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Endogenous Localization and Expression Patterns of Aurora Kinases B and C in Mouse Oocytes and Early Embryos

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Endogenous Localization and Expression Patterns of Aurora Kinases B and C in Mouse Oocytes and Early Embryos

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Abstract

The Aurora Kinase proteins are a family of serine/threonine kinases that have been shown to play fundamental roles in controlling M phase progression in somatic cells. Aurora Kinase A protein is known to be vital for proper spindle assembly and therefore, chromosome segregation. Previous reports have shown that Aurora Kinase B is vital for proper completion of karyokinesis and cytokinesis in somatic cells. The role of Aurora Kinase C in somatic cells has been found to be less clear; however it appears to play an important role in spermatogenesis. Little is known about the role of these Aurora Kinase proteins mouse oocytes during oogenesis, and even less is known about them in embryos during early development. The objective of these studies was to characterize the presence, localization, and function of Aurora Kinase B and Aurora Kinase C protein and mRNA in mouse oocytes and early embryos.

Oocytes and embryos were collected from hormone stimulated CF-1 mice and cultured for varying amounts of time. Cumulus denuded oocytes were either fixed for immunofluorescence microscopy studies, lysed for analysis of mRNA levels through the use of reverse transcription PCR (rtPCR) and quantitative rtPCR (q-rtPCR), lysed for protein analysis employing Western blotting, treated with Aurora Kinase protein inhibitor drugs, or microinjected with a siRNA pool targeting Aurora Kinase B. Samples were processed for immunofluorescence analysis using markers of spindle morphology (tubulins), Aurora Kinase B, Aurora Kinase C, and Aurora Kinase B activity (phospho Histone H3). Analysis of relative levels of Aurora Kinase B and Aurora Kinase C mRNA were assessed by rtPCR and q-rtPCR methods. Western blotting was performed on oocytes and early embryos to quantitate Aurora Kinase B and C protein levels. Aurora Kinase inhibitors, Hesperadin and ZM447439, were added to culture medium with mouse oocytes to determine the effects of the loss of Aurora Kinase activity. siRNAs were used to inhibit Aurora Kinase B mRNA in early embryos to ascertain the effect of functional loss of this transcript on embryo development.

Marked differences were observed in the localization of Aurora Kinase B when unfertilized oocytes or pre-zygotic genome activation (ZGA) embryos were compared to post-ZGA samples. There was no evidence of Aurora Kinase B protein localized to the mitotic spindle or resultant midbody in oocytes and blastomeres of early embryos. Western blotting results supported this data. Embryos fixed post-ZGA demonstrated Aurora Kinase B localization at midbodies between dividing cells, as was found in mouse embryonic fibroblast control cells. Aurora Kinase C protein was not demonstrable in mouse oocytes, embryos, or control cells using immunocytochemistry or Western techniques. In contrast, Aurora Kinase B and Aurora Kinase C mRNAs were both found to be present in mouse oocytes and early embryos. q-rtPCR data further supported this finding for Aurora Kinase B and revealed that the mRNA level of this transcript is relatively constant until ZGA at which point a decrease relative to the earlier stages was observed. Transcript levels recovered post-ZGA and were comparable to the pre-ZGA levels. Functional inhibition of the Aurora Kinase family through the use of Hesperadin or ZM447439 demonstrated the importance of these proteins for proper microtubule

and spindle organization, as these drugs disrupted both karyokinesis and cytokinesis in mouse oocytes and blastomeres of early embryos. Aurora Kinase B targeting siRNA also established a role for Aurora Kinase mRNA in embryos at the 2-cell stage based on the disruption of the cell cycle that was observed in treated embryos.

Given earlier reports showing the vital role of the Aurora Kinase proteins in proliferating somatic cells, knowledge of the expression and localization of these proteins in oocytes and early embryos is vital for the understanding of cell cycle control during oogenesis and early embryogenesis. Our data indicate that Aurora Kinase B mRNA may also play a role in early embryogenesis, demonstrating a need for analysis of transcript as well as protein. Our results, as well as outcomes of future experiments suggested by our work, may provide significant insight into cell cycle regulation differences between somatic and embryonic cells. These differences may have a profound impact upon manipulated embryos including those reconstructed through somatic cell nuclear transfer.

