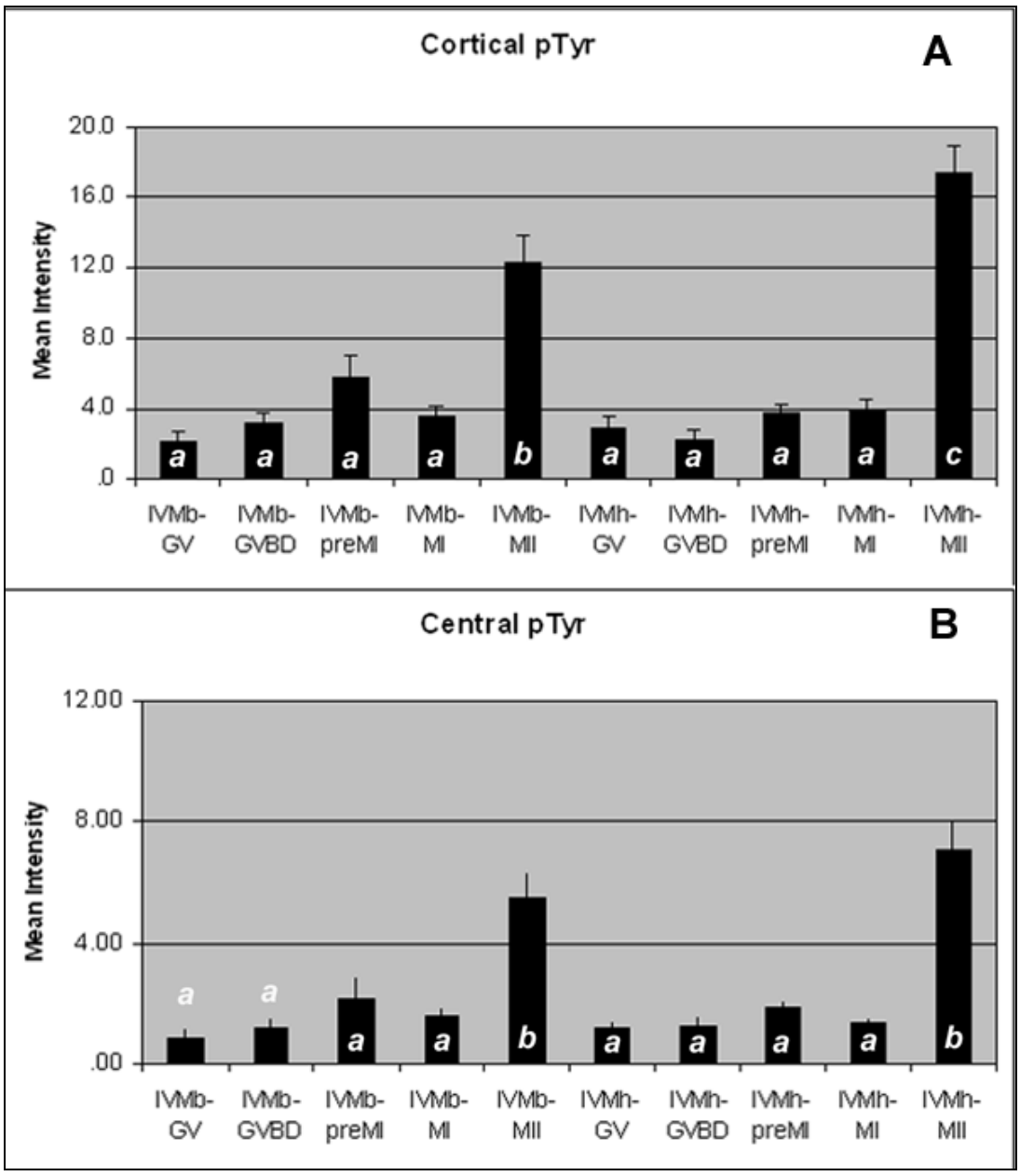
Figure 3

Figure 3. Linescans demonstrate regional localization of tyrosine phospho-proteins. Metamorph imaging software was used to produce linescans of fluorescently labeled oocytes. Linescans were drawn on the image from the central plane (from serial z-stacks) of each oocyte from the pTyr labeled sets (fig.1). Two linescans (7 μm thick) were taken from each oocyte including (1) a straight line across the entire diameter of each oocyte at the median and (2) circumferential scan around the entire surface of each oocyte to include the oolema and cortical region. Linescans across the median (A, C, E and F) show low levels of pTyr proteins across the cytoplasm with increased intensity at the cortex. The intensity of spikes at the cortex (*) and cytoplasm (*****) increase at MII. The cortical (B, D, F and H) scans further show with an increase at MII. (* oolema/cortex; ***** central cytoplasm; A-B=GV, C-D=pre-MI, E-F=MI, G-H=MII)


Cortical pTyr
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IVMb-GV
IVMb-GVBD
IVMb-preMI
IVMb-MI
IVMb-MII
IVMh-GV
IVMh-GVBD
IVMh-preMI
IVMh-MI
IVMh-MII
Central pTyr
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Mean Intensity
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IVMb-GV
IVMb-GVBD
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IVMb-MI
IVMb-MII
IVMh-GV
IVMh-GVBD
IVMh-preMI
IVMh-MI
IVMh-MII

Figure 4

Figure 4. Levels of pTyr proteins is increased significantly at metaphase-II. The same oocytes used for the linescan analysis were used for statistical comparisons of average fluorescent intensity. This included the cortical (A) measurements from linescans seen in fig. 3 plus the central cytoplasm (B), ie that region of the cytoplasm not included in the cortical scans. pTyr protein concentration in both the cortex and the cytoplasm were significantly increased at MII. Cortical pTyr levels were higher in oocytes culture in IVMh as compared to IVMb (a, b, c are significantly different, $P<0.05$).

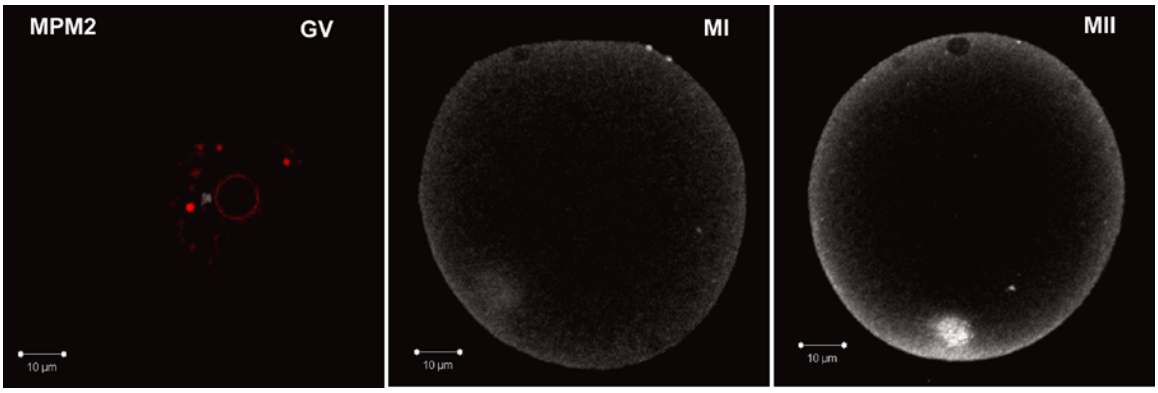

Figure 5

Figure 5. Cytoplasmic MPM2 proteins increased steadily during maturation. Oocytes from the same cohorts as were used for the pTyr experiments were also sorted into sets, labeled and imaged for MPM2 epitope proteins. Oocytes in this figure represent single sections at the central plane of oocytes from a single set all imaged together in order to show the changes in fluorescence intensity at different stages of maturation. Almost no MPM2 was detectable in the cytoplasm of GV stage oocytes. The level of intensity increased steadily from GV to MI to MII. MPM2 proteins did not associate specifically with the oocyte cortex.

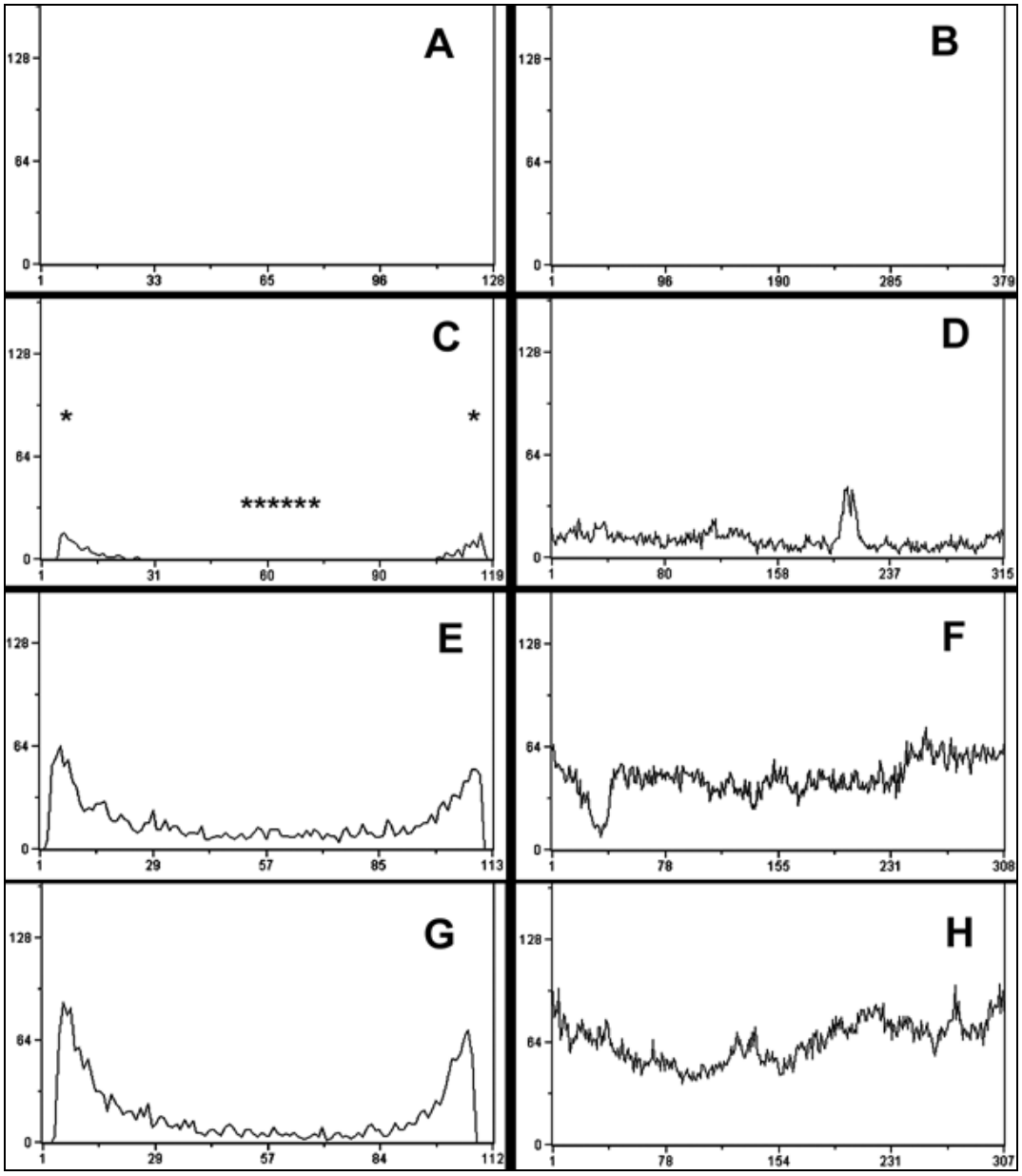
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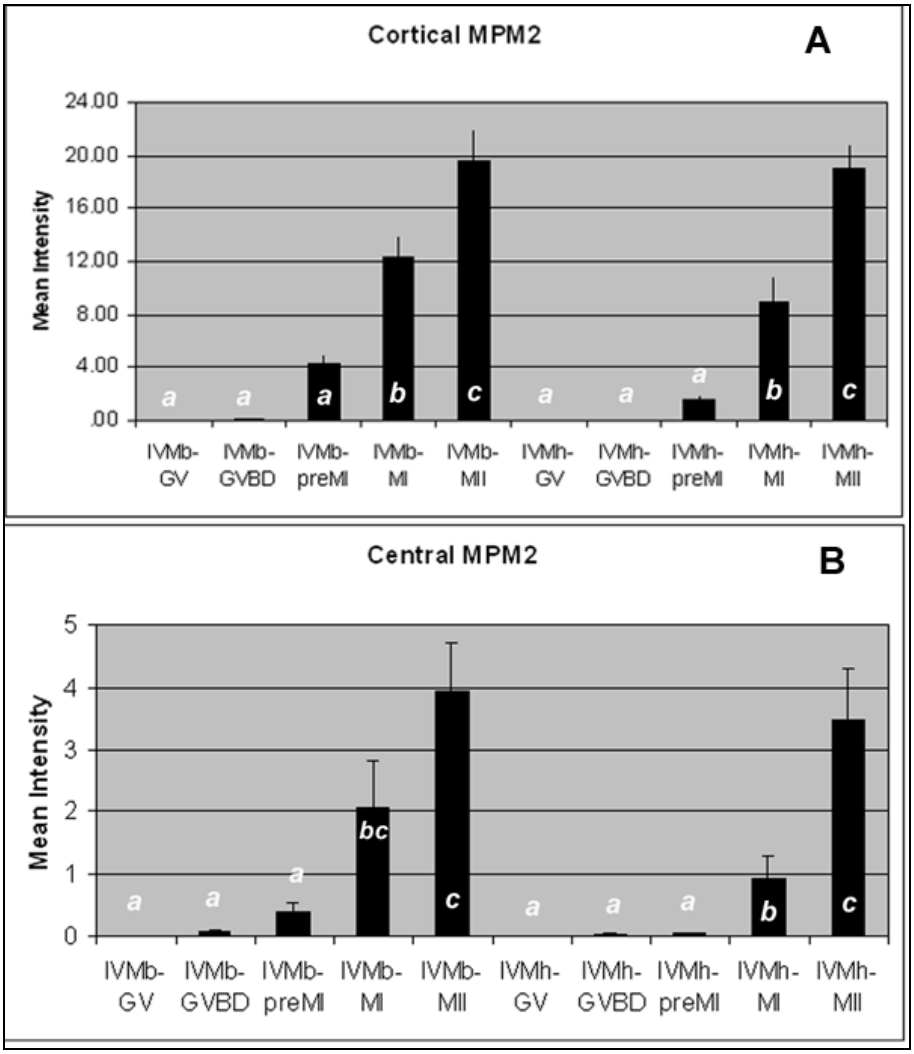
Figure 6

