Aurora Kinase-A Regulates Microtubule Organizing Center (MTOC) Localization, Chromosome Dynamics, and Histone-H3 Phosphorylation in Mouse Oocytes

JUN DING,[1] JASON E. SWAIN,[1,2] AND GARY D. SMITH[1,2,3,4]*

[1] Department of Obstetrics and Gynecology, University of Michigan, Ann Arbor, Michigan

[2] Reproductive Sciences Program, University of Michigan, Ann Arbor, Michigan

[3] Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, Michigan

[4] Department of Urology, University of Michigan, Ann Arbor, Michigan

SUMMARY

Aurora kinases (AURKs) are conserved serine/threonine kinases, crucial in regulating cell cycle events. Mammalian oocytes express all three *Aurk* isoforms throughout meiosis, with *AurkA* being the predominant isoform. Inhibition of all AURK isoforms by pharmacological means disrupts oocyte meiosis. Therefore, *AurkA* short interfering RNA (siRNA) was performed to silence *AurkA* gene expression in mouse oocytes and to further assess the function of *AurkA* during meiosis by analyzing subsequent loss-of-function oocyte phenotypes. Results indicated that *AurkA* siRNA applied in our experiments specifically knocked down both *AurkA* gene and protein expression without influencing transcript levels of *AurkB/AurkC* and other endogenous protein expression, such as GAPDH and ERK-2. AURKA was not essential for resumption of meiosis, but it potentiated oocyte meiotic progression. Knockdown of *AurkA* led to a significant reduction in the number of oocytes proceeding to metaphase II (MII). *AurkA* siRNA resulted in abnormal spindle assembly, improper localization of microtubule organizing centers (MTOCs) and misalignment of chromosomes in metaphase I (MI) oocytes. Co-immunoprecipitations demonstrated that AURKA was physically associated with phospho-Histone H3 ser10 in meiotic oocytes. *AurkA* siRNA dramatically reduced Histone H3 ser10 phosphorylation, but not ser28, and resulted in a significant increase of abnormal chromosome segregation in MII oocytes. In conclusion, as a predominant isoform among *Aurks* in oocytes, *AurkA* plays critical roles in mouse oocyte meiosis by regulating spindle and chromosome dynamics.

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* Corresponding author:
  6428 Medical Science I
  1150 W. Medical Center Drive
  Ann Arbor, MI 48109-0617.
  E-mail: smithgd@umich.edu

INTRODUCTION

Aurora kinases (AURKs) are an evolutionary conserved serine/threonine kinase family that plays a pivotal role in regulating cell cycle dynamics. In mitosis, AURKs are directly involved in regulation of mitotic entry, centrosome

Abbreviations: AURK, aurora kinase; dsRNA, double-stranded RNA; GVI, germinal vesicle-intact; GVBD, germinal vesicle breakdown; IBMX, isobutyl-methylxanthine; MI, metaphase I; MII, metaphase II; MTOC, microtubule organizing center; RNAi, RNA interference; RISC, RNA-induced silencing complex; siRNA, short interfering RNA.

maturation and separation, mitotic bipolar spindle assembly, chromosome segregation and cytokinesis (Crane et al., 2003; Ducat and Zhen, 2004; Meraldi et al., 2004). Three AURK isoforms, A, B, and C, are characterized in mammals based on their distinct subcellular localization and function in the cell cycle (Crane et al., 2003). AURKB localizes to chromosome kinetochores and the midbody during mitosis, and appears essential for chromosome condensation and cytokinesis (Ditchfield et al., 2003; Goto et al., 2003). AURKC is a chromosome passenger protein (Li et al., 2004) that associates with AURKB and survivin in mitotically dividing cells (Yan et al., 2005). Finally, AURKA localizes to centrosomes and spindle poles, and is primarily responsible for centrosome maturation and separation, and for bipolar spindle assembly during mitosis (Marumoto et al., 2005; Ulisse et al., 2006). In addition, AURKA regulates other important mitotic events, such as spindle checkpoint, mitotic entry, kinetochore function, cytokinesis, cell asymmetric division and cell fate determination (Barr and Gergely, 2007). Both depletion and overexpression of *AurkA* results in abnormal mitosis, and leads to genomic instability, aneuploidy, and tumor formation (Anand et al., 2003; Lu et al., 2008).

Though defined roles for AURKs exist for mitotically dividing cells, their functional roles during oocyte meiosis are just emerging. Accumulating evidence demonstrates that AURKs regulate meiotic progression, spindle dynamics and chromosome remodeling in both lower vertebrate and mammalian oocytes (Swain et al., 2008; Uzbekova et al., 2008; Lane et al., 2010). All three *Aurk* isoforms are expressed in mammalian oocytes throughout meiotic progression (Swain et al., 2008; Uzbekova et al., 2008; Shuda et al., 2009). Inhibition of all three oocyte AURKs using inhibitor ZM447439 results in meiotic arrest at a metaphase I (MI)-like stage, displaying irregular spindle formation, inhibition of histone H3 phosphorylation and abnormal chromosome condensation (Swain et al., 2008; Eichenlaub-Ritter, 2009). However, little information exists regarding the function of individual *Aurks* during mammalian oocyte meiosis. A recent paper using mRNA microinjection describes a role for AURKC in mouse oocytes (Yang et al., 2010) and two papers have attempted to delineate roles for AURKA. Yao et al. (2004) utilized antibody neutralization in mouse oocytes and found that AURKA inhibition interfered with oocyte meiotic resumption and distorted MI spindle organization. Saskova et al. (2008) reported that overexpression of AurkA led to the formation of an abnormal MI spindle, while reduction of *AurkA* via long double-stranded RNA (dsRNA) interfered with oocyte meiotic resumption and spindle assembly. Although AURKA was demonstrated to co-localize with MTOCs and chromosomes during oocyte meiosis (Yao et al., 2004; Saskova et al., 2008), no detailed information regarding the impact of *AurkA* on MTOCs and chromosome dynamics during oocyte meiotic progression were reported, and the specificity of previously used knockdown approaches is also questionable.