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Acknowledgements

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Chapter 1: Introduction & Background

The Aurora Kinase family is comprised of 3 serine/threonine kinase proteins in mammals – Aurora Kinase A, B, and C. While each member is a distinct protein, a high degree of homology exists within the family and their functions in the cell are similar, but not entirely complimentary. All three kinases have integral roles in proper assembly of the tubulin spindle, accurate segregation of chromosomes, and completion of cytokinesis to form two daughter cells (Carmena and Earnshaw, 2003; Bolanos-Garcia, 2005). The respective roles of the Aurora Kinases are fairly well established in somatic cells, but far less is known about their function or importance in gametes and early embryo genesis (Carmena and Earnshaw, 2003). Given that cell cycle defects are often observed in early development when oocytes and embryos are grown *in vitro* (Rizos *et al*., 2008), a greater understanding of the roles of Aurora Kinases in these cells may be significant in the pursuit to develop more successful oocyte and embryo manipulation techniques including culture and somatic cell cloning.

The Aurora Kinase Family

The first Aurora Kinase was found in *Saccharomyces cerevisiae* yeast and dubbed "increase in ploidy 1" or Ipl1 due to the uneven chromosome numbers found in mutants (Bolanos-Garcia, 2005). Slightly higher order organisms, such as Drosophila, *C. elegans*, and Xenopus, have evolved two distinct Aurora Kinase proteins, A and B, which function independently from one another. In this group of organisms Aurora Kinase A is most similar in sequence and function to the original yeast Ipl1. Mammals have three Aurora Kinases, known as Aurora Kinase A, B, and C (Carmena and Earnshaw, 2003). The three kinases share activating phosphorylation sites in the T-loops, D-box domains to promote degradation of the proteins at the end of mitosis, and N-terminal regulatory domains that control the activity of the Aurora Kianses (Fig 1, Quintás-Cardama *et al*., 2007). Aurora Kinase A in mammals appears to have maintained many of the same properties found in lower order organisms. Mammalian Aurora Kinases B and C have an extremely high degree of homology, 75% in humans, and seem to share the role of Aurora Kinase B observed in organisms lacking Aurora Kinase C (Quintás-Cardama *et al*., 2007). The high degree of homology shared amongst the mammalian Aurora Kinases is suggestive of their common ancestral background, although they have been found to each play a distinct and important role in the proper progression of M phase (Bolanos-Garcia, 2005).

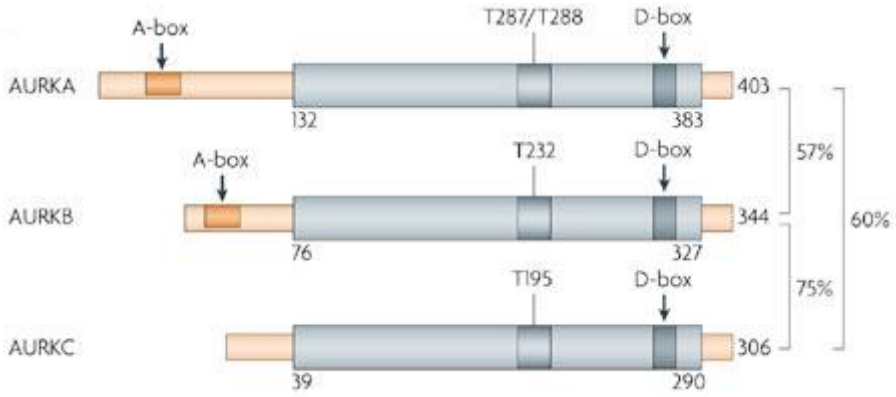

Figure 1: Homology of human Aurora Kinase proteins. Adapted from Quintás-Cardama *et al*., 2007.