Figure 6. MPM2 proteins increase during maturation and concentrate in the cytoplasm beneath the oolema. Oocytes labeled for MPM2 proteins were compared by linescans similar to pTyr images (see Fig. 3). Linescans across the median (A, C, E and F) demonstrate the steady increase of MPM2 proteins in both the central cytoplasm (*****) and the cortex (*) as oocytes mature from GV to MII. Fluorescence intensity was too low to measure at GV stage (A-B). The cortical (B, D, F and H) scans further demonstrate the increase in MPM2 proteins especially at MI (F) and MII (H). (* oolema/cortex; ***** central cytoplasm; A-B=GV, C-D=pre-MI, E-F=MI, G-H=MII)


Cortical MPM2
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IVMb-GVBD
IVMb-preMI
IVMb-MI
IVMb-MII
IVMh-GV
IVMh-GVBD
IVMh-preMI
IVMh-MI
IVMh-MII
Central MPM2
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Mean Intensity
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IVMb-GV
IVMb-GVBD
IVMb-preMI
IVMb-MI
IVMb-MII
IVMh-GV
IVMh-GVBD
IVMh-preMI
IVMh-MI
IVMh-MII

Figure 7

Figure 7. The rise in MPM2 proteins is significant at MI and MII stages of maturation. The average fluorescence intensity of MPM2 labeled oocytes were analyzed similar to pTyr protein analysis in fig. 4. MPM2 protein concentration increased significantly in both the cytoplasm (A) and cortical (B) region. Levels at MI and MII were significantly higher than earlier times. MII levels were significantly higher than MI. No differences were seen between culture treatments (IVMh, IVMb). (a, b, c are significantly different, $P<0.05$).

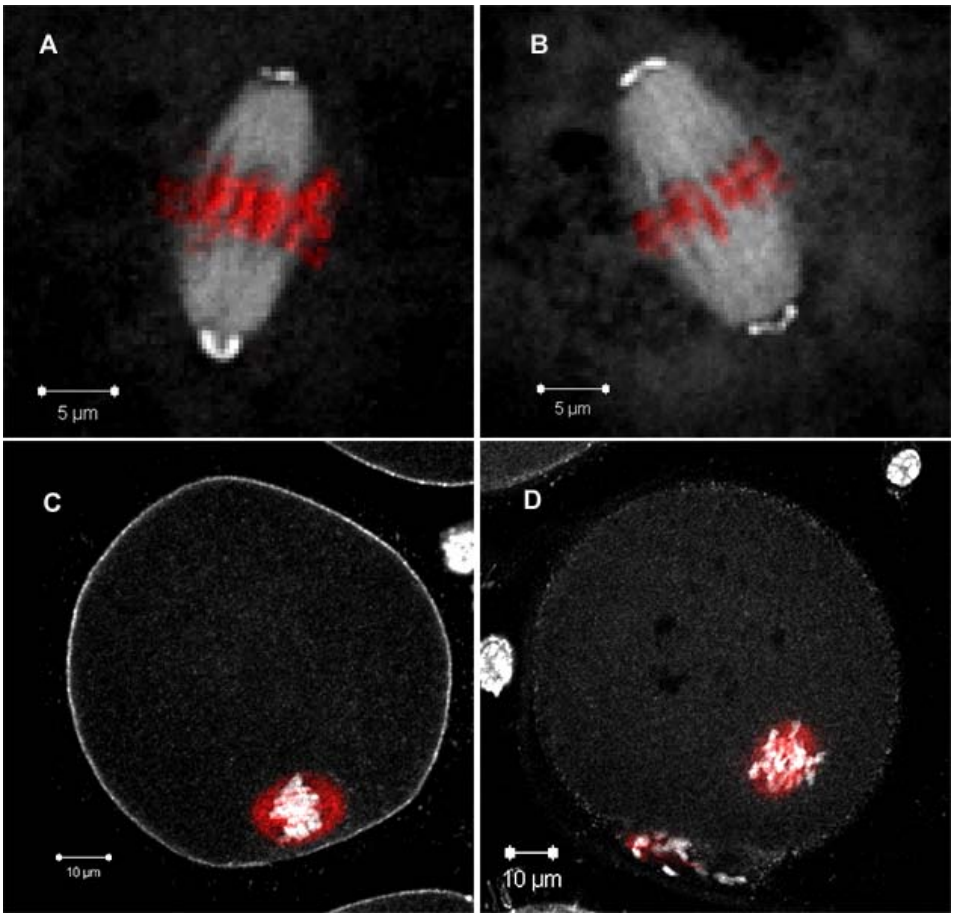

Figure 8

Figure 8. Fyn (-/-) oocytes exhibit reduced levels of pTyr proteins in the cortex while MPM2 proteins appear to localize normally to the spindle and spindle poles. Fyn (-/-) and wildtype oocytes were collected at the GV stage and matured in vitro for 17 h. Oocytes were then fixed, labeled with MPM2 or pTyr and imaged similar to those in the earlier studies. The images of MPM2 labeled spindles (A-B) are compressions of 2-3 sections however, pTyr images (C-D) are single sections from the central plane of each oocyte. There was no difference in MPM2 labeling on MII spindles or spindle poles of wildtype (A) or Fyn (-/-) (B) oocytes. Most (11/12; 92%) wildtype control oocytes (C) exhibited intense cortical pTyr as seen in previous experiments (fig. 1-2). However, only 4/13 (31%) Fyn (-/-) oocytes (A) had dense cortical pTyr similar to controls; 9/13 (69%) had greatly reduced levels of pTyr proteins in the cortex (D). Oocyte (B) has severely disorganized chromosomes which are frequently seen in Fyn (-/-) oocytes [McGinnis et al 2009 in press]. (A-B; white=MPM2, red=DNA: C-D; white=pTyr, red=tubulin, white on red=DNA)

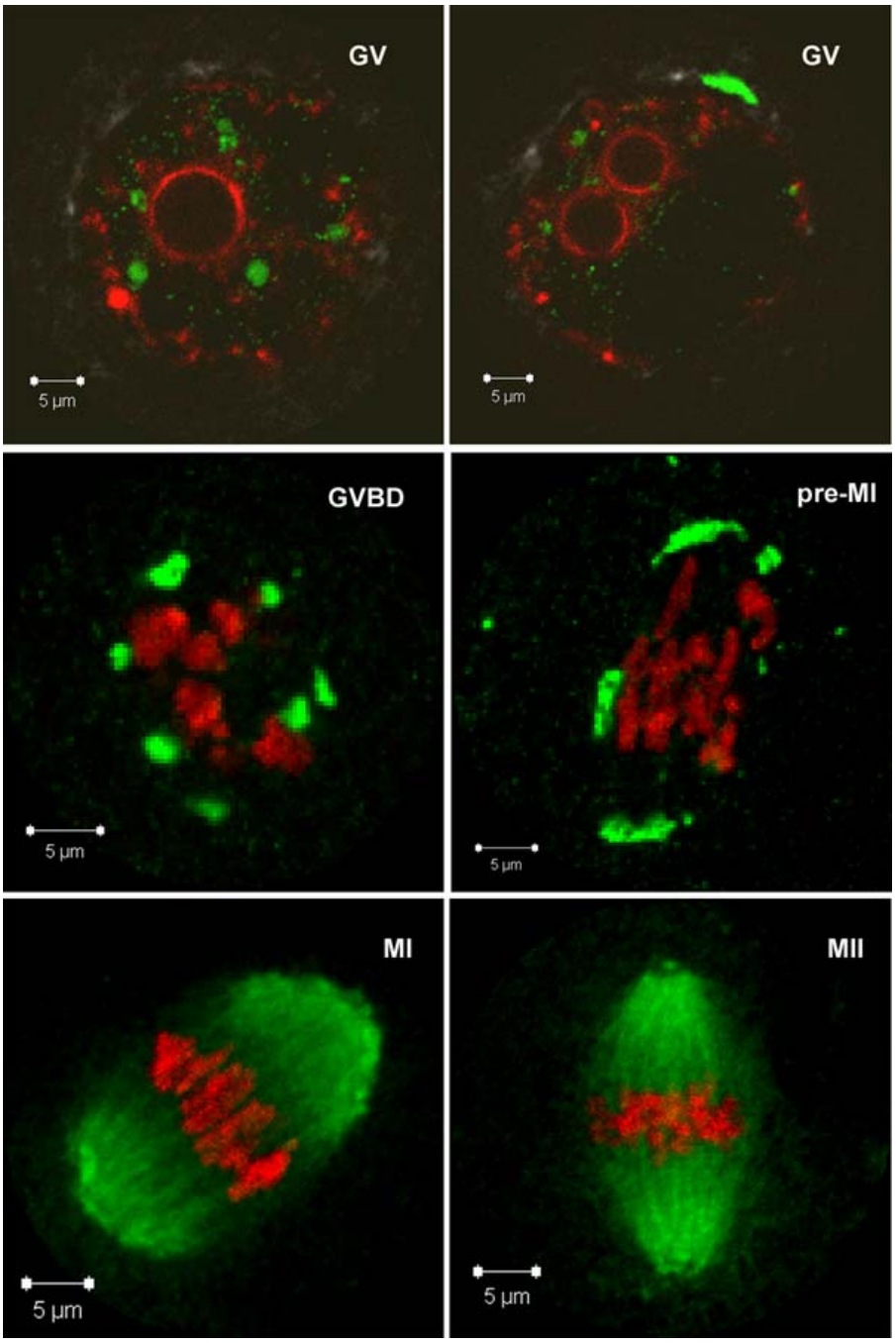

Figure 9

Figure 9. MPM2 proteins aggregate near to chromatin within the GV and associate with spindle microtubules and spindle poles. Oocytes from the MPM2 fluorescence intensity comparisons were examined for localization of MPM2 proteins to intracellular structures. These images were extracted from these data sets including compressions of 2-3 sections to enable visualization of the entire structure. Dense patches of MPM2 proteins were seen to associate with chromatin within the GV stage oocytes. The specific pattern of this labeling was diverse. As chromosomes condensed at GVBD and began to organize at pre-MI, MPM2 patches were seen to remain near to the chromosomes. At MI and MII, MPM2 localized to the spindle microtubules and the spindle poles. (green=MPM2, red=DNA)

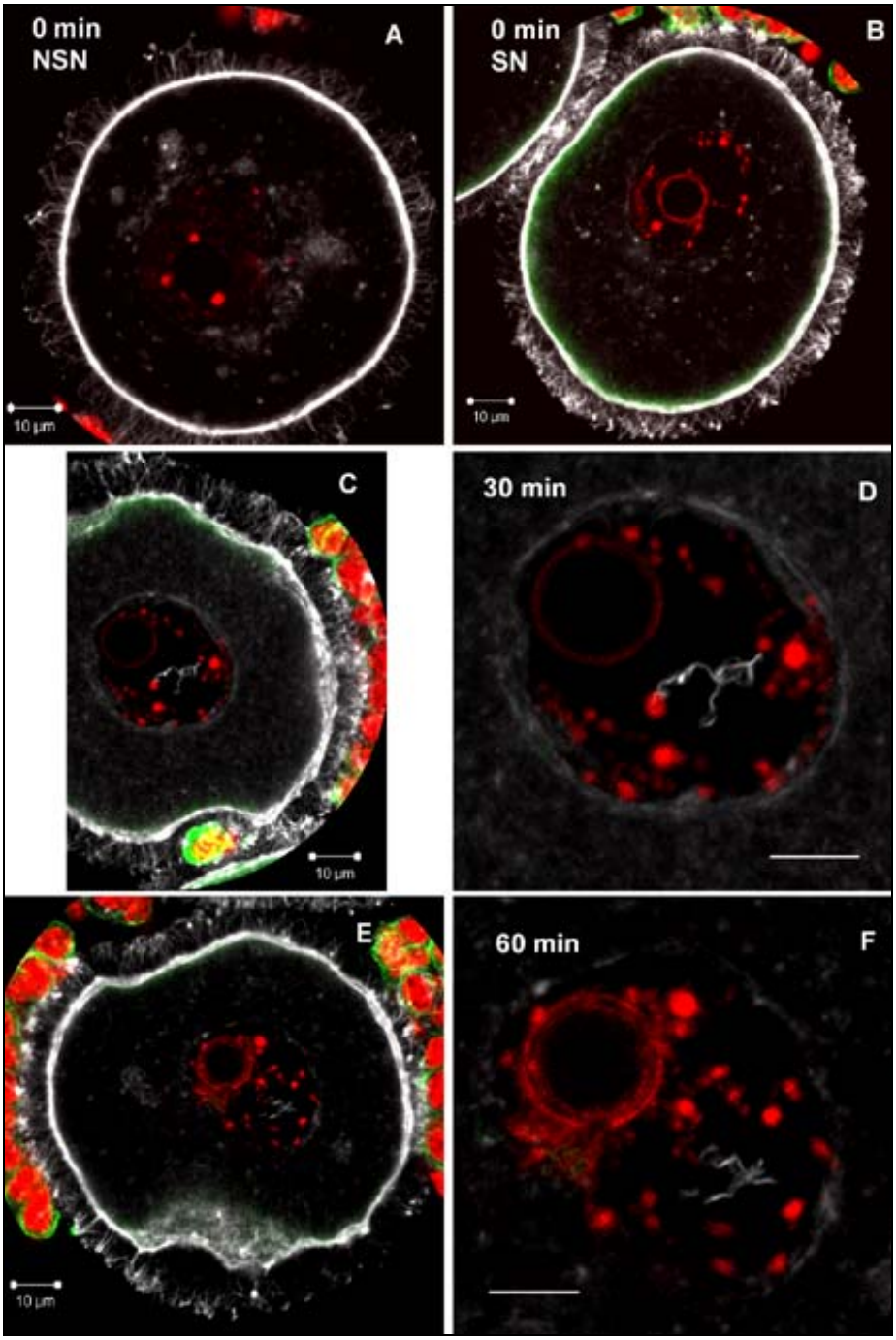

Figure 10

Figure 10. A single branched *f*-actin filament forms within the nucleus at diakinesis. Oocytes cultured for 0, 30, 60, 90 or 120 minutes in IVMh medium were fixed, labeled and imaged by confocal serial z-scans. For this figure, 3 sections (1 μm each) were selected through the center of the GV then compressed into a single image. A thickness of 3 sections (total 3 μm) was used so that each image could include the entire length of the nuclear actin filaments. Fully arrested oocytes fixed at time 0 in cAMP media contained no actin filament: (A) GV stage oocyte with non-surrounded nucleolus (NSN) collected directly from the ovary and fixed at time 0 minutes; (B) GV oocyte with surrounded nucleolus (SN) at time 0 min. Oocytes fixed after 30 (C-D) or 60 (E-F) minutes of maturation had entered diakinesis and contained a single branching f-actin filament within the nucleus. At 90 and 120 minutes of culture, chromosomes had condensed and no actin filament was visible (not shown). D and F are enlargements of the nuclear regions of C and E respectively. (green=tubulin, white=f-actin, red=DNA)