One method to determine function of specific intracellular regulators is RNAi. RNAi is a procedure that leads to gene silencing through degradation of the target transcript (Fire et al., 1998; Hammond et al., 2000). This mechanism is initiated by dsRNA homologous to the gene being silenced. The dsRNAs are processed by an enzyme named Dicer to generate duplexes of 21 nt with 3′-overhangs called small interfering RNA, or siRNA. The siRNA assembles into RNA-induced silencing complexes (RISCs) and directly mediates the sequence-specific mRNA degradation (Meister and Tuschl, 2004; Mello and Conte, 2004). Though fully grown oocytes progressing through meiosis historically have been thought to be transcriptionally inert, it has been confirmed that synthetic siRNA can be delivered manually into oocytes during this developmental period to silence specific genes (Svoboda et al., 2000; Wianny and Zernicka-Goetz, 2000; Wang et al., 2007; Solc et al., 2008). However, validation of specificity is crucial before specific functions can be attributed to various genes, especially in closely related genes such as the three *Aurk* isoforms.

To begin to delineate *Aurk* isoform-specific function during mammalian oocyte meiosis, *AurkA* siRNA was microinjected into mouse oocytes to silence gene expression, and specificity of the approach was thoroughly validated to ensure no impact on other *Aurk* isoforms. The functions of *AurkA* during mouse oocyte maturation was then determined by examining loss-of-function phenotypes, focusing on microtubule organizing center (MTOC) arrangement, spindle organization, and chromosome dynamics.

RESULTS

AurkA siRNA Specificity and the Influence on Oocyte Meiotic Progression

AurkA siRNA specificity was determined on both *AurkA* transcriptional and translational levels in germinal vesicle-intact (GVI) oocytes just before re-initiation of meiosis with IBMX withdrawal after 24 hr incubation. The real-time PCR analysis demonstrated that, in comparison to control oocytes, *AurkA* siRNA could specifically knockdown *AurkA* transcript levels 59- to 67-fold (upper 98% efficiency) without significantly affecting the other two *Aurk* isoforms (*AurkB* and *AurkC*) (Fig. 1A); and Western blot analysis further confirmed that AURKA protein expression levels were dramatically reduced in oocytes before meiotic resumption by *AurkA* siRNA, without significantly influencing other oocyte endogenous protein expression, represented by GAPDH and ERK2 assessments (Fig. 1B).

To determine if *AurkA* knockdown impacts oocyte meiotic resumption and progression, oocytes undergoing GVBD and development to metaphase II (MII) in control and *AurkA* siRNA groups were compared at 2 and 17 hr after release from meiotic arrest, respectively. Results demonstrate that *AurkA* knockdown had no significant influence on oocyte meiotic resumption or GVBD. Rates of GVBD were 94%, 95%, and 90% for buffer (n = 358), non-targeted siRNA (n = 132), and *AurkA* siRNA (n = 355), respectively (Table 1). However, *AurkA* knockdown significantly reduced the rate of MII development (58%; $P < 0.05$)
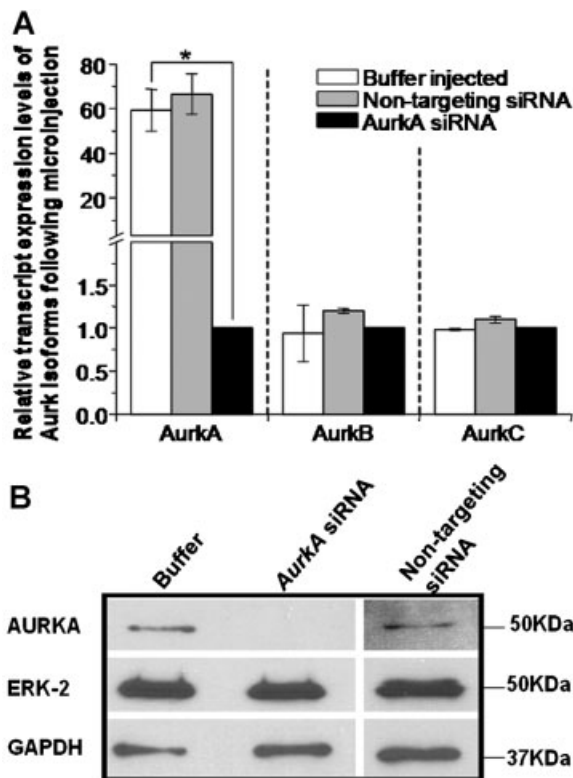
Figure 1. A: *AurkA* transcript level was significantly reduced in *AurkA* siRNA injected oocytes compared with buffer and non-targeting siRNA injected controls ($P < 0.01$), whereas *AurkB* and *AurkC* transcript levels are not significantly influenced. Data are presented as mean $\pm$ SEM and statistical significance was determined using unpaired Student's *t*-test, $P < 0.01$. Oocyte samples were collected at the GVI stage just before re-initiation of meiosis with IBMX withdrawal. **B**: Representative Western blot analysis demonstrating that AURKA protein expression was significantly knocked down by *AurkA* siRNA, but not with non-targeting siRNA or buffer injection. Equal numbers of GVI oocytes were loaded (n = 130 each lane). Experiments were repeated three times.

compared to buffer (80%) and non-targeting siRNA (81%) microinjection (Table 1).