Aurora Kinase A has been extensively studied in somatic cells (Bolanos-Garcia, 2005). Human Aurora Kinase A is found on the centrosomes at initiation of mitosis and at the poles of the tubulin spindle once it has been assembled (Fig 2A & B). Observations of Drosophila cells with a mutated form of the Aurora homologue were found to display monopolar spindles. These deformed spindles were deemed responsible for the aberrant chromosome numbers in daughter cells (Glover *et al*., 2005). In mammals the role of Aurora Kinase A is very similar to what has been found in yeast and flies, despite the presence of three distinct Aurora proteins. Aurora Kinase A is known to be found on centrosomes in early M-phase and remains centrosome associated throughout the completion of cell division. Mammalian somatic cells lacking functional Aurora Kinase A exhibit abnormally shaped spindles without two distinct poles, which eventually lead to improper segregation of the chromosomes at anaphase (Gopaland *et al*., 1997).

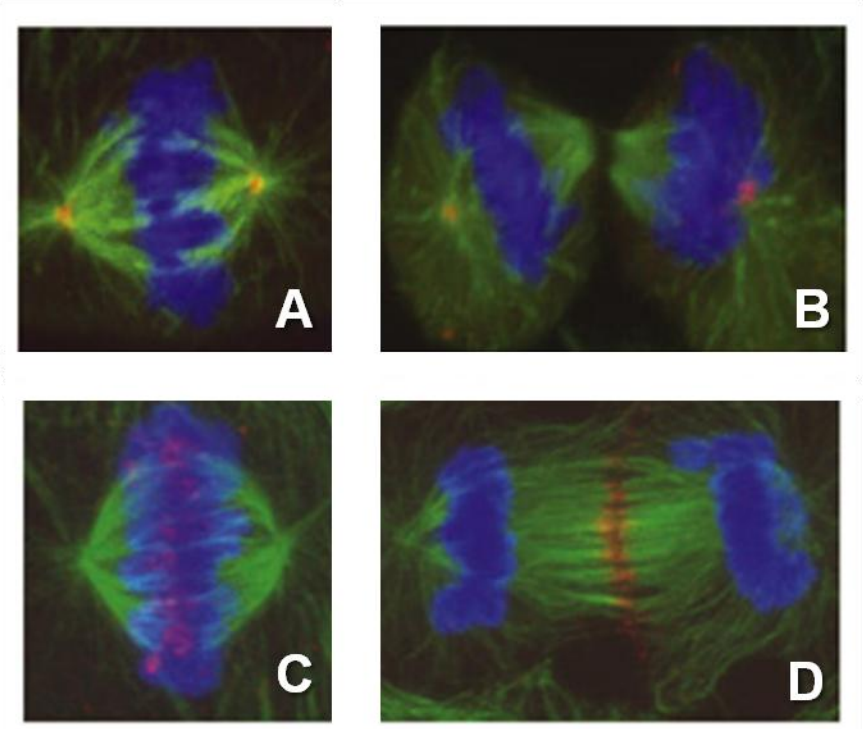

Figure 2: Localization of the Aurora Kinase proteins in somatic cells. Tubulin is shown in green, chromatin in blue. A, Aurora Kinase A is shown in red at the poles of the spindle in a metaphase stage cell. B, Aurora Kinase A is shown in red at the poles of the disintegrating spindle in a late telophase cell. C, Aurora Kinase B is shown in red co-localized with chromatin at the metaphase plate in a metaphase stage cell. D, Aurora Kinase B is shown in red at the center of the spindle in an anaphase cell. Aurora Kinase C is not shown, but is identical to the Aurora Kinase B localization depicted. Adapted from Vader and Lens, 2008.