Chapter Four

Localized Activation of Src-Family Protein Kinases in the Mouse Egg

ABSTRACT

Recent studies in species that fertilize externally have demonstrated that fertilization triggers localized activation of SFKs in the egg cortex. However, the requirement for SFKs in activation of the mammalian egg is different from lower species and the objective of this study was to characterize changes in the distribution and activity of SFKs during zygotic development in the mouse. Immunofluorescence analysis of mouse oocytes and zygotes with an anti-phosphotyrosine antibody revealed that fertilization stimulated accumulation of pTyr-containing proteins in the egg cortex and that their abundance was elevated in the region overlying the MII spindle. In addition, the poles of the MII spindle exhibited elevated pTyr levels. As polar body extrusion progressed, pTyr containing proteins were especially concentrated in the region of cortex adjacent to the maternal chromatin and the forming polar body. In contrast, pTyr labeling of the spindle poles eventually disappeared as meiosis II progressed to anaphase II. In approximately 24% of cases, the fertilizing sperm nucleus was associated with increased pTyr labeling in the overlying cortex and oolemma. To determine whether SFKs could be responsible for the observed changes in the distribution of pTyr containing proteins, an antibody to the activated form of SFKs was used to localize activated Src, Fyn or Yes. Activated SFKs were found to be strongly associated with the meiotic spindle at all stages of meiosis II; however, no concentration of labeling was evident at the egg cortex. The absence of cortical SFK activity continued until the blastocyst stage when strong cortical activity became evident. At the pronuclear stage, activated SFKs became concentrated around the pronuclei in close association with the nuclear envelope. This pattern was unique to the earliest stages of development and disappeared by the eight

cell stage. Functional studies using chemical inhibitors and a dominant-negative Fyn construct demonstrated that SFKs play an essential role in completion of meiosis II following fertilization and progression from the pronuclear stage into mitosis. These data suggest that while SFKs are not required for fertilization-induced calcium oscillations, they do play a critical role in development of the zygote. Furthermore, activation of these kinases in the mouse egg is limited to distinct regions and occurs at specific times after fertilization. *[McGinnis, LK, Albertini DF and Kinsey WH. Localized activation of Src-family protein kinases in the mouse egg. Dev Biol (2007) 306:241-254]*

INTRODUCTION

Src-family protein tyrosine kinases (SFKs) are cytoplasmic enzymes that can be targeted to plasma membrane microdomains where they typically act to transduce signals from external stimuli [289]. Signal transduction cascades involving SFKs such as Fyn, Src and Yes have been shown to play a major role during egg activation and early development in species that fertilize externally such as marine invertebrates, amphibians and fish [290-292]. In these species, SFKs are activated rapidly after fertilization and function in triggering the sperm-induced calcium transient that initiates the egg activation process [290, 293-297]. In the zebrafish oocyte, kinase activation was shown to be initiated at the point of sperm−egg fusion and to progress through the egg cortex [298]. Later stages of egg activation such as pronuclear fusion and mitosis also require PTK activity although the specific kinases involved in these steps have not been identified [299, 300]. Once development has begun, Fyn and Yes are required for cell movements involved in epiboly [1, 301] while Src and Yes function during cell intercalation and blastopore closure [302]. The role of SFKs in mammalian fertilization is clearly different from that in externally fertilizing species. For example, while mammalian eggs express Fyn, Yes and in some cases, Src [242, 243] these kinases are not required for the unique sperm-induced calcium oscillations [241, 303, 304] which trigger egg activation in mammals [305]. Instead, these

calcium oscillations are initiated directly by a sperm-borne phospholipase that does not require PTK regulation [306]. The function of SFKs in later stages of mammalian fertilization has been addressed primarily through the use of parthenogenetic activation. Studies in mouse and rat demonstrate that agents which suppress SFK activation also inhibit the MII/anaphase transition induced by parthenogenetic activation *in vitro*. In addition, microinjection of active Fyn kinase has been shown to stimulate meiosis resumption in mouse and rat [242, 307]. A second requirement for SFK activity at S or S/G2 phase of the first mitotic division has been demonstrated through the use of chemical inhibitors such as genistein [264, 308]. Further analysis using GST fusion proteins encoding the SH2 domain of Fyn have confirmed the importance of SFK activity for development past the pronuclear stage [142]. Together, these observations indicate that SFK such as Fyn may play an important role in development of the mammalian zygote, but it is unclear which specific pathways are regulated during zygotic development.

The objective of the present study was to determine whether fertilization of the mouse oocyte triggers the global activation of SFKs in the egg cortex as occurs in lower species [298]. The approach was to first use antibodies to phosphotyrosine to establish which parts of the egg contain elevated levels of phosphotyrosine, and secondarily to use an antibody to activated SFKs to establish the distribution and activation pattern of this family of kinases. The results indicate that fertilization does stimulate PTK signaling in localized regions of the mouse egg. The activated SFKs are associated with meiotic and mitotic spindle microtubules and the pronuclear envelope. The activation pattern of these kinases is different from that in lower species such as zebrafish since even though pTyr labeling indicated that some tyrosine kinase was active in the egg cortex, the activated SFKs were restricted primarily to the spindle and the pronuclear envelope. One potential application of these studies could be to evaluate the quality of egg activation and zygote development under different conditions used in assisted reproductive technologies. Therefore, we have focused our morphological studies primarily on zygotes produced

by *in vivo* fertilization and not exposed to *in vitro* culture during recovery and fixation.

MATERIAL AND METHODS

Embryo and oocyte collection

Oocyte cumulus complexes (OCC) and zygotes were collected from 6- to 7-week-old CF1 female mice (Harlan Sprague−Dawley, Indianapolis, IN, USA). Females were stimulated with 5 IU eCG (Calbiochem, San Diego, CA, USA) followed by 5 IU hCG 48 h later. To produce *in vivo* fertilized embryos, female mice were mated with mature B6D2F1 (C57BL/6× DBA/2) male mice. Embryos were collected every 15−30 min between 13.0 and 16.0 h post-hCG to provide a range of developmental stages from non-fertilized OCC to early pronuclei. Embryos were also collected at later stages of development (times post-hCG: 24 h=2 pronuclei, 48 h=late 2 cell, 72 h=compacting 8 cells, 120 h blastocyst). Embryos and OCC were released from the oviducts directly into fixative with phosphatase inhibitors to avoid the possibility that the ex vivo environment or culture conditions might influence the activity of protein kinases or phosphatases in the egg.

Western blot analysis

MII oocytes collected as above were incubated in FHM (HEPES-buffered KSOM, Specialty Media Phillipsburgh, NJ, USA) containing 0.3 mg/ml hyaluronidase and 40 μM phenylarsine oxide and 100 μM sodium orthovanadate to remove attached cumulus cells. The oocytes were then washed three times in FHM medium, then excess medium was removed with a pulled glass pipet. The oocytes were immediately solubilized in a final volume of 10 μl of SDS−gel sample buffer containing 40 μM phenylarsine oxide and 100 μM sodium orthovanadate and stored at −70 °C. Samples were resolved on a 10% SDS−PAGE with a 4% stacking gel. The wells in the stacking gel were formed with a comb containing teeth 1 mm in width,

which facilitated analysis of very small sample volumes (typically 1 µl). Immunoblotting and detection was performed as previously described [298].

Fixation and immunohistochemical staining

All eggs and embryos were fixed for 5 min at room temperature in FHM medium with 2% paraformaldehyde followed by 30 min at 35 °C in microtubule stabilization buffer (0.1 M PIPES, pH 6.9, 5 mM MgCl2·6H2O, 2.5 mM EGTA) containing 2% formaldehyde, 0.1% Triton X-100, 1 µM Taxol, 10 U/ml aprotinin and 50% deuterium oxide [247]. After fixation, eggs and embryos were transferred into wash solution (PBS containing 2% BSA, 2% powdered milk, 2% normal goat serum, 0.1 M glycine and 0.01% Triton X-100) and held overnight at 4 °C. All fixatives and wash solutions were supplemented with 40 µM phenylarsine oxide and 100 µM sodium orthovanadate to inhibit phosphatase activity. Cumulus cells were removed after fixation and immediately before staining by adding 0.3 mg/ml hyaluronidase to the wash solution for less than 1 min. Embryos were washed twice without hyaluronidase before labeling.

To limit the potential effects of storage time on phosphorylated epitopes, all embryos were labeled within 24 h of fixation and imaged within 2 days after labeling. The clone 28 mouse monoclonal antibody (Biosource International, Camarillo, CA, USA) was used to localize activated forms of Src, Fyn and Yes, while a monoclonal antibody to the autophosphorylation site at Tyr416 (Nonphospho-Src(Tyr416),Cell Signaling Technology Inc., Danvers, MA) was used to detect inactive Src-family PTKs. An anti-phosphotyrosine antibody (clone 4G10, Upstate, Lake Placid, NY, USA) was used to localize tyrosine phosphorylated proteins. Embryos were co-labeled with either rat monoclonal anti-tubulin (YOL 1/34, Abcam Inc., Cambridge MA, USA) or phalloidin conjugated with Alexa 568 (to label f-actin) to display cytoskeletal structures. Secondary antibodies were goat anti-mouse Alexa 488 or goat anti-rat Alexa 568 (Molecular Probes, Eugene OR USA). Negative controls were prepared identically to the labeled samples however primary antibodies were pre-

mixed with either phospho-L-tyrosine (clone 4G10) or clone 28 blocking peptide (EPQYQPGENL-COOH) synthesized by (Synpep Corp. Dublin, CA) at 1.0 μM. In experiments where both clones 28 and 4G10 were used, the embryos from each treatment were divided randomly after fixation and all embryos from each replicate were labeled and imaged at the same time. Embryos were labeled with primary antibodies at 35 °C for 1 h, washed 3× then labeled with secondary antibody for 1 h. After secondary labeling, embryos were transferred to a wash solution containing 1 μg/ml Hoechst 33258 with or without Alexa 568-phalloidin and stored in the dark overnight at 4 °C. Embryos were mounted the following morning and imaged (mounting medium consisted of 1:1 glycerol: PBS supplemented with 5 mg/ml sodium azide and 1 μg/ml Hoechst 33258). All chemicals, hormones and reagents were purchased from Sigma Chemical Company, St. Louis, MO, unless otherwise stated.

Imaging and data analysis

Samples were imaged by serial z-sections (8 μm depth) on an inverted Zeiss LSM500 confocal microscope. The serial z-sections were used to detect 3-dimensional relationships between the egg cortex, meiotic spindle and fertilizing sperm. Fluorescence intensity was quantitated by linescan and by area measurement analysis using Metamorph 6.2 (Universal Imaging Corp., Downington, PA).

Pharmacological treatment of eggs fertilized *in vitro*

In order to test the effects of Src-family PTK inhibitors on fertilization and initiation of embryonic development, a synchronized pool of zygotes was produced by *in vitro* fertilization, using methods previously published [309]. Briefly, oocyte cumulus complexes (OCC) were collected 14 h post-hCG from superovulated CF1 female mice. Sperm were collected from the cauda epididymis of mature B6D2F1 male mice and capacitated for 90 min in modified Tyrode's medium. OCC were released directly into fertilization drops of mKSOMaa medium (KSOMaa, Chemicon,

Temecula, CA) supplemented with 4 mg/ml BSA, 5.56 mM glucose and 1 mM glycyl−glutamine [310]. Capacitated sperm were added to OCC (1×106 /ml) and allowed 5 h for fertilization and early pronuclear formation. Several PTK inhibitors were tested including: PP2 (10 and 100 μM; Calbiochem, San Diego, CA), SKI-606 (1 and 10 μM; Calbiochem), PD168393 (0.5 and 5 μM; Calbiochem) and GTP-14564 (2 and 20 μM; Calbiochem). Inhibitors were prepared as stock solutions in DMSO and stored at −20 °C. Culture medium was equilibrated in the CO2 incubator in 15 ml tubes for at least 2 h before culture. Immediately before adding embryos, each inhibitor was thawed and mixed with pre-equilibrated mKSOMaa medium and 50 μl drops were placed into NUNC 4-well plates. Each treatment drop was overlain with mineral oil (sterile filtered Sigma Embryo Tested Mineral Oil, cat. M8410) containing the same concentration of the inhibitor. After 24 h culture, all embryos were fixed for immunofluorescence analysis as described above. Statistical analysis of developmental success was performed using SigmaStat software (Jandel Scientific, San Rafael, CA).