MTOC Localization, Spindle Assembly, and Chromosome Alignment in Metaphase I Oocytes

Confocal microscopic analysis showed that significantly more (86%) of control MI oocytes (n = 30) possessed regular spindles with aligned chromosomes and bipolar

MTOCs (Fig. 2A) than *AurkA* siRNA MI oocytes (32%, n = 35). Approximately 68% of AurkA siRNA oocytes showed irregular MI spindles with their MTOC improperly localized and their chromosomes misaligned (Fig. 2B, $P < 0.01$). Unlike control oocytes containing irregular MI spindles with the random appearance of mislocalized MTOCs or scattered chromosomes or both improperly localized MTOCs and chromosomes, AurkA siRNA oocyte containing irregular spindles normally accompanied misaligned chromosomes and improperly localized MTOCs. Our additional analysis examining structural components of MTOCs through co-localization of two major components, γ-tublin and pericentrin, further confirmed that *AurkA* knockdown significantly disrupted the bipolar localization of MTOCs and resulted in MTOCs scattered around the spindle area in MI oocytes (Fig. 2C).

Oocyte Chromosome Segregation

AurkA knockdown led to a significant increase in MII oocytes with abnormal chromosome segregation (42% total; 34% hyperploidy +8% hypoploidy, n = 38) compared to controls (25% total; 18% hyperploidy +7% hypoploidy, n = 56, $P < 0.05$; Fig. 3A), and resulted in oocyte aneuploidy (Fig. 3B).

AURKA and Phospho-Histone H3 ser10 During Meiosis and Mitosis

To identify a substrate for AURKA that may explain the observed phenotypic defects following siRNA and to determine if *AurkA* can specifically influence chromosome dynamics up to MII, we analyzed oocyte histone H3 phosphorylation following *AurkA* siRNA. Results demonstrated that *AurkA* knockdown inhibited histone H3 phosphorylation at ser-10, but had no effect on the phosphorylation on the ser-28 (Fig. 4A,B).

To further confirm that AURKA is directly involved in the histone H3 phosphorylation under physiological conditions, coimmunoprecipitation using phospho-histone H3 ser10 antibody was performed in both meiotic oocytes and mitotic KGN cells. Our results demonstrate that AURKA can be specifically coimmunoprecipitated by phospho-histone H3 ser10 antibody in both mitotic KGN cells (Fig. 4C) and in vivo-matured MII oocytes (Fig. 4D), while other proteins, like β-tubulin, could not be coimmunoprecipitated under the same experimental condition. Since knockdown of AURKA caused inhibition of histone H3 ser10 phosphorylation, we concluded that AURKA acts as a histone H3 ser10 kinase in oocytes.

TABLE 1. Comparison of Germinal Vesicle Breakdown and MII Development in Control and AurkA Knockdown Oocytes

	GVBD $\pm$ SEM (%) 2 hr after meiotic resumption	MII $\pm$ SEM (%) 17 hr after meiotic resumption
Buffer control oocytes (n = 358)	94 $\pm$ 1.7[a]	80 $\pm$ 3.0[b]
Nontargeting siRNA oocytes (n = 132)	95 $\pm$ 0.84[a]	81 $\pm$ 1.6[b]
AurkA siRNA oocytes (n = 355)	90 $\pm$ 1.7[a]	58 $\pm$ 3.0[c]

All data were averaged and based on at least three independent experiments and expressed as a mean $\pm$ SEM. Data were statistically analyzed by unpaired Student *t*-test. Different superscripts (a, b, and c) within a column indicate significant differences between treatments, $P < 0.05$.
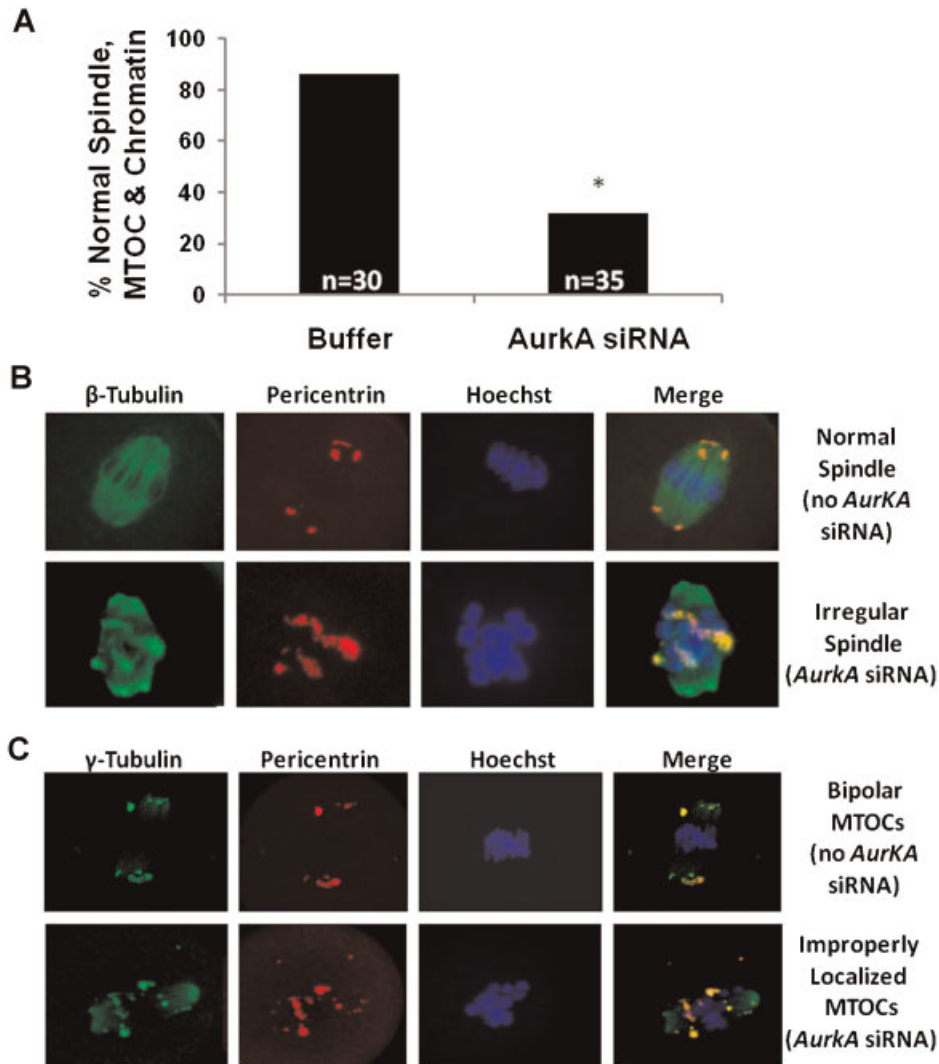
Figure 2. A: *AurkA* knockdown in mouse oocytes results in a significant reduction in normal spindle and chromatin formation/alignment in MI oocytes (86% vs. 32%). Statistical significance was determined using Chi-square analysis, $P < 0.01$. **B**: Representative micrographs resulting from confocal microscopic analysis demonstrating *AurkA* knockdown lead to improper localization of MTOCs (pericentrin, red), misaligned chromosomes (Hoechst, blue) and irregular shaped spindles (β-tubulin, green) in MI oocytes cultured for 7 hr. **C**: Additional analysis on impact of *AurkA* siRNA on MTOC structure. Representative micrographs using confocal microscopic analysis examining co-localization of key structural proteins, γ-tubulin (green) and pericentrin (red), with and without *AurkA* siRNA.