The role of Aurora Kinase A in mammalian oocytes has been investigated and found to be much the same as in somatic cells (Yao *et al*., 2004). In mouse oocytes prior to MI Aurora Kinase A is found enriched in microtubule organizing centers (MTOCs) and as the spindle is assembled it is then observed at the spindle poles. Levels of Aurora Kinase A expression appear to remain stable, but abundant throughout meiosis (Saskova *et al*., 2008). When the protein was inhibited with monoclonal antibodies a variety of deformed spindles were observed (Fig 3). The majority of spindles appeared to have no poles, and mono-polar and misshapen spindles were also seen. These spindle abnormalities ultimately lead to unequal chromosome migration and aneuploidy in the absence of Aurora Kinase A (Yao *et al*., 2004).

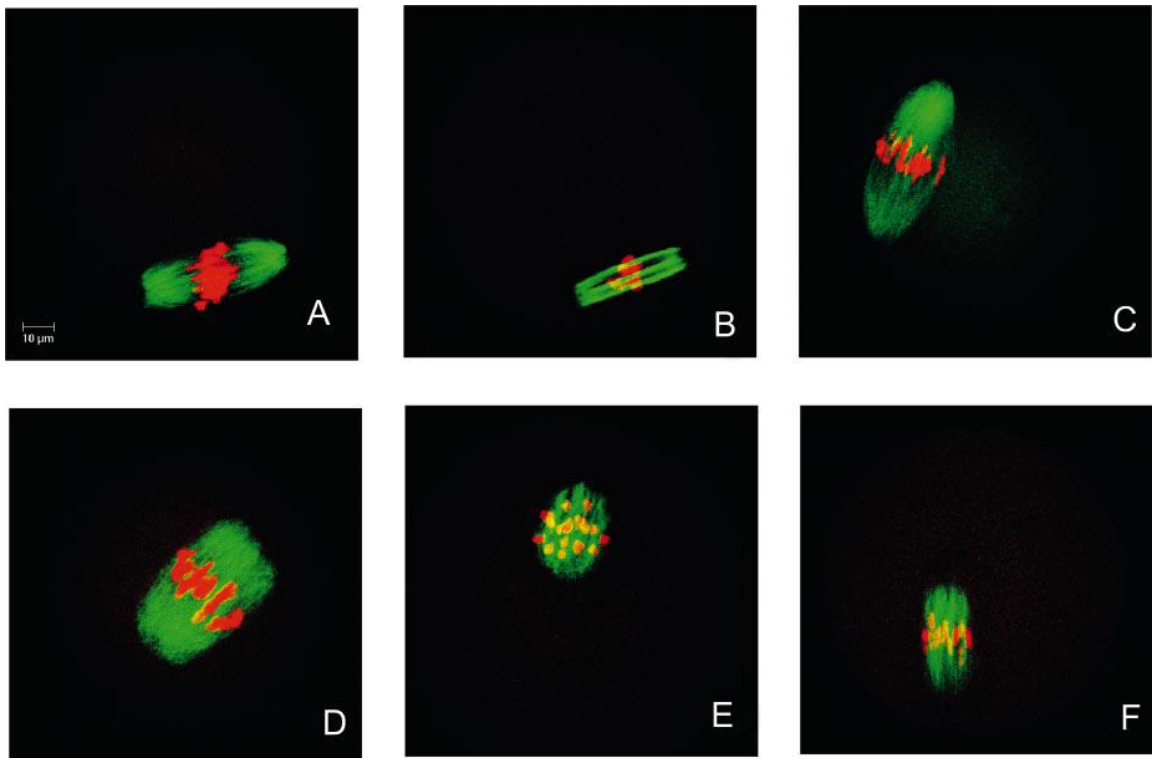

Figure 3: Mouse oocytes microinjected with Aurora Kinase A monoclonal antibody and stained for Tubulin (green) and chromatin (red). A, control oocyte injected rabbit IgG. Normal spindles observed in this group. B-F, experimental oocytes injected with Aurora Kinase A monoclonal antibody. Varied abnormal spindles observed in this group. Adapted from Yao *et al*., 2004.