RESULTS

Evidence for PTK activity in the mouse oocyte

It is well established that the mammalian oocyte expresses active protein tyrosine kinases with the result that pTyr containing proteins accumulate in the egg [264, 304]. In order to gain an insight into the regions of the egg that are actively involved in PTK signaling during fertilization, we used a well- established monoclonal antibody to phosphotyrosine (4G10) to localize pTyr in mouse oocytes and zygotes. Our procedure entailed the aggressive use of phosphotyrosyl phosphatase inhibitors at all stages of sample preparation to prevent dephosphorylation of tyrosine residues by phosphatases present in the egg a and in the reagents used for immunofluorescence. Initial observations recorded at lower magnification Fig. 1, revealed that pTyr residues were distributed uniformly in the cytoplasm of the MII oocyte with some concentration in the cortex adjacent to the

MII spindle (Fig. 1A, white arrows). Fertilization triggered accumulation of pTyr in the egg cortex which was evident by early anaphase (Fig. 1B), remained elevated through telophase (Fig. 1C) and became less intense by the pronuclear stage (Fig. 1D). Visual analysis of over 60 oocytes and zygotes indicated that fertilization triggered increased accumulation of pTyr-containing proteins in the egg cortex. These changes in fluorescence intensity were quantitated in 22 oocytes or zygotes that were oriented such that the meiotic spindle or polar bodies could be clearly identified. The ratio of anti-pTyr fluorescence intensity in the egg cortex relative to that in the central cytoplasm was determined by quantitation of average pixel intensity (integrated pixel intensity/ pixel number). These measurements were made in the cortex (egg surface and cytoplasm 5 μm deep) and the central cytoplasm (region deep to the cortex) using Metamorph 6.2. As seen in Table 1, fertilization resulted in a 1.5-fold increase in the concentration of pTyr-containing proteins detected in the cortex over that in the central cytoplasm. The magnitude of the changes in pTyr content of the egg cortex relative to the central cytoplasm was also evident when fluorescence intensity was quantitated by linescan analysis through the equator of the egg. As seen in Fig. 2, fluorescence intensity was not concentrated in the egg cortex prior to fertilization. However, zygotes collected at anaphase II and telophase II exhibited a marked concentration of pTyr proteins in the egg cortex represented in Fig. 2 as the left and right extremes of the x axis.

In most cases, the cortical fluorescence intensity was not uniform over the entire egg, but appeared more intense over the half of the zygote containing the meiotic spindle (Fig. 1). In order to demonstrate the asymmetric nature of the cortical pTyr-specific fluorescence, the fluorescence intensity of the entire egg cortex was quantitated by circumferential linescan analysis and presented as a two dimensional graph in Fig. 3.

Linescans were initiated in the cortex of the egg 180° opposite the meiotic spindle and progressed clockwise around the egg. The pixel intensity was averaged over a region of cortex approximately 5 μm deep beginning at the egg surface. In this

analysis, the cortex adjacent to meiotic spindle appears near the middle of each graph and is indicated by arrows. These measurements were not intended to compare fluorescence intensity from egg to egg but rather to show the changes in relative fluorescence intensity from region to region in a single egg cortex. As predicted from the images presented in Fig. 1, the pTyr- specific fluorescence in the cortex appeared as a collection of microspikes of varying intensity across the circumference of the egg. In unfertilized oocytes, the amplitude of the microspikes near the meiotic spindle was, for the most part, similar in intensity to those elsewhere in the cortex. However, during anaphase/telophase, cortical fluorescence intensity was 2- to2.5-fold higher in the hemisphere containing the maternal chromatin and spindle (arrows). Highly localized concentrations of pTyr containing proteins were consistently observed at the margins of the site of polar body extrusion possibly reflecting the remnants of the contractile ring.

Table 1
Comparison of immunofluorescence intensity in the cortical and central ooplasm

Sample	n	Cortical/central	SEM
Unfertilized	7	1.144	0.0284
Fertilized	12	1.671	0.0721

In order to compare the level of bound anti-P-Tyr antibody in MII oocytes with that of zygotes fixed prior to the pronuclear stage, the fluorescence intensity per unit area of the egg cortex was calculated and expressed as a ratio to the intensity of the central cytoplasm of each egg or zygote. Fluorescence intensity was quantitated as pixel intensity in the green fluorescence channel using the region measurement tool in Metamorph 6.2. The region of each egg cortex was traced by hand and included the plasma membrane and the underlying cytoplasm approximately 5 μm deep. The region of the central cytoplasm was the remainder of the egg. The integrated pixel intensity of each region was divided by the number of pixels in each region. The ratio of cortical fluorescence intensity to central cytoplasmic fluorescence intensity was then calculated and the values represent the mean obtained from (n) oocytes or zygotes and is expressed ±SEM. Analysis by t-test revealed that there is a statistically significant difference between the input groups ($P=<0.001$).

At the pronuclear stage, fluorescence intensity in the cortex showed little evidence of polarity and was highly variable with scattered regions of more intense fluorescence distributed around the entire egg. Further observations made at higher magnification showed more clearly that, in the unfertilized egg, pTyr was concentrated in the cortex overlying the MII spindle (Fig. 4A). In addition, pTyr was concentrated in regions of cytoplasm adjacent to the poles of the MII spindle (Figs. 4B−D). pTyr was found concentrated at the spindle poles in the majority (19 of 24, 79%) of unfertilized eggs and completely disappeared after fertilization by anaphase (0 of 18). Another feature that became obvious at higher magnification was the observation of intense pTyr fluorescence in the plasma membrane or cortex immediately overlying some sperm that had recently fused with the egg (Fig. 5). Sperm in which the head appeared to be external to the egg plasma membrane (Fig. 5A) were not associated with increased cortical fluorescence (0 of 7). However, approximately 24% (5 of 21) of the cases in which the sperm head was positioned just beneath the egg plasma membrane exhibited elevated pTyr labeling in the overlying egg cortex and plasma membrane (Figs. 5B−E). The fact that this feature was detected in only a subset of eggs that had incorporated sperm indicated that the pTyr accumulation may be a transient event. Examination of the sperm themselves indicated that while little or nor pTyr was detected in the head region, the entire sperm flagellum labeled with the pTyr antibody which was especially pronounced in the region of the mid-piece (Fig. 5A).

In summary, these results clearly indicate that the MII oocyte responds to fertilization with intense PTK signaling that is localized to the cortex overlying the spindle as well as specific sites associated with the spindle poles, the site of sperm incorporation and the site of polar body extrusion. These signaling events are transient and are no longer detected at the mid−late pronuclear stage.

Evidence for activation of Src-family PTKs in the egg

In order to determine whether the above increase in pTyr containing proteins could result from localized activation of SFKs, we used a phosphorylation site-specific antibody to detect activated Src-family members in the egg by immunofluorescence. The clone 28 antibody recognizes the dephosphorylated, C-terminal tyrosine (QYQPG) and flanking sequence common to several SFKs [249]. This antibody can detect activated Src, Fyn and Yes, and possibly other Src-family members [291], and we have used it recently to detect activated SFKs in the zebrafish egg [298]. The specificity of this antibody in the mouse egg system was demonstrated by Western blot analysis of MII oocytes (Fig. 6) which showed that the clone 28 antibody bound a single band of 59−60 kDa and that binding was blocked by excess peptide epitope (EPQYQPEGNL).

Immunofluorescence analysis of mouse oocytes before and at different times after fertilization demonstrated that activated SFKs were distributed uniformly throughout the cytoplasm of MII oocytes (Fig. 7A) and early zygotes (Figs. 7D−G). In distinct contrast to the pTyr labeling pattern, clone 28 binding exhibited no significant concentration in the egg cortex. However, the meiotic spindle was heavily labeled by the clone 28 antibody in all eggs examined (n=59) as seen at higher magnification in Fig. 7B. The localization of SFKs to the spindle resembled the results of a recent report [191] in which a monoclonal antibody (Nonphospho-Src (Tyr416)) against the autophosphorylation site of SFKs labeled the spindle in mouse oocytes. We have repeated their results (Fig. 7C) and it is clear that the clone 28 antibody and the Nonphospho-Src(Tyr416)antibody label the spindle with similar morphology. Since the Nonphospho-Src(Tyr416) antibody binds to inactive SFKs, the result presented in Figs. 7B and C demonstrate that the spindle is associated with a population of SFKs that includes both inactive and active kinases. As development of the zygote progressed, the distribution of clone 28 labeling did not change during anaphase II (Fig. 7D), telophase (Fig. 7E) and the early pronuclear stage (Fig. 7F). The specificity of clone 28 binding to the spindle was demonstrated by incubating the

antibody with a blocking peptide (Fig. 7G) duplicating the epitope against which the antibody was designed.

Once meiosis was complete and zygotes reached the late pronuclear stage, the clone 28 antibody was found to label the pronuclear envelope (Figs. 8A and D). In cleavage stage mouse embryos, activated SFKs continued to be associated with the nuclear envelope of the 2-cell embryo (Figs. 8B and E) although the labeling was less intense than at the pronuclear stage. Embryos that were fixed during the process of mitotic division displayed activated SFKs associated with the mitotic spindle and midbody (Figs. 8C and G).

At compaction, activated SFKs were no longer associated with the nuclear envelope (Figs. 9A and D) and the cortex remained devoid of SFK activity. Blastocyst stage embryos also exhibited activated SFKs associated with mitotic spindles and midbodies (Figs. 9B and E, arrows). In addition, the blastocyst was the first developmental stage in which active SFKs were concentrated in the cortical cytoplasm of individual cells. This was most prominent in the inner cell mass cells but also visible in the trophoblast cells (Figs. 9E and F). Mitotic cells exhibited increased SFK activity in all regions of the cytoplasm (Figs. 9E and F, arrows).

In summary, even though the zygote cortex displayed fertilization-dependent accumulation of pTyr-containing proteins, activated SFKs were not detected in the zygote cortex. Instead, activated SFKs were localized to the meiotic and mitotic spindles as well as the nuclear envelope at the late pronuclear stage and early 2-cell stages. Once the embryo reached the blastocyst stage, cortical localization of activated SFKs was obvious in all cells of the blastocyst. Blastocysts also exhibited intense activation of SFKs in cells undergoing mitosis.

Functional requirement for Src-family PTKs in zygote development

In order to determine whether the catalytic activity of SFKs plays an important role in development of the mammalian zygote, we tested the effect of several PTK inhibitors as well as a dominant-negative mutant Fyn construct on

zygote development. These experiments were performed on eggs fertilized *in vitro* to ensure a synchronized population. MII oocytes were fertilized by incubation with capacitated sperm for a period of 5 h, then transferred to culture drops containing different concentrations of PTK inhibitor and overlain with oil equilibrated with the same concentration of inhibitor. Zygotes were cultured for an additional 24 h then scored for developmental progress. As seen in Table 2, 80% of the control zygotes treated with DMSO as a solvent control had reached the two cell stage. Zygotes treated with GTP14564 [311], an inhibitor of class III receptor tyrosine kinases (IC50 0.3 μM), or PD168393, an inhibitor of the EGFr kinase (IC50 0.7 nM) [312], successfully developed to the two cell stage within 24 h. The SFK inhibitor PP2 had a small, but significant effect on zygote development at concentrations between 10 and 100 μM, a range at which it could have non-specific effects on other protein kinases [313, 314]. However, the recently developed SFK inhibitor SKI-606 was much more effective at concentrations known to exhibit specificity in cell culture systems. Zygotes treated with SKI-606 exhibited a reduced rate of cleavage at 1 μM and were almost completely inhibited at 10 μM. Cell division was reduced to 44% at 2.5 μM, a concentration similar to the IC50 reported to inhibit proliferation of cultured somatic cells [250, 315]. Examination of the zygotes that failed to cleave as a result of treatment with SKI-606 revealed that most were arrested prior to completion of second polar body emission. Typically, the MII spindle had rotated until it was perpendicular to the egg surface and all zygotes exhibited misplaced chromosomes and aberrant spindle microtubules. In addition, the treatment seemed to cause disruption of the microtubule dynamics in the egg resulting in some monopolar structures and astral arrays of microtubules under the egg cortex (Fig. 10). Zygotes remained arrested in this configuration for as long as 24 h. When SKI-606 was added after meiosis was complete and two pronuclei had formed, development was still arrested prior to the first mitotic division (Table 2) demonstrating that zygotes require SFK activity at a second point during the first cell cycle.

In an effort to confirm the role of SFKs in zygotic development without the use of chemical inhibitors, we tested the effect of a dominant-negative form of the Fyn kinase produced by mutation of Lysine 399 which is critical to catalytic activity [1]. As seen in Table 2, injection of pronuclear stage zygotes with mRNA encoding the dominant negative Fyn blocked development to the two cell stage with the majority of zygotes arrested with intact pronuclei (not shown). Those zygotes injected with mRNA encoding native Fyn as a control reached the two cell stage normally. Together, these results indicate that the observed association of activated SFKs with the MII spindle and pronuclear envelope are likely to represent functional signaling events important for zygote development.

Table 2. Effect of different PTK inhibitors on zygote development

Treatment	Stage treated	n	% 2-cell	SEM
DMSO	MII/anaphase	80	82.1	0.045
PD168393 (5 μM)	″	49	80.1	0.071
GTP14564 (20 μM)	″	50	78.9	0.071
PP2 (10 μM)	″	34	72.5	0.185
(100 μM)	″	37	33.7*	0.086
SKI-606 (1 μM)	″	72	72.3	0.154
(2.5 μM)	″	21	43.5*	0.165
(10 μM)	″	89	14.9*	0.086
(10 μM)	Pronuclear	106	13.3*	0.033
c-Fyn RNA (1.5 μg/μl)	″	77	83.2	0.650
dn-Fyn RNA (1.5 μg/ml)	″	69	37.9*	4.550

Mature, MII oocytes were collected from superovulated females and fertilized by incubation with capacitated sperm for 4.5 h as described in Materials and methods. The eggs were then transferred to small droplets of mKSOMAA containing the indicated inhibitor and overlain with oil equilibrated with the same inhibitor. This transfer was done either immediately after fertilization (MII/anaphase) or at the early pronuclear stage (pronuclear). For RNA injection, mRNA was transcribed in vitro and prepared as previously described [1], then pronuclear stage zygotes were injected with approximately 1−2 pg of RNA encoding native Fyn kinase (c-Fyn) or a catalytically inactive mutant kinase FynK299M [1] and cultured in normal mKSOMAA. The status of the zygotes was assessed at 24 h post-fertilization by examination by Hoffmann modulation or confocal fluorescence microscopy to establish whether sperm incorporation did occur and whether cell division had occurred. Values represent the mean from at least two experiments. *Indicates that the value is significantly different from the DMSO control group as determined by t-test ($P<0.05$).