DISCUSSION

AURKA has been localized to nuclei in GV-intact mouse oocytes; co-localized with MTOCs and condensing chromosomes at prophase oocytes; found at the spindle pole, MTOCs and condensed chromosomes in MI oocytes (Yao et al., 2004; Saskova et al., 2008); and further concentrated to spindle poles at MII oocytes (Yao et al., 2004). Recent comparative studies on levels of three *Aurk* isoform transcripts demonstrated that *AurkA* was the predominant form in meiotic competent bovine and mouse oocytes, and its transcript levels were much higher than *AurkB* and *AurkC*

(Swain et al., 2008; Uzbekova et al., 2008; Shuda et al., 2009). These data suggest a crucial role of *AurkA* among the three isoforms in regulating the progression of oocyte meiosis in mammals.

In this study, mouse *AurkA* sequence-specific siRNA was utilized to assess the function of *AurkA* during mouse oocyte meiosis. Importantly, results demonstrated that *AurkA* siRNA could specifically inhibit *AurkA* gene transcription and translation without influencing *AurkB*, *AurkC* transcript levels. Furthermore, because no AURKB or AURKC antibodies could be found to give reliable results in mouse oocytes, we also utilized GAPDH and ERK-2 antibodies as
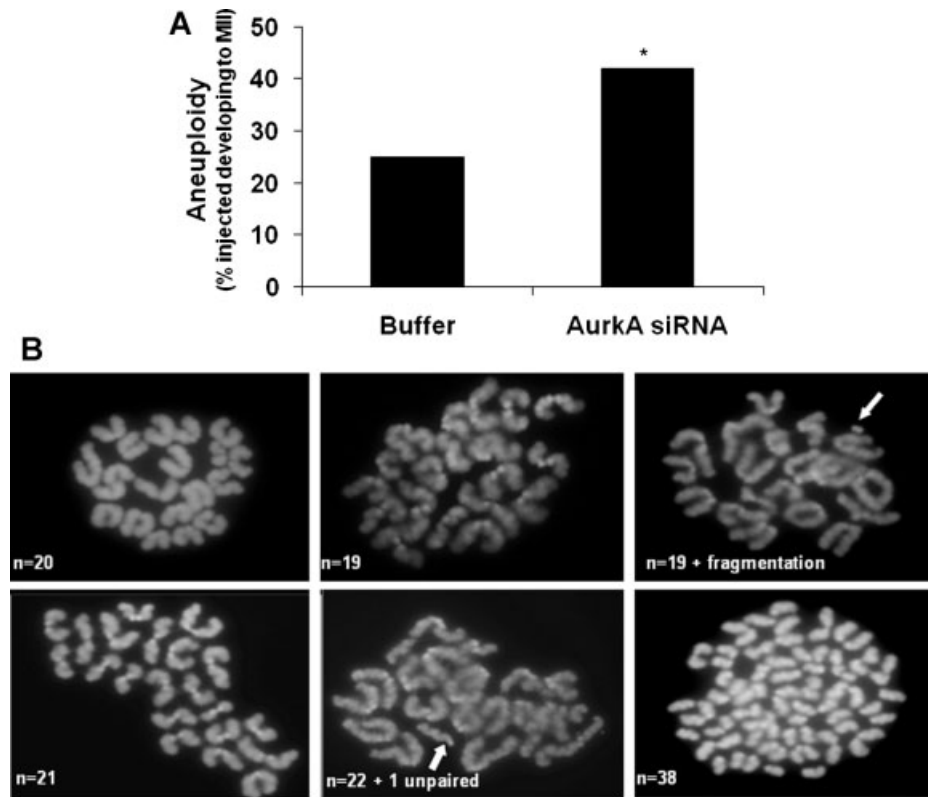
Figure 3. A: *AurkA* knockdown significantly inhibits normal chromosome segregation in oocytes, as 42% of MII oocytes displayed abnormal chromosome complement compared to 25% of controls. Statistical significance was determined using Chi-square analysis, $P < 0.05$. **B**: Representative chromosome spread micrographs demonstrating the aneuploidies observed in MII oocytes cultured for 17 hr after *AurkA* knockdown (n = number of chromosomes in image).

indicators to verify that endogenous protein levels in our experiment were not impacted by AurkA siRNA. In contrast, two prior studies examining *AurkA* function in oocytes using antibody neutralization (Yao et al., 2004) and long double strand *AurkA* RNAi (Saskova et al., 2008), failed to ensure that the antibody and dsRNAi applied in their experiments were *Aurk* isoform-specific and had no impact on overall endogenous protein expression. For instance, antibody neutralization studies utilized rabbit IgG injected oocytes as controls, but no further experiments were performed to confirm the specificity of AurkA antibody (Yao et al., 2004) while AurkA RNAi studies used GFP dsRNA as controls but did not confirm whether AurkA dsRNA impacted transcript levels of Aurk B and C (Saskova et al., 2008). Lack of specificity in prior studies may be one explanation for observed differences between studies. Use of rescue studies utilizing *AurkA*, similar to those conducted with *AurkB* (Shuda et al., 2009), could help validate findings.