In somatic cells Aurora Kinase B is an important member of the chromosomal passenger complex (CPC), along with Survivin, INCENP, and Borealin. While the individual members of the complex are responsible for localization, interaction with targets, and complex morphology, Aurora Kinase B is responsible for phosphorylating and activating a wide variety of substrates including serine 10 of Histone H3, mitotic centromere-associated kinesin, and vimentin among others (Vader *et al*., 2006). Immunohistochmistry and Western blotting have shown that Aurora Kinase B protein levels are at their peak in cells that are currently undergoing M phase. Staining shows that it is found on condensed chromosomes in the kinetochore region though metaphase (Fig 2C, Fig 4Ba). At the kinetochore, Aurora Kinase B plays a pivotal role in the progression of M phase by recognizing monotelic and syntelic attachment of sister chromatids to the spindle microtubules. Advancement of mitosis beyond metaphase is prevented until these attachments are corrected (Tanaka *et al*., 2002). After metaphase is exited, Aurora Kinase B protein can be found in the center of the

spindle and on the spindle midbody as cytokinesis occurs (Fig 2D, Fig 4Ca & Da). This dynamic localization has much to do with the protein's several roles as M-phase progresses. A role for Aurora Kinase B in cytokinesis completion has also been demonstrated. Aurora Kinase B is responsible for phosphorylating several proteins at the cleavage furrow including MgcRacGP which is essential for the final steps of cytokinesis (Carmena & Earnshaw, 2003).

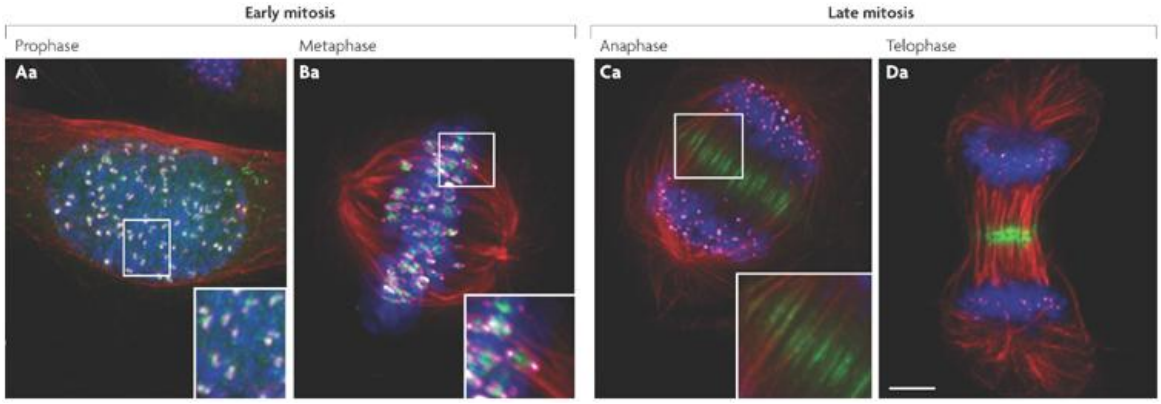

Figure 4: Aurora Kinase B (green) localization in HeLa cells undergoing mitosis. Tubulin (red) and chromatin (blue) are also stained. Scale bar represents 5 µm. Adapted from Ruchaud et al., 2007.

Aurora Kinase B has been studied in the oocytes of several species. Through the use of a fluorescent recombinant Aurora Kinase B protein, the localization of the protein has been studied in mouse oocytes. The protein was found co-localized with centromeres during metaphase I and as meiosis I progressed was found to remain localized to the midbody. Localization was not found to be the same through meiosis I, but rather the protein was distributed throughout the cytoplasm (Shuda *et al*., 2009). In *Xenopus laevis* oocytes, Aurora Kinase B activity, via Histone H3 phosphorylation, was found to rapidly increase after treatment with progesterone and subsequent germinal vesicle breakdown. Although a functional role and localization of the protein was not described, it was determined that the activity of Aurora Kinase B is activated by the presence of INCENP, one of the other CPC proteins (Yamamoto *et al*., 2008). The role of Aurora Kinase B in meiosis has also been studied in oocytes of surf clams (*Spisula solidissima*). It was found that inhibition of Aurora Kinase B through the use of Hesperadin, a pharmaceutical Aurora Kinase inhibitor, resulted in chromatin decondensation, Histone H3 dephosphorylation, chromosome segregation defects in meiosis II, and finally the absence of the formation of the second polar body (Georget *et al*., 2006). Taken as a whole, these studies show some similarities of Aurora Kinase B function and localization in cells undergoing meiosis and mitosis. There are also