DISCUSSION

Numerous studies involving chemical inhibitors, dominant negative fusion proteins and exogenous, recombinant kinases have demonstrated that PTKs including SFKs, play an important role in activation of eggs from non-mammalian species. These species typically exhibit a rapid activation of SFKs which may play a role in sperm−egg fusion [316] and are required for the rapid, high amplitude calcium transient that triggers egg activation [292]. In addition, PTK activation has been observed later in zygotic development and is required for steps involved in pronuclear migration and fusion [299, 300] as well as developmental competence [1, 317]. The extent to which these pathways are required in mammalian fertilization is now the subject of much investigation. Recent studies have clearly shown that SFK activity is not required for the repetitive calcium oscillations that trigger activation of the mouse oocyte [241, 304] highlighting differences between fertilization in mammals and lower species that fertilized externally. However, the experiments to date do indicate that PTKs are important for later aspects of zygotic development. Analysis of mammalian egg activation through the use of chemical inhibitors and SH2 domain-containing fusion proteins has demonstrated that MII resumption induced parthenogenetically required SFK activity [242, 307]. However, MII resumption induced by sperm injection has proven more difficult to inhibit with these reagents [142]. Evidence for PTK functions later in zygote development was first obtained with the chemical inhibitor genistein [264, 308], which blocked development prior to the exit from S-phase of the first zygotic cell cycle. Similarly, microinjection of a fusion protein encoding the SH2 domain of Fyn kinase caused developmental arrest at the late pronuclear stage [142]. Together these studies suggest that, while SFKs may not be critical for calcium signaling at fertilization in mammals, they do play significant roles in later events critical to development of the mammalian zygote.

The signaling mechanisms present in the mammalian oocyte prior to fertilization include receptor protein tyrosine kinases such as EGF receptor [318] and c-Kit [319], as well as the SFKs Yes, Fyn and, in some cases, Src [241, 242]. While

direct measurement of PTK activity in mammalian eggs has proven difficult [243], the presence of active PTK signaling (phosphorylation greater than dephosphorylation) can also be inferred by the accumulation of the pTyr reaction product which can be detected either by chemical means [264] or with anti-pTyr antibodies [304]. These studies have indicated that since the amount of pTyr in the egg increased after fertilization, the balance between PTK and PTPase shifts after fertilization such that pTyr is allowed to accumulate. In the present study, we have used immunofluorescence localization of pTyr-containing proteins as a method to detect increased PTK signaling within different subcellular compartments of the zygote. This study was made possible by careful attention to controlling post-fixation dephosphorylation of egg proteins through the use of the covalent PTPase inhibitor phenylarsine oxide at all stages of fixation and processing. The results demonstrated that increased PTK signaling occurs in several different subcellular compartments following fertilization of the mouse egg.

Immunofluorescence analysis of pTyr accumulation during fertilization and zygote development revealed that fertilization is followed by highly localized changes (both increases and decreases) in PTK signaling in the egg. The unfertilized MII oocyte was relatively quiescent with a low level of pTyr distributed fairly evenly throughout the oocyte with only a slight concentration of pTyr in the cortex adjacent to the MII spindle and at the spindle poles. Fertilization resulted in a significant accumulation of pTyr in the zygote cortex indicating that increased PTK signaling was occurring in this compartment. The distribution of cortical pTyr exhibited a distinct polarity in most zygotes with the highest concentration of pTyr in the hemisphere associated with the meiotic spindle. During anaphase, the region directly overlying the spindle (corresponding generally with the cortical granule free domain [320] was usually most intensely labeled by the anti-pTyr antibody. This suggests that PTK signaling may play an important role in either actin-mediated events that modify this region of cortex, or in other signaling pathways such as MAPK [321] or PAR-3 [322], which characterize this important region. Within the zygote cortex, further

concentrations of pTyr were detected at the shoulders of the emerging polar body associated with the region occupied by the contractile ring. The contractile ring in sea urchin embryos has been associated with ganglioside-1 and cholesterol-rich microdomains that are characterized by intense PTK signaling [323], and it is likely that a similar mechanism is employed during polar body extrusion.

While fertilization caused an increase in PTK signaling in the egg cortex, the highly localized accumulation of pTyr at the spindle poles decreased as anaphase progressed and disappeared at telophase. The fact that PTK signaling at the spindle poles declined as meiosis progressed indicates that this pathway is likely linked to the cell cycle events that control spindle function. Enzymes such as MAPK [324] and polo like kinase [325-327] which are substrates for PTKs and have been localized to spindle poles are likely targets for PTK signaling in this region.

Another highly localized PTK signaling event was detected in the cortex overlying the site of sperm incorporation. Since pTyr concentration was found in only 24% of the sperm incorporation sites observed, we infer that it could represent a transient signaling event. This event was not detected in the early stages of sperm incorporation such as sperm−egg attachment or formation of the fertilization cone. Instead it was apparent only after the sperm head was fully incorporated and generally when nuclear decondensation had begun. It is not clear whether the pTyr containing proteins observed overlying the sperm head were egg proteins or were contributed by the sperm. It is also unclear whether they were phosphorylated by a PTK derived from the egg or the sperm. The timing of this localized signaling event indicates that it does not represent the phosphorylation of uroplakin III, which occurs rapidly in the Xenopus oocyte and is thought to function in sperm−egg fusion [316]. In any case, this interesting finding demonstrates that the site of sperm incorporation/decondensation is associated with highly localized PTK signaling.

The above results raise the question of which PTKs in the egg are responsible for the localized changes in protein tyrosine phosphorylation. The recent development of phosphorylation site-specific antibodies that recognize the activated form of

different PTKs has provided a method to identify which PTKs are activated in different subcellular compartments. As an initial step, we have used the clone 28 antibody [249] which is specific for the activated form of SFKs and has been used to demonstrate highly localized changes in SFKs activity in tissues ranging from kidney, peripheral nerve, endometrium and various cancers [328-330]. One limitation is that since the sequence of amino acids flanking the C-terminal tyrosine is highly conserved among Src-family members, this antibody cannot differentiate among the different family members. However, only a limited number of SFKs have been detected in eggs of different species [242, 331, 332]. Therefore, the kinases detected by the clone 28 antibody in the egg probably include Fyn, Yes and possibly Src, although other SFKs may contribute to our results. The antibody would not be able to detect the closely related Abl, the receptor type PTKs such as Kit or EGF receptor or other PTKs such as FAK or JAK.

The most surprising observation was that activated SFKs were not highly localized to the zygote cortex which exhibited such a dramatic increase in pTyr after fertilization. We have previously shown that Fyn kinase is concentrated at the cortex of the mouse egg [142] but the present results indicate that in the mouse egg, Fyn must remain inactive in this compartment until later in development. This observation contrasts with our results in the zebrafish egg which exhibited cortical activation of SFKs at fertilization [298]. Instead, the results in mouse demonstrated a very striking association of activated SFKs with active spindle structures (both meiotic and mitotic) as well as midbodies. This finding confirms earlier results obtained with this same antibody in somatic cells [333-335]. Localization of active SFKs to the spindle also correlated well with the demonstration that Fyn kinase was tightly bound to the meiotic spindle in rat oocytes [129]. A similar approach was used by Zheng et al., 2007 [191] to detect SFKs in the mouse oocytes. Their results demonstrated that an antibody to non-activated SFKs (Nonphospho-Src (Tyr416)) also bound to the MII spindle with a morphology similar to that reported here for the clone 28 antibody. The fact that both active and inactive SFKs were associated with

the spindle at all stages of spindle function suggests that this signaling mechanism may play a role in maintenance of the spindle structure or function. For example, tyrosine phosphorylation of CDC2 has been linked to its ability to drive assembly of the mitotic spindle in yeast [336].

In addition to the spindle, the second most obvious site of SFK activation was at the pronuclear envelope of late pronuclear stage zygotes. We have previously demonstrated that Fyn kinase associated with the pronuclear envelope [142], so it is likely that Fyn represents some of the activated SFKs detected by the clone 28 antibody near the pronuclear membranes. The association of active PTKs with the nuclear envelope remained high at the 2-cell stage but was much reduced by the four-cell stage and was not evident in the nuclei of blastocysts or in somatic cells such as cumulus cells. The demonstration of activated SFK located at the surface of the nuclear envelope during pronuclear through the two cell stage correlates well with functional data demonstrating that PTK activity and SFK activity specifically is required at this stage. For example, chemical PTK inhibitors have been shown to cause zygotic arrest at this stage [264, 308] and GST fusion proteins encoding the SH2 domain of Fyn caused mouse zygotes to arrest at the late pronuclear or two cell stage. While SFK activation has been shown to play a role in cell cycle events in somatic cells [337], the fact that intense, nuclear envelope-associated PTK activation was primarily observed at the 1−2 cell stage indicates that this must represent a zygote-specific function, possibly involving alterations in nuclear structure associated with zygotic gene activation.

The use of pharmacological inhibitors specific for SFKs has included studies of the PP2 which blocks MII resumption in response to parthenogenetic stimuli but only at high concentrations [242, 307]. SU6656, which appears to become sequestered in vacuoles in the egg, has little effect on fertilization [241]. In the present study, we have tested the recently developed quinolinecarbonitrile derivative SKI-606 which combines high specificity for SFKs with superior aqueous solubility and stability [250]. The compound inhibits Src *in vitro* with an IC 50 of 1.2 nM,

while it exhibited an *in vitro* IC50 of 2.6 μM for the receptor PTK ErbB-2, and 19 μM for the Ser/Thr kinase Cdk4 [251]. Like PP2 (Clark and Peterson, 2003), SKI-606 also inhibits the closely related Abl kinase with an IC50 of 1.0 nM. The compound readily penetrates the plasma membrane, blocking Src kinase activity in cultured cells with an IC50 of 250 nM and Src-dependent cell proliferation with an IC50 of 1.5−2.5 μM. The stability of this compound in aqueous media leads to its ability to inhibit tumor growth *in vivo* with an IC50 of 250 nM [315] and the compound is now undergoing clinical trials for treatment of human cancer (ClinicalTrials.gov). We found this compound to block meiosis II completion and cause abnormal spindle structures and a high frequency of misplaced chromosomes. When added after meiosis was complete, it blocked development to the two cell stage. In order to confirm this result by another means and begin the process of determining which SFKs are involved in different stages of zygotic development, we used a dominant-negative form of Fyn kinase introduced by RNA injection. The results of this experiment indicated that Fyn is likely to be a key component of those SFKs required for zygotic development. The use of kinase-inactivating mutations to produce dominant-negative forms of SFKs has been successfully employed in developmental studies before [302]. The kinase inactivating point mutation used in FynK299M has the advantage that the U, SH3 and SH2 protein interaction domains remain intact and can compete with the native Fyn for protein interactions occurring both upstream and downstream. To the extent that the specificity of these interactions is common to other Src-family members, the dominant-negative construct can be expected to compete with other Src-family members and thereby block compensation by these kinases. This provides an advantage over single gene knockout or RNAi knockdown studies but makes it more difficult to identify the role of each specific kinase. While SFKs are well known to share overlapping specificity, it is unlikely that non-Src-family PTKs would be affected by this dominant-negative construct since they would not share the combination of SH2, SH3 and U domain specificities.

In summary, the present study has demonstrated that fertilization in mammals results in highly localized PTK signaling events associated with specific regions of the egg cortex, meiotic spindle and pronuclear envelope. Their localized nature and differential timing indicates that they are likely under different control mechanisms in the zygote. The SFKs, which are highly concentrated in the egg cortex, were not activated significantly in this compartment and it is likely that other PTKs are responsible for the intense tyrosine phosphorylation of proteins in the cortex of the mouse egg. Instead, the SFKs appeared to play roles in spindle structure or function as well as nuclear events unique to the zygote and early cleavage stages.