Developmental data demonstrated that *AurkA* is not essential for spontaneous oocyte meiotic resumption (e.g., GVBD). Though both *AurkA* transcript and protein in GVI oocytes before meiotic resumption were significantly reduced after 24 hr *AurkA* siRNA process, oocyte meiotic resumption/GVBD did not differ significantly from controls.

This result is in agreement with the findings that inhibition of bovine and mouse oocyte AURKs with inhibitors VX680 or ZM447439, which inhibit all AURKs, had no significant impact on oocyte GVBD (Swain et al., 2008; Uzbekova et al., 2008). This is also well supported by the recent report that overexpression of *AurkA* could not override the inhibitory effect on oocyte meiotic resumption caused by high cAMP level (Saskova et al., 2008), while other essential factors (cdc25A and cdc25B) could promote GVBD under similar culture conditions (Solc et al., 2008). However, these results are in contrast to the previous work presented by Yao et al. (2004) and Saskova et al. (2008) who demonstrated that microinjecting AURKA antibody or *AurkA* long double strand RNA in mouse oocytes inhibited oocyte GVBD. As mentioned, variations in technique or lack of specificity of antibody or RNAi could explain these differences.

Though *AurkA* knockdown had no impact on meiotic resumption/GVBD at 2 hr, *AurkA* siRNA did lead to a significant reduction in the number of oocytes proceeding to MII and extruding the first polar body after 17 hr of in vitro maturation. This result is consistent with other studies examining roles for all *Aurks* or *AurkA* in oocytes (Yao et al., 2004; Saskova et al., 2008; Swain et al., 2008; Uzbe-
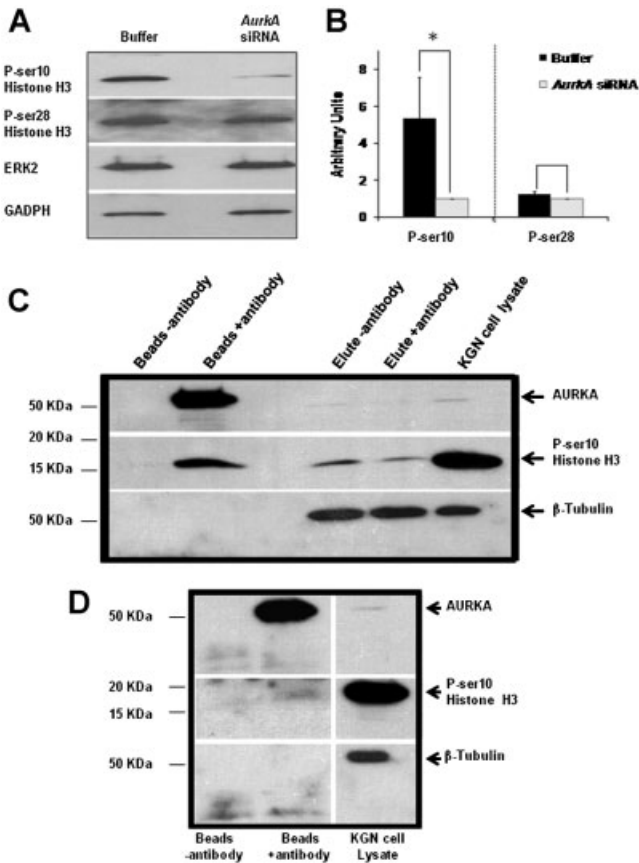
Figure 4. A: Representative Western blot and (**B**) desitometric analysis demonstrating that *AurkA* knockdown significantly inhibited the phosphorylation of histone H3 at ser-10, but not at ser-28. Equal numbers of oocytes (n = 120) were loaded in each lane and the membrane was stripped and re-probed for ERK-2 and GAPDH. Experiments were repeated three times. **C,D**: Representative co-immunoprecipitation results demonstrating that AurkA can be specifically co-immunoprecipitated by phospho-histone H3 ser10 antibody in (C) KGN cells and (D) MII oocytes (n = 283/lane), while β-tubulin cannot.

kova et al., 2008). This inhibition may be due to direct effects on chromosomes or to regulation of the meiotic spindle and its components during earlier stages of meiosis, such as microtubules or MTOCs.