several distinct differences noted in the protein's role in meiosis I and II revealing that meiosis and mitosis are not controlled or orchestrated identically.

In comparison to Aurora Kinase A and B, less is known about the roles and functions of Aurora Kinase C. This is in part due to the fact that Aurora Kinase C is very highly homologous to Aurora Kinase B, making it difficult to accurately target in experimental studies. It has been demonstrated in HeLa cells that Aurora Kinase C protein can also be found as a member of the CPC, but is expressed in markedly lower abundance than Aurora Kinase B protein. Aurora Kinase C also has a much lower affinity for the other CPC members, further contributing to its more minor role. In the absence of Aurora Kinase B, Aurora Kinase C can compensate to some degree, but not completely. Some cells depleted of Aurora Kinase B protein are able to successfully divide and perpetuate, while the vast majority fail to perform a proper mitosis. This partial complementation is thought to be partly due to the lack of Aurora Kinase C protein quantity as well as the reduced complex affinity (Sasai *et al*., 2004; Slattery *et al*., 2009). Aurora Kinase C has also been shown to have a role in the development of male gamete cells (Fig 5). Mouse sperm cells formed in the absence of Aurora Kinase C were found to have significantly more defects including decreased chromatin condensation and abnormal morphology than wildtype counterparts (Kimmins *et al*., 2007). A similar role for Aurora Kinase C in mouse oocytes has not been found. It remains unclear if Aurora Kinase C is vital for control of meiosis progression in all cells undergoing the process (oocytes and sperm), or if this activity is found in developing sperm cells alone.