ACKNOWLEDLGEMENTS

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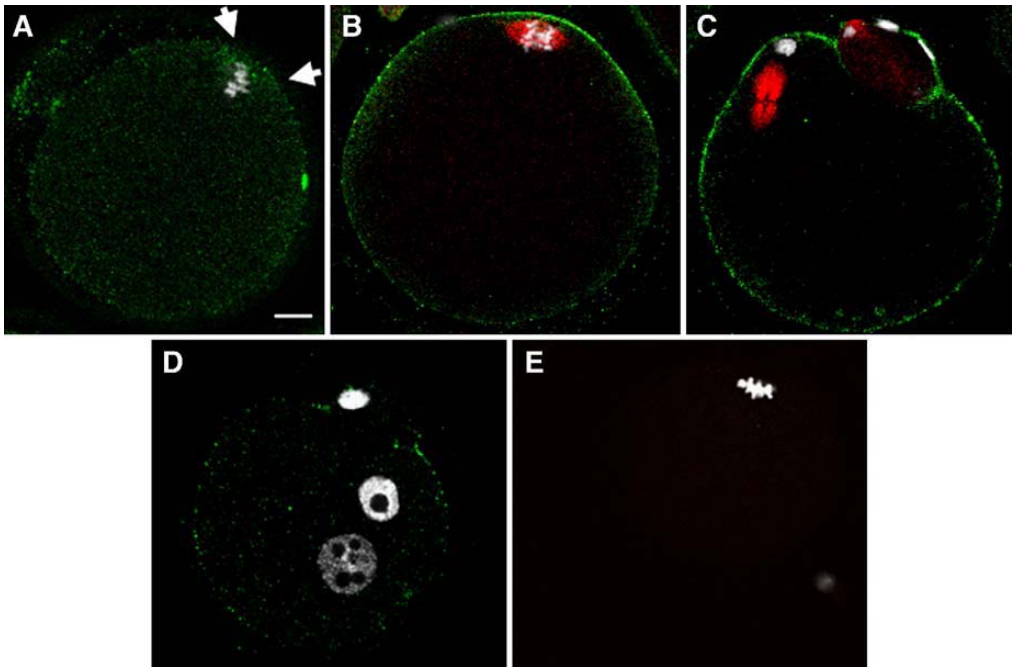

Figure 1

Fig. 1. Effect of fertilization on the distribution of pTyr-containing egg proteins. MII oocytes were recovered from the oviducts of superovulated mice at 14 h post-hCG and fertilizing eggs and zygotes were collected from superovulated mated females at 13.0−16.0 h post-hCG. The oocytes or zygotes were dissected free of the oviduct in the presence of fixative and processed for immunofluorescence as described in Materials and methods. The samples were stained with the mouse anti-pTyr monoclonal 4G10 followed by Alexa-488-goat anti-mouse IgG (green) as well as Hoechst 33258 (white) to detect DNA. In some cases, the spindle was stained with rat monoclonal YOL 1/34 anti-tubulin, which was detected by Alexa-568-goat anti-rat (red). Samples were examined on a Zeiss LSM500 confocal microscope as described in Materials and methods. (A) MII oocyte; (B) fertilized, early anaphase; (C) fertilized telophase; (D) fertilized, early pronuclear stage (16 h post-hCG). Specificity was demonstrated by incubating eggs in the presence of the 4G10 antibody+1 mM pTyr (E). Magnification is indicated by the bars tt represent 10 μm.

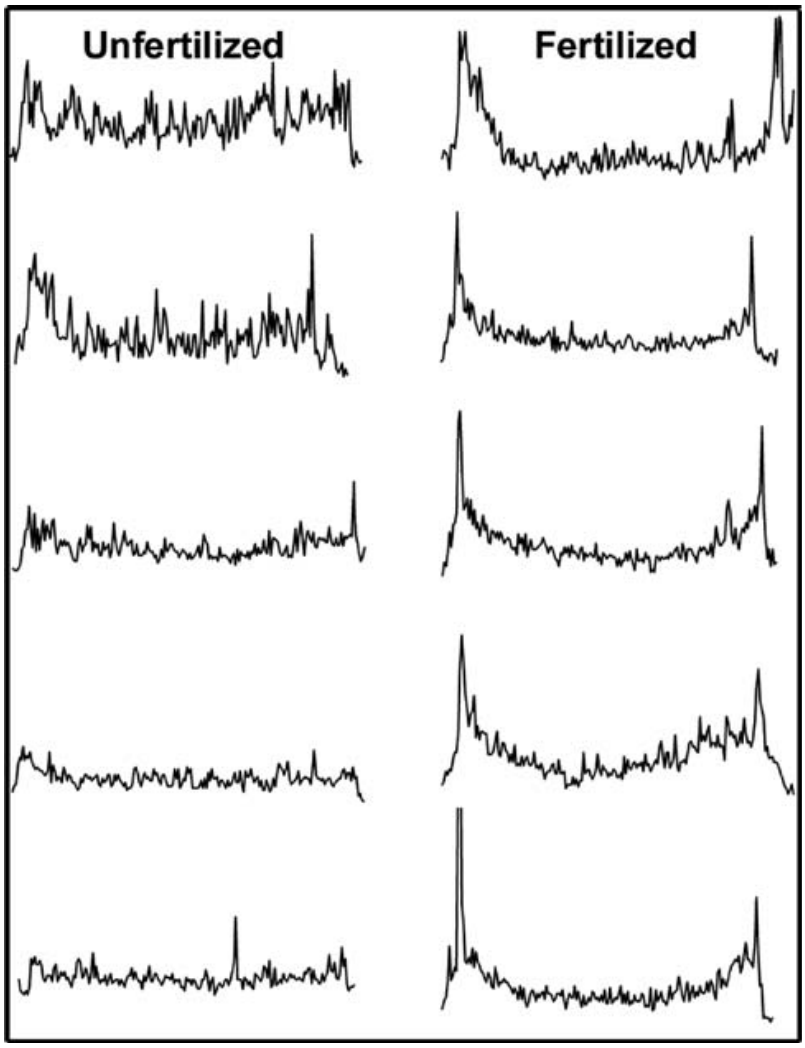

Figure 2

Fig. 2. Changes in the relative distribution of pTyr in response to fertilization. In order to demonstrate the effect of fertilization on the distribution of pTyr residues, transverse linescan analysis was performed on images from eggs and zygotes labeled with the anti-pTyr antibody as in Fig. 1. Measurement lines were drawn originating from a point adjacent to the meiotic spindle and passing through the center of the egg to the opposite side. Pixel intensity along the course of the lines is presented in the vertical axis. Measurements made from representative unfertilized eggs are presented on the left and those made from different stages of zygote development up to the pronuclear stage.

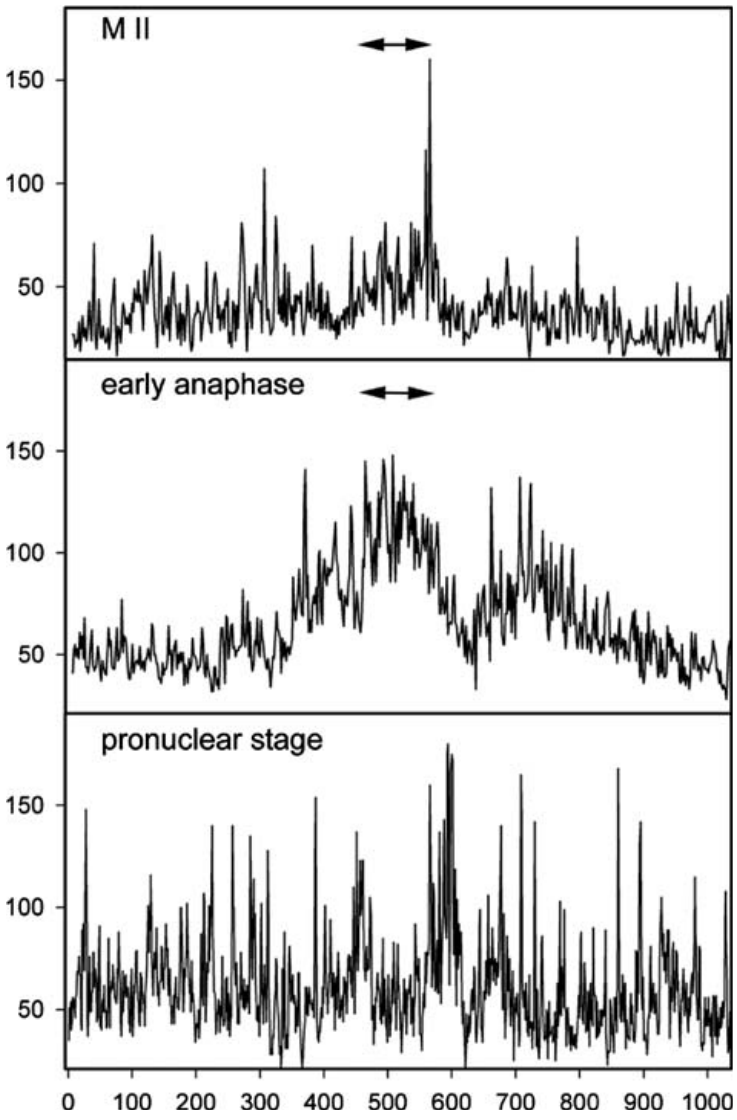
M II
150
100
50
early anaphase
150
100
50
pronuclear stage
150
100
50
0
100
200
300
400
500
600
700
800
900
1000

Figure 3

Fig. 3. Distribution of pTyr-containing proteins in the egg cortex. In order to demonstrate the asymmetric distribution of P-Tyr in the egg cortex, the fluorescence intensity of eggs and zygotes labeled with the anti-P-Tyr antibody as in Fig. 1 was quantitated by circumferential linescan analysis using Metamorph 6.2 and is indicated on the vertical axis. A measurement line was traced on images beginning from a position at the egg surface opposite the meiotic spindle and progressed clockwise around the egg cortex to include the entire cortex (horizontal axis). The position of the spindle is therefore near the center of each scan and is indicated by the arrows.

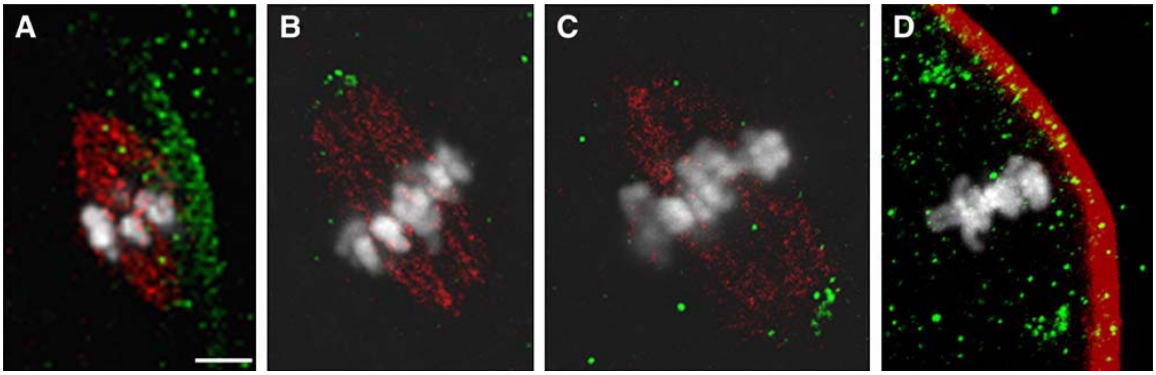

Figure 4

Fig. 4. Localization of pTyr proteins in the region of the meiotic spindle. Oocytes were collected from the same replicates as shown in Fig. 1. Panel A is a compression of 3 serial confocal images showing the overlying cortex and the full length spindle with a small spot of pTyr label visible at the bottom spindle pole (A). Panels B and C are two individual scans of a single MII spindle showing pTyr-specific labeling at both spindle poles, top (B) and bottom (C). Panel D is an MII spindle from a second egg in which the layers have been compressed to allow visualization of both spindle poles on a single image. Panels A−C were co-labeled with YOL 1/34 antitubulin and Alexa-568 (red) to show spindle microtubules, while Panel D co-labeled with Alexa 568-phalloidin to identify f-actin. Magnification is indicated by the bar

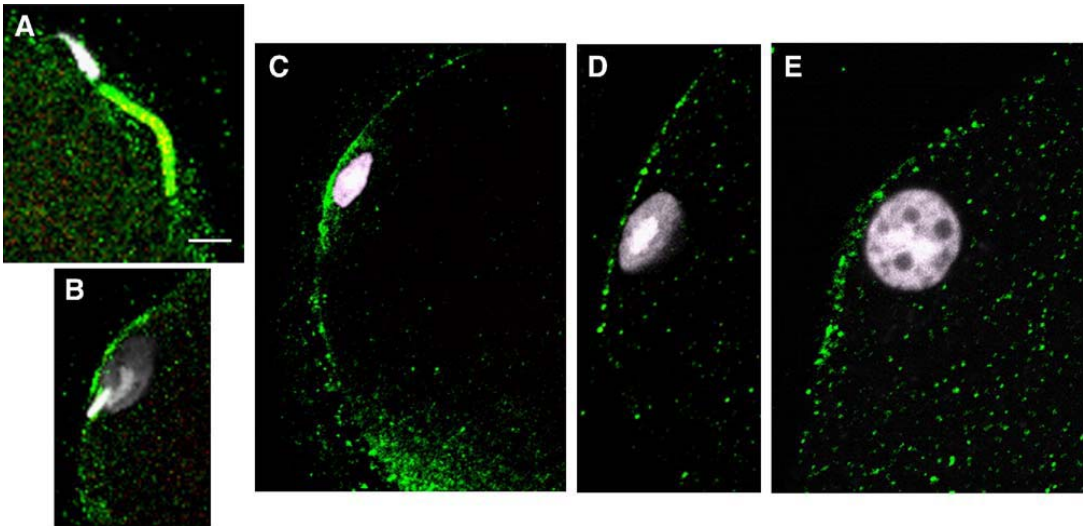

Figure 5

Fig. 5. pTyr-containing proteins in the egg cortex overlying the decondensing sperm head. Samples prepared as in Fig. 1 were stained with anti-pTyr followed by Alexa-488-goat anti-mouse IgG (green) and Hoechst 33258 (white) to label DNA. Fertilizing (capacitated) sperm bound to the oolemma exhibited pTyr proteins in the sperm midpiece; however, specific pTyr label was not detected in the head region (A). As fertilization progressed, decondensing sperm heads located deep to the oolemma were associated with an increase in pTyr-containing proteins within the egg cortex immediately overlying the sperm nucleus (B−D). Occasionally, the male pronucleus formed and remained adjacent to the egg cortex for some time and the pTyr label continued to be detected in the cortex near the paternal chromatin (E). As the sperm DNA migrated away from the cortex, the pTyr label disappeared (not shown). Magnification is indicated by the bar that represents 10 μm.