Previous reports indicate that *AurkA* directly regulates centrosome functions in mitotic cells (Crane et al., 2003; Barr and Gergely, 2007). In mouse oocytes, there are no centrosome structures with centrioles, rather MTOCs are reported to function like centrosomes (Schuh and Ellenberg, 2007). AURKA co-localizes with MTOCs during the entire meiotic process in mouse oocytes (Yao et al., 2004; Saskova et al., 2008). This tight structural connection suggests that AURKA may also be functionally associated with MTOCs during oocyte meiosis. Our siRNA studies demonstrated that *AurkA* knockdown disrupts MTOC location in MI oocytes. To further explore the influence of *AurkA*

on MTOCs, we stained MI oocytes for two MTOC proteins, gamma tubulin and pericentrin, and examined MTOC organization. Our confocal microscopic analysis is the first evidence demonstrating that highly specific *AurkA* siRNA in mouse oocytes indeed disrupts the proper localization of these key MTOC proteins. Instead of bipolar localization, MTOCs in *AurkA* knockdown oocytes were scattered in the middle part of the spindle area. Recent studies indicated that MTOC self-organization and polar migration or pole ejection was critical to bipolar spindle assembly in mouse oocytes (Schuh and Ellenberg, 2007). Therefore, this improper localization of MTOCs might cause the aberrant spindle assembly in MI *AurkA* siRNA oocytes. Interestingly, evidence suggests that AURKA is an activator of Kinesin-5 (Eg-5) (Crane et al., 2003; Barr and Gergely, 2007), and activation of Eg-5 is a critical regulator of pole ejection and spindle bipolarization in mouse oocytes (Schuh and Ellenberg, 2007).

In addition to its localization with MTOCs, AURKA was also found to co-localize with chromosomes during mouse oocyte meiosis (Yao et al., 2004; Saskova et al., 2008). AURKA initially localized in nuclei before meiotic resumption, then associated with condensing chromatin after GVBD (Yao et al., 2004). Like MTOCs, this structural correlation also suggests a potential role of *AurkA* in regulating chromatin remodeling during oocyte meiosis. In support of this developmental pathway, immunocytochemistry and chromosome spreads demonstrated that *AurkA* siRNA significantly increased the number of oocytes with abnormal spindles and chromosome segregation or misalignment on the metaphase plate. Though we only examined MI development at 7 hr, there is little reason to think that observed alignment defects were simply due to delayed meiosis. No delay was observed in GVBD and observed defects were severe, unlike organizational structuring of the oocyte meiotic spindle at other time points reported in the existing literature.

It should be noted that our chromosome analysis experiment did yield an ~25% aneuploidy rate in vehicle microinjected control oocytes, which is much higher than those in the naturally in vivo matured MII oocytes (Hunt et al., 2003; Liu et al., 2008). As evidenced by the elevated hyperploidy rate, this high aneuploidy rate is not due to chromosome loss (hypoploidy) that sometimes occurs during the fixation process. Possible explanations for the elevated rates of aneuploidy observed in control oocytes include the use of equine chorionic gonadotropin (eCG) and/or follicle stimulating hormone (FSH) treatment, which has been reported to increase MII oocyte aneuploidy (Sato and Marrs, 1986; Roberts et al., 2005). Furthermore, there may be an impact from the act of microinjection as well as 24 hr IBMX incubation. Recent publications, however, also demonstrated no dramatic impact on oocyte aneuploidy after FSH treatment (Duncan et al., 2009; Chiang et al., 2010; Lane et al., 2010) or even microinjection (Reis et al., 2007). Importantly, our current experiment was carefully controlled, as both control and *AurkA* siRNA oocytes were manipulated under the same conditions and the rates of aneuploidy in *AurkA* siRNA MII oocytes were statistically significantly higher

than those in control oocytes. Thus, we conclude that *AurkA* siRNA induced MII oocyte aneuploidy.

In an effort to explain how *AurkA* regulates oocyte chromosome dynamics, histone H3 phosphorylation was investigated. Inhibition of histone H3 phosphorylation, especially histone H3 ser10 and ser28 phosphorylatyion, is essential in controlling chromosome condensation/ segregation in mitotic cells or mammalian oocytes (Wei et al., 1999; Hsu et al., 2000; Bui et al., 2004; Wang et al., 2006; Swain et al., 2007, 2008). Importantly, AURKA is a potent histone H3 ser10 kinase in mitotic cells (Crosio et al., 2002). Our Western blot analysis revealed that *AurkA* knockdown significantly inhibited histone H3 ser10, but not ser28, phosphorylation in oocytes. Our coimmunoprecipitation further demonstrate, for the first time, that AURKA specifically interacts with phospho-histone H3 ser10 in mammalian oocytes. This is interesting, considering that the previous studies using pharmacologic inhibitors of all AURKs inhibited histone H3 phosphorylation on both ser 10 and ser 28 (Swain et al., 2008). We therefore conclude that AURKA is acting as a histone H3 ser10 kinase in meiotic oocytes, while either AURKB or AURKC regulate Ser28 phosphorylation. However, it should be noted that both AURKB and AURKC may also play a role in ser10 phosphorylation. AURKB has been shown to regulate both ser10 and ser28 in mitotic cell extracts (Goto et al., 2003), and its localization to condensed chromatin in oocytes suggests a possible role in meiosis as well. While AURKB has been reported in bovine oocytes (Jelinkova and Kubelka, 2006), conflicting results exists in regard to its presence in mouse oocytes (Shuda et al., 2009; Yang et al., 2010). Previous studies attempting to examine AURKB function in oocytes rely on the use of low doses of broad pharmacologic inhibitors, which inhibit all AURKs and thus make it impossible to designate any specific roles for AURKB (Jelinkova and Kubelka, 2006; Shuda et al., 2009; Lane et al., 2010), though over expression of *AurkB* did rescue defects in chromosome alignment following broad AURK inhibition using ZM447439 (Shuda et al., 2009). Additionally, AURKC was found to regulate histone H3 ser10 phosphorylation during mouse oocyte meiosis, though the specificity of the AURKC knockdown approach was not validated and the impact on AURKA levels were not examined (Yang et al., 2010). Thus, it is possible that a redundancy system exists, which permit multiple AURKs to regulate ser10 phosphorylation. It is also important to note that other relevant substrates likely exist that help explain observed chromosomal defects following *AurkA* knockdown. AURKA phosphorylates several other proteins, such as the kinetochore-specific protein CENP-A in mitotic cells due to a sequence motif similarity to the phosphorylation motif of histone H3 ser10 (Zeitlin et al., 2000). Inhibition of CENP-A phosphorylation resulted in chromosomal misalignment on metaphase plate in mammalian cells (Cleveland et al., 2003; Kunitoku et al., 2003). Future studies examining additional substrates of AURKA may be informative.