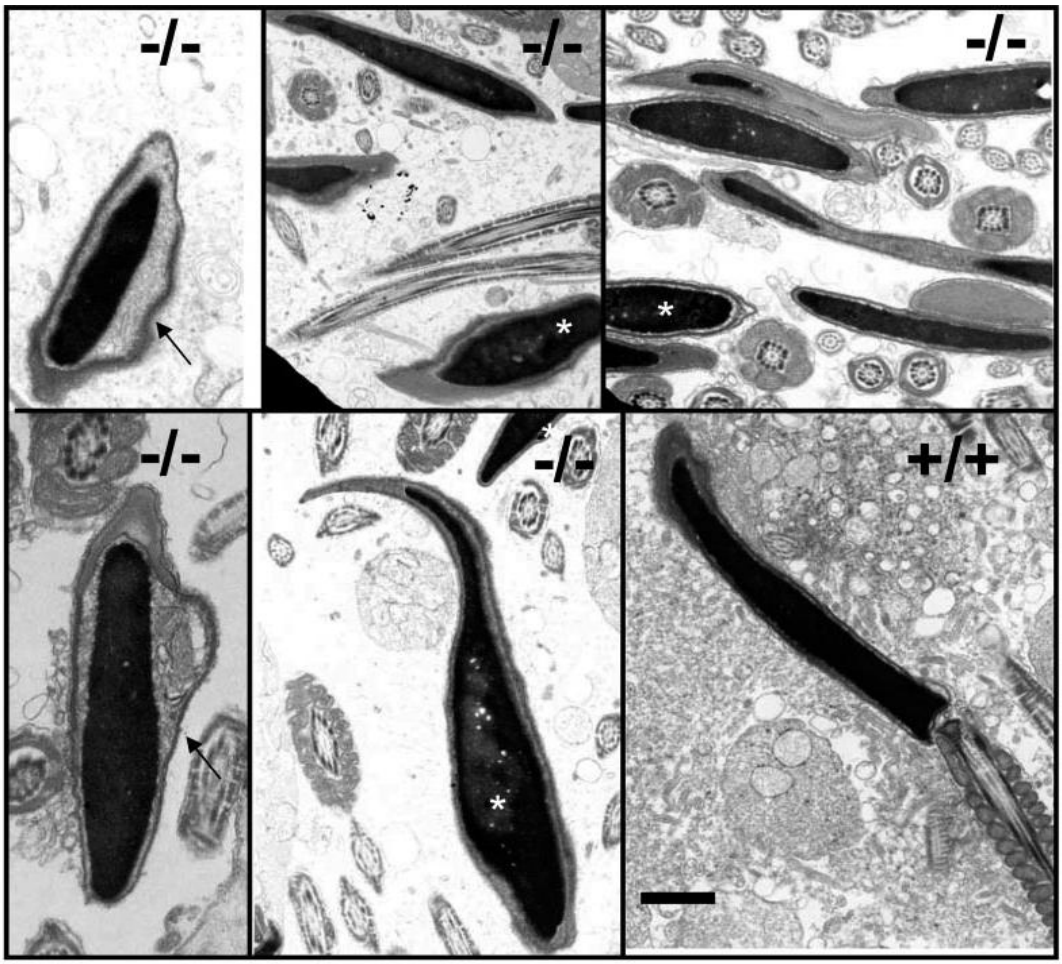

Figure 5: Sperm observed by electron microscopy from either WT (+/+) or Aurora Kinase C mutant (-/-) mice. Asterisks show heterogeneous chromatin condensation, arrows show acrosome/head defects. Scale bar, 0.5 µm. Adapted from Kimmins *et al*., 2007.

Given the integral roles that the Aurora Kinase family proteins play in the process of cell division, it is not surprising that they have been found to be involved in cell cycle defects. Aurora Kinases were first implicated as potential oncogenes in 1998 when Aurora Kinase A was found to be overexpressed in more than 50% of primary human colorectal cancer cells (Bischoff *et al*., 1998). Numerous cancer cell types have since been investigated and all found to have abnormally high levels of Aurora Kinase proteins (Tbl 1). All three human Aurora proteins appear to have an association with cancer as each has been found altered in at least two types of cancer (Kollareddy *et al*., 2008). It is not clear if the upregulation is the cause of the disease or a result of it. Regardless, the Aurora Kinase proteins are currently a popular target for many promising new cancer treatments. The inhibition of the Aurora Kinase proteins through the use of several different pharmaceuticals has proven to be

sufficient to prevent further growth of tumors *in vivo*. Whether this avenue of treatment continues to be advantageous remains to be seen, but for now the Aurora Kinase proteins are of great interest in the fight against cancer (Gautschi *et al*., 2008).

Aurora Kinase	Tumor Type
Aurora Kinase A	Breast Cancer Human Gliomas Ovarian Cancer Prostrate Cancer Cervical Cancer Colon Cancer Pancreatic Cancer Lung Cancer
Aurora Kinase B	Colon Cancer Thyroid cancer Oral Cancer Non small cell lung carcinoma Breast cancer
Aurora Kinase C	Breast Cancer Liver Cancer

Table 1: Over-expression or amplification of Aurora kinases has been found in a wide variety of tumors types, making them attractive targets for cancer therapy. Adapted from Kollareddy *et al*., 2008.