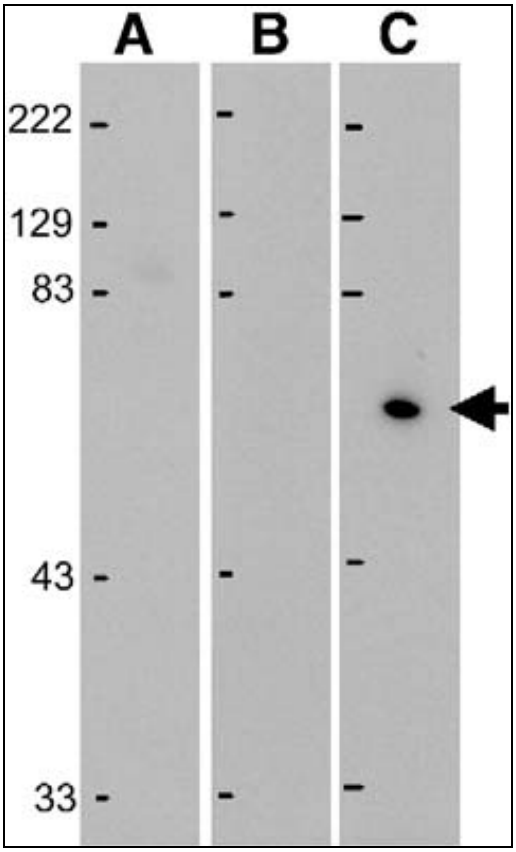

Figure 6

Fig. 6. Detection of Src-family PTKs by Western blot of mouse oocytes. Samples of unfertilized, cumulus-free oocytes were loaded on multiple lanes of a 10% SDS−PAGE gel (7.5 eggs/lane) and electrophoresed, then blotted to a nylon membrane and blocked with TTBS+5% dried milk containing phosphatase inhibitors as described in Materials and methods. Lanes were incubated with a control mouse monoclonal IgG (lane A), clone 28 IgG+1 mM blocking peptide (lane B) or clone 28 IgG (lane C), at a concentration of 1 μg/ml overnight. The blots were then w shed, incubated with goat anti-mouse IgG peroxidase, and bound antibody was localized by chemiluminescence. The position of molecular weight standards is indicated (in kDa) at left, and the position of the Src-family PTK(s) detected with the clone 28 antibody is indicated by the arrow at right.

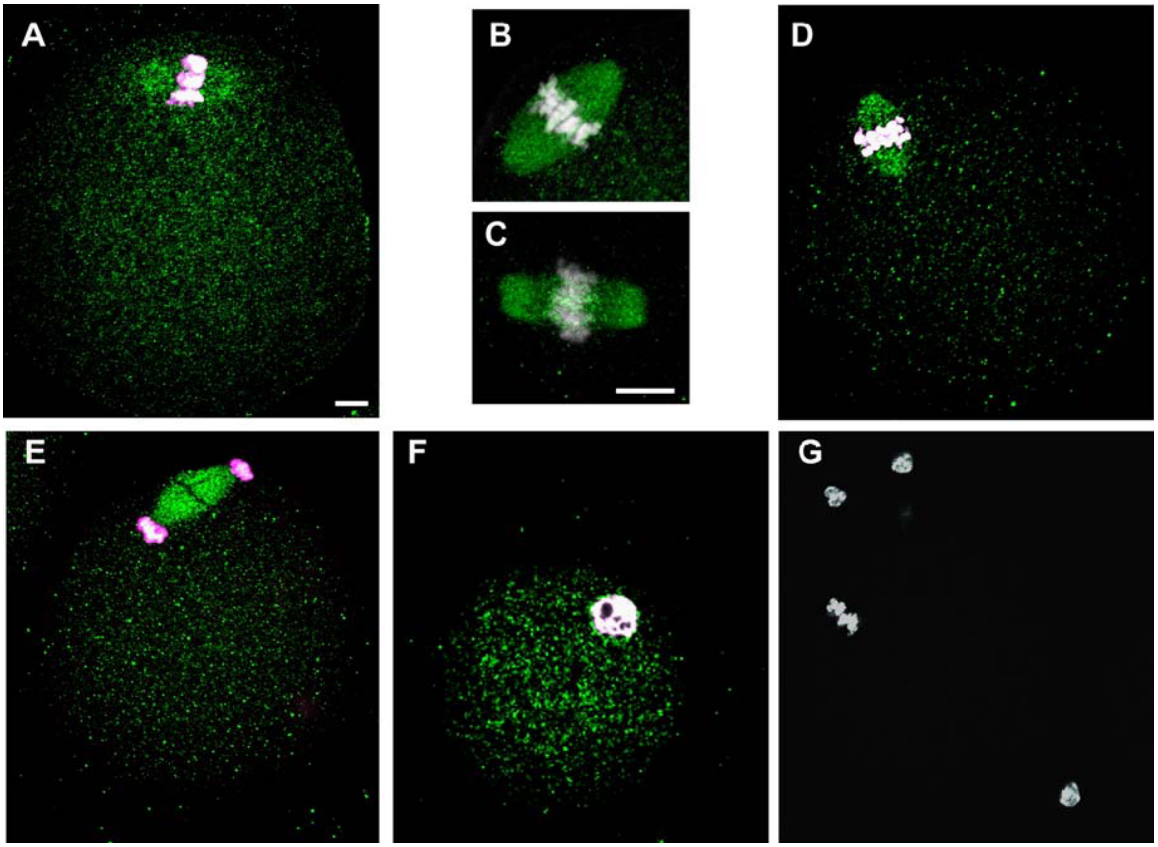

Figure 7

Fig. 7. Active Src-family PTKs associate with spindle microtubules. Oocytes and zygotes fixed at different stages of zygote development were labeled with a monoclonal antibody against activated Src-family PTKs (clone 28) or with the Nonphospho-Src (Tyr416) antibody specific for inactive Src-family PTKs (Panel C only). Bound antibody was detected with Alexa-488-goat anti-mouse IgG (green) and Hoechst 33258 (white) was used to demonstrate chromatin. Active SFKs were detected as a low level of uniform fluorescence in the cytoplasm of oocytes as well as zygotes up to the early pronuclear stage. In contrast, high levels of both activated SFKs (A, B) and inactive SFKs (C) were associated with the spindle microtubules of the unfertilized MII oocytes indicating that the spindle is associated with a population of SFKs, some of which are active. Active Src-family PTKs detected with the clone 28 antibody remained associated with the spindle from early anaphase (D) and telophase (E). When the male and female pronuclei formed, the active SFKs became uniformly distributed in the cytoplasm again (F). Specificity was demonstrated by incubating the sample with the clone 28 antibody in the presence of the synthetic peptide (EPQYQPGENL) at 1 mM (G). Magnification is indicated by the bar that represents 10 μm.

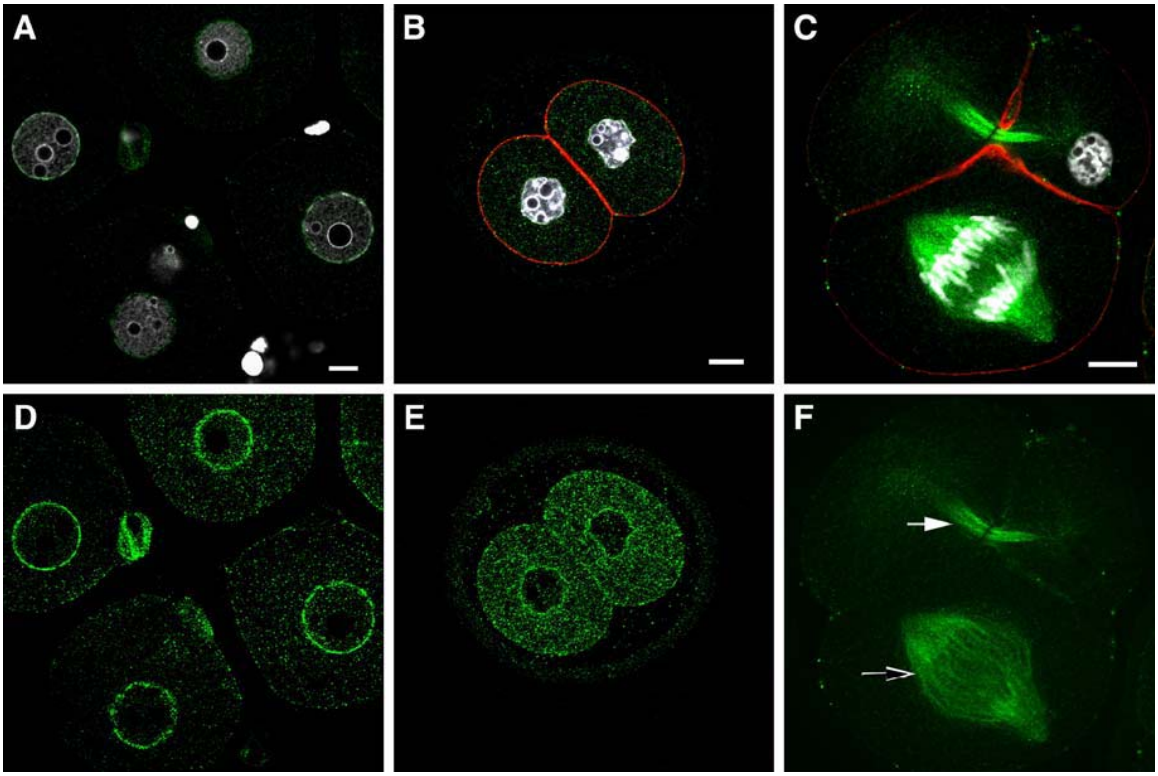

Figure 8

Fig. 8. Activated SFKs associate with the nuclear envelope at the late pronuclear stage. Later pronuclear stage zygotes (24 h post-hCG), 2-cell (48 h) and 3-to 4-cell (60 h) embryos were fixed and labeled with the clone 28 antibody as described for Fig. 5. Activated SFKs were localized at or near the pronuclear envelope (A, D). This peri-nuclear localization was still detectable at the 2-cell stage (B, E). During mitosis (C, F), activated SFKs were associated with the spindle (open arrow) and midbody microtubules (closed arrow). Magnification is indicated by the bar that represents 10 μm.

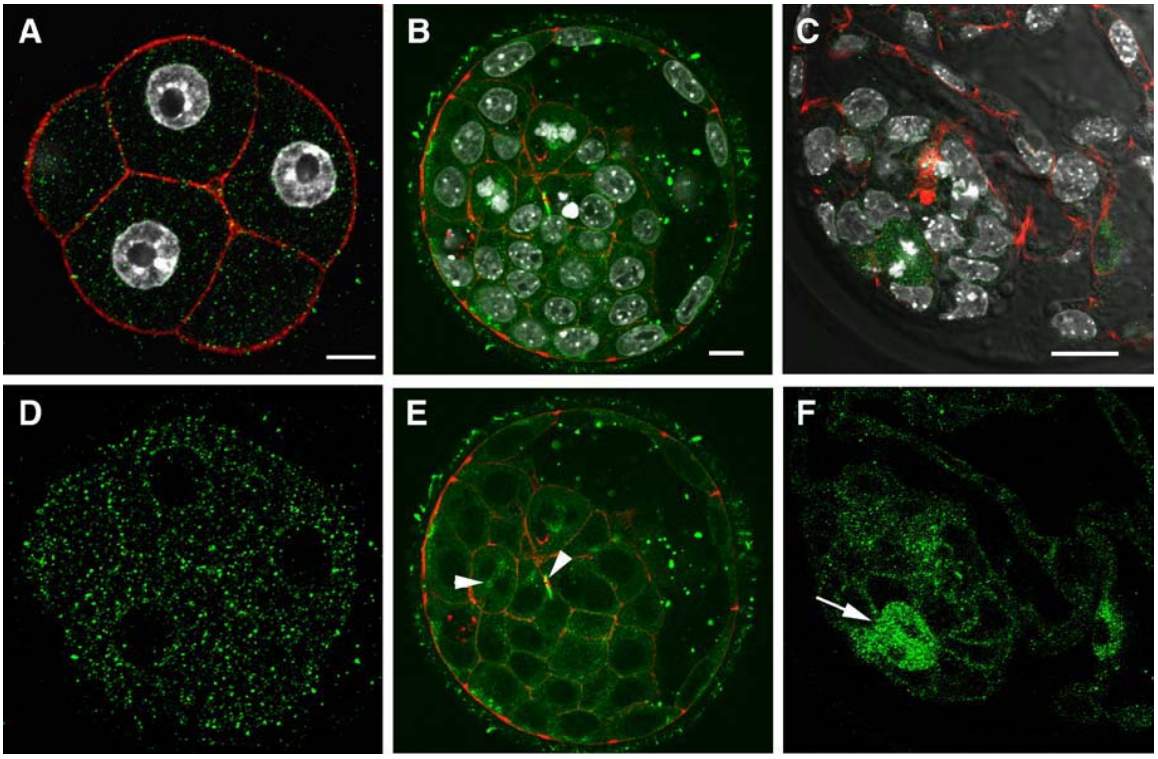

Figure 9

Fig. 9. Distribution of activated SFKs in the morula and blastocyst. Embryos were fixed at the compaction (48 h post-hCG) and the expanded blastocyst (120 h post-hCG) stages, then labeled with the clone 28 antibody (green) as well as Alexa 568-phalloidin (red) to visualize f-actin. At the 8-cell compacted stage, activated SFKs were no longer associated with the nuclear envelope and were evenly distributed throughout the cytoplasm. In blastocyst stage embryos (B, C, E, F), activated Src-family PTKs were concentrated at the cortex of most inner cell mass and trophoblast cells as well as at mitotic spindles and midbodies (arrowheads). At higher magnification (C, F) activated Src-family PTKs were concentrated in cells actively undergoing mitosis (arrows). Magnification is indicated by the white bar that represents 10μm.