In summary, these studies describe functional roles for *AurkA* in mouse oocyte meiosis utilizing a highly specific siRNA approach. *AurkA* did not appear to be essential for oocyte meiotic resumption; however, it did play a crucial role in regulating mouse oocyte meiotic progression, with reduced AURKA levels disrupting spindle assembly, chromosomal alignment, and normal chromosome segregation when protein levels were reduced. This is the first report demonstrating a critical role for *AurkA* in proper oocyte MTOC organization/localization, as well as being a histone H3 kinase that regulates histone H3 activity through ser10 phosphorylation. Because of the apparently high overlap between all 3 *Aurk* isoforms in their targets and observed phenotypes, future studies must be careful to confirm specificity of knockdown approaches. Future studies will focus on additional substrates of *AurkA* involved in regulation of MTOC localization/organization.

MATERIALS AND METHODS

All procedures described within were reviewed and approved by The University Committee on Use and Care of Animals at the University of Michigan, and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

Mouse Stimulation and Oocyte Collection

Female CF-1 mice (Harlan, Indianapolis, IN), 20–21 days old, were primed with 5 IU equine chorionic gonadotropin (eCG) (Sigma, St. Louis, MO), and fully-grown, meiotically competent germinal vesicle intact (GVI) oocytes were collected after 42–44 hr hormone stimulation. Cumulus-enclosed oocytes were isolated by manually rupturing of antral follicles in human tubual fluid + Hepes media (HTFH; Irvine Scientific, Santa Ana, CA) supplemented with 0.3% bovine serum albumin (BSA) and $40\,\mu M$ isobutylmethylxanthine (IBMX) (Sigma) to maintain meiotic arrest for subsequent siRNA. Oocytes were then denuded, washed, and kept in HTF/0.3% BSA/$40\,\mu M$ IBMX at $37^{\circ}C$, 5% CO_2 and air incubator prior to microinjection.

AurkA siRNA Designing, Synthesis, Preparation, and Microinjection

AurkA siRNA was designed based on the NCBI mouse *AurkA* sequence (NM_011497.2) using Dharmacon Customer Chicago, IL siRNA Design Tool and synthesized by Dharmacon (Chicago, IL) siRNA Technologies. 5′ATACGTACCAGGAGACTT Att 3′; 3′ttTATGCATGGTCC ttTATGCATGGTCC T CTGAAT 5′. The nontargeting, scrambled siRNA was purchased from Dharmacon (D001210-01-05). Both *AurkA* siRNA and nontargeting siRNA were reconstituted in Dharmacon siRNA buffer to a final concentration of $20\,\mu M$ and stored at $-80^{\circ}C$ until utilization.

Approximately 20 pl *AurkA* siRNA, nontargeting siRNA or Dharmacon siRNA buffer was microinjected into the cytoplasm of denuded GVI oocytes, and cells were cultured for 24 hr in HTF/0.3% BSA/$60\,\mu M$ IBMX to prevent meiotic progression and to allow for siRNA processing. The meiotic inhibitor IBMX was removed after triple washing in HTFH + 0.3% BSA media, and oocytes were allowed to proceed with meiosis in HTF + 0.3% BSA in a 5% CO_2 incubator for 2 hr

to assess germinal vesicle breakdown (GVBD), 7 hr to assess metaphase I (MI) development, and 17 hr to categorize metaphase II (MII) development. Data were collected from three different individual experiments. The number of GVBD and MII oocytes was calculated and analyzed statistically.

Total RNA Isolation, Reverse Transcription, and Real-time PCR

Total RNA was extracted from 20 oocytes in buffer, nontargeting siRNA and *AurkA* siRNA microinjected groups respectively using Picopure RNA Isolation kit (Arcturus Bioscience, Mountain View, CA) following manufacturer's instructions. Oocyte cDNA was synthesized using 125 pmol random hexamer, 500 mM dNTP, 20 IU RNase inhibitor and 62.5 IU MultiScribe™ reverse transcriptase (ABI systems) in a final volume of 50 μl. Primers for mouse *AurkA*, *AurkB*, and *AurkC* were designed with no sequence overlap between isoforms (Swain et al., 2008). Real-time PCR was performed on Applied Biosystems 7300 Real-Time PCR system using SYBR Green PCR Master Mix (Applied Bio-Systems, Foster City, CA). Each PCR reaction was performed with 1.5 oocyte equivalents of cDNA. Real-time PCR reactions were carried out for 40 cycles (95°C for 15 sec, 60°C for 1 min) after initial 10 min incubation at 95°C. Data were collected over three replicates with triplicate samples for each isoform. Fold decreases were based on the transcript level of *AurkA* in siRNA microinjected oocytes, which were normalized to 1. Comparative Ct method was used for data analysis and β-actin was used for internal control. Statistical significance was determined using the unpaired Student's *t*-test.