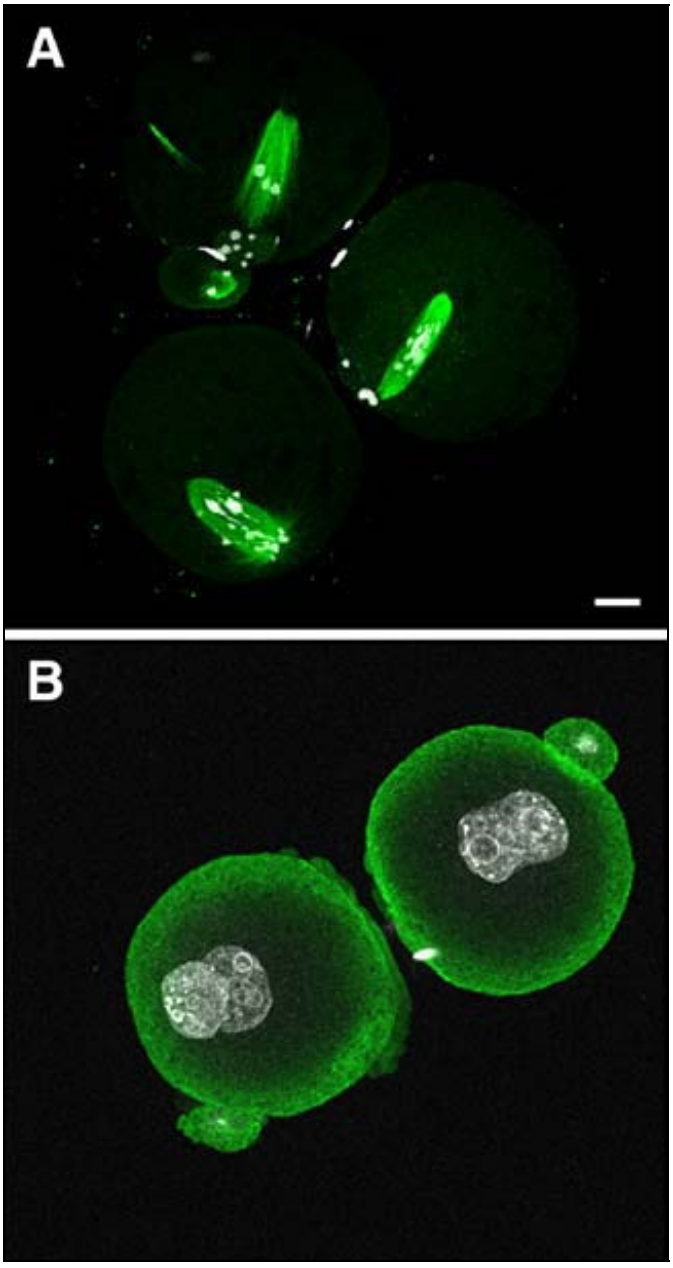

Figure 10

Fig. 10. Inhibition of Src-family PTK activity disrupted normal microtubule dynamics during meiosis II. Mature, MII oocytes were fertilized in vitro for 5 h, then transferred to medium containing SKI-606 (10 μm) (A) or DMSO (B). Zygotes were examined at 24 h post-IVF when most controls were at the two cell stage. All embryos that failed to cleave to the two cell stage were fixed and stained with anti-tubulin (green) and Hoechst 33258 (white). Panel A demonstrates typical SKI-606-treated zygotes, and Panel B demonstrates the morphology of DMSO-treated controls that failed to cleave within 24 h. Magnification is indicated by the bar which represents 10 μm.

Chapter Five

Conclusions

Mammalian oocytes engage in a remarkable series of cytoskeletal and cell cycle based modifications that prepare the oocyte for the initiation and continuance of development. Multiple signaling pathways appear to operate during the process of oocyte maturation to ensure that the quality of the cytoplasm and genome will meet the standards required to initiate and complete development.

Oocyte maturation invokes complex signaling pathways to achieve cytoplasmic and nuclear competencies for fertilization and development. The Src-family kinases FYN, YES and SRC are expressed in mammalian oocytes but their function during oocyte maturation remains an open question. Using chemical inhibitor, siRNA knockdown, and gene deletion strategies the function of Src-family kinases was evaluated in mouse oocytes during maturation under *in vivo* and *in vitro* conditions. Suppression of SFKs as a group with SKI606 greatly reduced meiotic cell cycle progression to metaphase-II. Knockdown of FYN kinase expression after injection of FYN siRNA resulted in approximately 50% reduction in progression to metaphase-II similar to what was observed in oocytes isolated from FYN (-/-) mice matured *in vitro*. Meiotic cell cycle impairment due to a Fyn kinase deficiency was also evident during oocyte maturation *in vivo* since ovulated cumulus oocyte complexes collected from FYN (-/-) mice included immature metaphase-I oocytes (18%). Commonalities in meiotic spindle and chromosome alignment defects under these experimental conditions demonstrate a significant role for Fyn kinase activity in meiotic maturation.

Kinases and their targets require specific regional distributions to effect changes in cell function. To examine this process during meiotic maturation, the protein phosphorylation patterns were mapped topographically over the course of meiotic cell cycle progression in *in vitro* matured mouse oocytes. Oocytes collected at the GV stage were matured *in vitro* in either a basal embryo medium (IVMb) or a medium designed to improve oocyte developmental quality (IVMh). After meiotic

reinitiation, oocytes matured for 0, 2, 4, 8 and 16h were fixed and probed for phosphotyrosine (pTyr) or mitosis associated phosphoserine/threonine (MPM2) epitopes. Quantitative and qualitative analyses were performed on oocytes at progressive stages of maturation using confocal microscopy and image analysis. pTyr epitope was evident throughout the cytoplasm at all stages with intense staining at the cortex. Cytoplasmic and cortical labeling increased significantly between MI and MII. Supplemented media significantly increased cortical levels of pTyr proteins at MII when compared to oocytes matured under IVMb conditions. In contrast, MPM2 epitope was distributed in patterns distinctly different from those bearing pTyr. Cytoplasmic MPM2 epitope localized to the meiotic spindle, spindle poles and cytoplasmic MTOCs but was not detectable in the cortex. Cytoplasmic MPM2 localized in a subcortical region. In meiotic spindles, pTyr epitope was concentrated at spindle poles whereas MPM2 was distributed throughout the spindle in a pattern distinct from pTyr. Both cortical and spindle pole pTyr epitopes were absent in oocytes obtained from Fyn (-/-) oocytes suggesting a role for the Fyn tyrosine kinase in meiotic spindle integrity and cortical remodeling. These findings demonstrate that tyrosine kinases impose spatially discrete patterns of protein phosphorylation that may underlie the distinction between nuclear and cytoplasmic maturation in mammalian oocytes.

Recent studies in species that fertilize externally have demonstrated that fertilization triggers localized activation of Src-family protein kinases in the egg cortex. However, the requirement for Src-family kinases in activation of the mammalian egg is different from lower species. Therefore, we examined changes in the distribution and activity of SFKs during zygotic development in the mouse. Immunofluorescence analysis of mouse oocytes and zygotes with an anti-phosphotyrosine antibody revealed that fertilization stimulated accumulation of pTyr-containing proteins in the egg cortex and that their abundance was elevated in the region overlying the MII spindle. In addition, the poles of the MII spindle exhibited elevated pTyr levels as seen in our earlier studies. As polar body extrusion

progressed, pTyr containing proteins were especially concentrated in the region of cortex adjacent to the maternal chromatin and the forming polar body. In contrast, pTyr labeling of the spindle poles disappeared as meiosis II progressed to anaphase II. In approximately 24% of cases, the fertilizing sperm nucleus was associated with increased pTyr labeling in the overlying cortex and oolema. To determine whether SFKs could be responsible for the observed changes in the distribution of pTyr containing proteins, an antibody to the activated form of SFKs was used to localize activated Src, Fyn or Yes. Activated SFKs were found to be strongly associated with the meiotic spindle at all stages of meiosis II; however, no concentration of labeling was evident at the egg cortex. The absence of cortical SFK activity continued until the blastocyst stage when strong cortical activity became evident. At the pronuclear stage, activated SFKs concentrated around the pronuclei in close association with the nuclear envelope. This pattern was unique to the earliest stages of development and disappeared by the eight cell stage. Functional studies using chemical inhibitors and a dominant-negative Fyn construct demonstrated that SFKs play an essential role in completion of meiosis-II following fertilization and progression from the pronuclear stage into mitosis. These data suggest that while SFKs are not required for fertilization-induced calcium oscillations, they do play a critical role in development of the zygote. Furthermore, activation of these kinases in the mouse egg is limited to distinct regions and occurs at specific times after fertilization.

Thus, previously unanticipated functions for SFKs have been identified for the first time that mediates the spatial and temporal remodeling of cytoskeleton and cell cycle during oocyte maturation and early development. These findings will have an immediate impact on the field of human ARTs as this pathway has been completely overlooked up to now.

Chapter Six

Future Directions

The GV stage oocyte contains large amounts of stored maternally derived mRNA. These maternal mRNAs are selectively translated at specific times during oocyte maturation and embryonic development. Many mRNAs are degraded during maturation from GV to MII stages while others are recruited so that their protein products enable essential processes during fertilization and later embryonic development. In vitro maturation of oocytes has been shown to cause misregulation of these processes leading to aberrant levels of mRNA at MII and early embryonic stages [338, 339]. The most stable transcripts are molecules involved in kinase signaling pathways [339]. Therefore preservation of kinase signaling pathways is required for the maintenance of proper oocyte maturation leading to healthy offspring. The following kinase pathways have all been implicated in oocyte maturation: Cdk1/Cyclin B, MAPK, PI3K, hormone receptors (androgen, estrogen and progesterone), integrin receptors, growth factor receptors (EGF, PDGF, Kit, IGF1) and PKA. Interestingly, all of these pathways intersect with the SFK signaling pathway.

Our studies have identified SFKs, especially Fyn kinase as an essential signaling pathway during oocyte maturation and embryonic development. Evidence suggests a role for SFKs in chromatin and spindle configurations during meiosis and the first mitotic cell cycle and events occurring at the cortex of the oocyte during maturation and fertilization.

I see two primary directions for future studies based on this evidence. First, since IVM has significant effects on overall levels of mRNA in oocytes and since proper regulation of mRNA levels in oocytes is essential for healthy embryonic development, it would be of particular interest to know if there is a specific effect of IVM on levels of SFK mRNA and proteins. Concentration and activity levels of SFK proteins would also be of interest. Unfortunately, these types of studies are difficult

because very large numbers of oocytes are required to detect small changes in mRNA and protein concentration. However, it could feasibly be done. Our studies have already demonstrated a requirement for SFKs in both oocyte maturation and embryonic development therefore changes in the levels of mRNA caused by IVM could have significant effects on the resulting offspring. Knowledge of these in vitro effects would be beneficial to clinical ARTs and would lead to the production of better in vitro conditions for IVM.

The second future direction would determine the protein targets of Fyn kinase thus enabling the identification of specific events regulated by SFKs during maturation, fertilization and early embryonic development. The use of phospho-specific antibodies and immunohistochemical labeling with confocal microscopy has identified the metaphase spindle poles and the egg cortex as sites of intense tyrosine phosphorylation events. Active SFKs have been found in association with microtubules in the spindle and cortex at all stages of oocyte maturation, embryonic development and even in the companion cumulus cells. Therefore there seems to be two distinct locations of SFK activities: the spindle and the cortex.

The targets for SFKs at spindle poles are unknown. Spindle poles of mammalian oocytes are different than those of mitotic cells in that they are formed of acentriolar centrosomes. However, even in the absence of true centrioles, many of the somatic cell centrosomal milieus are present at the poles of oocytes. These include pericentrin, γ-tubulin and other γ-tubulin-ring-complex proteins [119, 340, 341]. Interestingly, γ-tubulin is a known target of Fyn kinase although the purpose of this modification is not known [131, 133]. Changes in γ-tubulin concentrations in mouse oocytes during maturation induces changes in spindle size, demonstrating a role for γ-tubulin during spindle formation [248]. Precise timing and coordination of nuclear and cytoplasmic maturation is required for proper spindle and chromatin organization [342]. Interestingly, chromosome modifying proteins also associate with centrosomes in mouse oocytes [343]. This suggests a correlation between the pTyr activities at the spindle poles with our data that shows errors in chromosome

organization and chromatid separation following loss of Fyn kinase [344]. Identification of Fyn kinase targets in centrosome, chromatin and spindle associated proteins will help to determine the specific rolls for Fyn kinase in the progression of meiotic maturation as well as the early divisions of the embryo.

Our studies have also identified tyrosine phosphorylation in the egg cortex in association with chromosome and spindle proximity. pTyr proteins localize specifically over both male and female chromatin following fertilization [134] and activated SFKs were found associated with cortical microtubules [134, 344]. When chromatin moves close to the egg cortex, an actin cap and cortical granule-free domain is formed in the region above the meiotic chromatin [320, 345]. Ongoing studies in the Kinsey lab, of oocytes following fertilization have found changes in the structure of this cortical actin matrix in Fyn (-/-) oocytes. Oocytes from Fyn (-/-) mice or wildtype eggs treated with SKI606 form abnormally small actin caps and fail to relocate cortical granules (Luo, McGinnis and Kinsey, submitted). Interestingly, this region is the same area where we have identified the appearance of tyrosine phosphorylation overlying the chromatin and suggests at least one roll for Fyn kinase in the egg cortex: cortical actin organization.

Several proteins associated with the actin cytoskeleton and cortical dynamics are known targets of SFKs. For instance, cortactin, tensin, focal adhesion kinase (FAK) and paxillin are all phosphorylated by SFKs in somatic cells [282] and phosphorylated FAK localizes to the actin cap in oocytes (Kinsey, unpublished data). Cortical actin caps with associated pTyr proteins also form on the dorsal surface of cultured HELA cells. Inactivation of SFKs in these cells prevents the formation of the actin cap while dissociation of the actin cap causes the loss of the associated phosphotyrosine labeling [346]. Although the mechanisms have not been identified, it is tempting to suggest that the mechanisms are similar between the actin cap of somatic cell and mammalian oocytes. Other important members of the cortex are also known Fyn targets including β-catenin, α-catenin and p120catenin [281]. Phosphorylation of β-catenin causes it's dissociation from the cell cortex.

Interestingly, β-catenin associates to the cortex surrounding the MII oocyte but is distinctly absent from the region of the actin cap (McGinnis, unpublished data). SFKs also bind the microtubule associated protein Tau. This binding causes an increase in SFK regulation of cortical actin dynamics [347]. This correlation of SFKs with proteins associated with both microtubules and actin are tantalizing. It would be interesting to know if these same associations control the apparent effects between the meiotic spindle, metaphase chromatin and cortical actin dynamics in the mammalian oocyte. Determining what oocyte proteins bind directly with SFKs and which proteins are phosphorylated in response to SFK activation would form a foundation towards solving the riddle of SFK control of oocyte maturation and embryonic development.

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