Western Blot Analysis

Western blot analysis was performed to determine the effects of *AurkA* siRNA on the protein expression levels of AURKA and other downstream signaling molecules. Because nontargeted siRNA and vehicle microinjection were similar in lack of influence on *Aurk* expression and meiotic progression, subsequent studies were performed with vehicle microinjection as control. Equal numbers of MI and MII oocytes in control and *AurkA* knockdown groups were compared. Oocytes were placed in Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, CA) according to manufacturer's instruction, vortexed and placed on ice for 15 min. Following sonication on ice for 10 sec, samples were denatured at 90°C for 10 min and loaded on 12% gel for electrophoresis. Total protein from equal numbers of mouse oocytes in control and *AurkA* knockdown groups was loaded in each lane and separated by one-dimensional SDS–PAGE. Gels were equilibrated and transferred to Hybond-P PVDF transfer membrane (Amersham Life Sciences, Little Chalfont Buckinghamshire, UK) by Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories) according to the manufacturer's instructions. Blots were blocked in 5% nonfat milk in Tris-buffered saline (TBS) with 0.1% Tween (TBST) at room temperature

for 1 hr, and incubated with the appropriate primary antibody diluted in TBST/5% BSA at least overnight at 4°C with agitation. The antibodies included anti-AURKA (1:500, Cell Signaling, Danvers, MA), anti-phospho-ser10-histone H3 (1:500, Cell Signaling), anti-phospho-ser 28-histone H3 (1:500, Upstate, Billerica, MA), anti-ERK-2 (1:1,000, Santa Cruz, Santa Cruz, CA) and anti-GAPDH (1:1,000, Novus, Littleton, CO). After complete washing in TBST, blots were incubated with appropriate peroxidase-conjugated IgG secondary antibody (Amersham Life Sciences) at room temperature for 2 hr, washed in TBST and developed with ECL Plus reagents (Amersham Life Sciences) according to the manufacturer's instructions. Membranes were re-probed using different primary antibodies after stripping at 50°C for 30 min in stripping buffer (62.5 M Tris–HCl, pH 6.7, 2% SDS, and 100 mM β-mercaptoethanol).

Immunocytochemistry and Confocal Microscopic Analysis

To examine the effect of *AurkA* siRNA on spindle formation, MTOC localization and chromosomal alignment, MI oocytes in control and knockdown groups were attached to poly-lysine coated coverslips separately, fixed in 2% (w/v) paraformaldehyde for 1 hr at 37°C, blocked overnight with PBST/0.3% BSA at 4°C and incubated with primary antibodies at 37°C for 1 hr. Antibodies used for experiments included anti-β-tubulin to visualize microtubules (Sigma, 1:200); anti-pericentrin (Covance, Princeton, NJ, 1:200) and γ-tubulin (Sigma, 1:100) to visualize MTOCs. Chromatin was stained with Hoechst 33342 (5 μg/ml). After washing of primary antibodies, samples were reacted with the appropriate Alexa568 and 488 conjugated secondary antibodies (Molecular Probes, Carlsbad, CA) at a 1:1,000 dilution for 1 hr at 37°C. Coverslips were then mounted on glass slides for visualization under 1,000× confocal microscope (Olympus Fluoview 500). For confocal microscopic analysis, Z-stacks were captured with 0.3 μm steps through the entire spindle, MTOC, and chromosome area in each oocyte, then images were reconstituted via Fluoview software. Images were taken and graded in blinded fashion as normal or abnormal. Criteria included normal shaped barrel spindle formation, proper localization of chromatin on the metaphase plate, and proper localization of MTOC proteins (pericentrin and γ-tubulin) at the spindle poles.

Chromosome Analysis

Metaphase II oocytes matured for 17 hr in control and knockdown groups were collected and chromosomal spreads prepared using 1% paraformaldehyde method (Hodges and Hunt, 2002) with slight modification. All treatments from the same day were prepared side-by-side to control for variability. Briefly, zona pellucidae of oocytes were removed by 0.6% proteinase K in PBS. Zona-free oocytes were then washed, treated with hypotonic buffer (0.075 M KCl) for 5 min, and fixed onto a microscope slide dipped in the solution of 1% paraformaldehyde/3 mM DTT/0.15% TritonX-100 (pH 9.2). Slides were then placed into

humidified chambers overnight, air dried at room temperature, stained by 5 µg/ml Hoechst 33342 for 5 min, triple washed with 1 × PBS and mounted in glycerol mounting solution. Chromosomal spreads were analyzed at 1,000× on a Leica DMR microscope and images taken so that the chromosome numbers could be counted in a treatment blinded fashion. Numbers of chromosomes were counted to determine segregation errors and data was statistically analyzed.

Co-Immunoprecipitation

To determine if AURKA was directly involved in the phosphorylation of histone H3 under physiological condition in cells and oocytes, co-immunoprecipitation experiments using phospho-histone H3 (ser10) antibody (Cell Signaling) were performed in MII oocytes and the human granulosa-like tumor cell line, KGN, following manufacture's instruction. Briefly, MII oocytes (n = 566) were collected from superovulated CF1 mice (8 weeks old) and KGN cells cultured in 75 cm^2 flask were harvested at their 90% confluence. Both cells were triple washed with cold 1 × PBS before transferring to or scrapping off in ice cold 1 × cell lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM Sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na$_3$VO$_4$ with proteinase inhibitor cocktail (Roche, Indianapolis, IN)]. Cells were sonicated on ice five times for 10 sec each, centrifuged at 12,000g for 10 min at 4°C, and their supernatants were recovered for co-IP experiment. The supernatants were divided into two equal parts and 1 × PBS or phospho-Histone H3 (ser10) antibody were added, respectively, in control or Co-IP groups (1:50 dilution), and incubated overnight at 4°C with gentle rocking. The following day, pre-washed protein A agarose beads (50% slurry, Millipore, Billerica, MA) were added into each group, incubated at 4°C with gentle rocking for 3 hr, then pelleted down at 12,000g for 30 sec at 4°C. The eluted supernatants were kept for Western blot analysis later and protein A agarose beads pellets were washed with 500 µl lysis buffer for four times at 4°C, then 3 × SDS sample buffer added and heated at 95–100°C for 5 min. Western blot analysis was carried out as described. The whole KGN cell lysate was served as non-IP control. Experiments were repeated twice.

Statistical Analysis

Unpaired Student *t*-test and Chi-square analysis was performed, as indicated in results, table or the figure legends, using GraphPad Software. Differences of $P < 0.05$ were considered to be significant.

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