In vitro Manipulations of Mammalian Oocytes and Pre-Implantation Embryos

In vitro maturation (IVM) of oocytes and subsequent culture of preimplantation stage embryos is an important technique for many reasons. The cattle industry relies upon gamete culture for embryo transfer between donors and recipients. This allows offspring to be produced from valuable donor animals without requiring down time for the donor. Superovulation of the donor prior to harvest and culture also makes it possible to produce several offspring per breeding cycle from a female that would normally produce only one oocyte. Any time pre-implantation manipulations of the oocyte or embryo are required, harvest and culture is also a necessity. These manipulations can include genetic screening, sex selection, disease prevention, somatic cell nuclear transfer, and many others. Human assisted reproduction clinics also regularly require successful culture of oocytes and embryos to produce viable embryos for transfer into recipients. Clearly being able to mature oocytes

and culture pre-implantation embryos *in vitro* and maintain viability is an essential technique for many applications related to fertility and infertility. Unfortunately, to date IVM is especially problematic in mammals and results in high loss rates (Hashimoto, 2009).

While *In vitro* maturation of oocytes and pre-implantation embryos is required in the many instances previously mentioned, the technique has not been perfected. Many oocytes and embryos must be discarded because they exhibit signs of cell cycle defects that render them non-viable. These defects can be observed as cell fragmentation, failure of cell cycle progression and lysis of individual cells within the embryo. While cells with these gross morphological abnormalities are easily culled, culture also induces defects that are not easily detected in live samples. This can include aberrant gene expression profiles, post-transcriptional changes, and other flaws that affect viability. The root cause of these defects is not entirely clear, although some gene expression abnormalities have been proven.

Studies have shown that bovine oocytes matured in vitro often give rise to offspring with "abnormal offspring syndrome" (AOS). AOS includes subtle defects such as increased placenta weight and abnormal placental blood vessel development as well as more obvious neonatal development flaws such as increased size and dystocia at birth. AOS is observed in a wide variety of severities, making diagnosis of specific genes involved difficult, especially prior to embryo transfer (Farin *et al*., 2006). Bovine oocytes have also been used to demonstrate the role of the cumulus-oocyte complex (COC) in the development of the oocyte. Oocytes denuded of cumulus cells immediate upon removal from the ovary, as is typically done as oocytes are harvested for research purposes, were shown to have significantly lower levels of luteinizing hormone receptor and follicle stimulating hormone receptor mRNA (Calder *et al*., 2005). Further studies on IVM in macaque oocytes has shown that insulin-like growth factor 2 protein, a growth factor required for early development, and steroidogenic acute regulator protein, a mitochondrial transport protein involved in steroid hormone production, are both significantly reduced in IVM oocytes. The reduction of both of these proteins has been remedied by supplementation with human recombinant growth factor and developmental competence in these treated oocytes is increased (Nyholt de Prada *et al*., 2010). *Given these and other common defects of cultured oocytes and pre-implantation embryos, elucidation of factors controlling the cell cycle in these cells is vital for understanding cell cycle regulation and future improvements in culture conditions that may yield a higher percentage of viable embryos.*

One of the reasons that the culture of oocytes and early embryos is particularly challenging is the enormous developmental changes that occur in the time period from ovulation to implantation (Fig 6). Ovulated oocytes must first resume meiosis, which occurs due to calcium oscillation signaling activated by sperm during fertilization (Ducibella *et al*.,

2002). Pro-nuclei are formed upon fertilization and eventually fuse to create one diploid nucleus in a one-cell embryo. Embryos then undergo another major modification – *zygotic genome activation*. This is the stage at which the embryo initiates mRNA transcription from its own genome rather than using maternal mRNA produced and stored in the oocyte during oogenesis. The exact stage at which this occurs is species dependant, but it is generally around the 2-cell stage (Schultz, 2005). In the stages just prior to implantation the embryo goes through further morphological changes and cell differentiation. Compaction occurs, which establishes gap connections between blastomeres so that material may be transported and shared. Once compaction is complete, due to the gap junctions between blastomeres, the outer blastomeres become polarized as does the distribution of cytoplasmic and membrane bound factors. From this stage forward, polarity of the outer blastomeres is maintained and the cells of the embryo no longer have equal developmental potential. Following compaction, blastulation will commence. In order to form a proper blastula the trophectoderm and the inner cell mass must differentiate to form two distinct cell lineages (Marikawa and Alarcón, 2009).

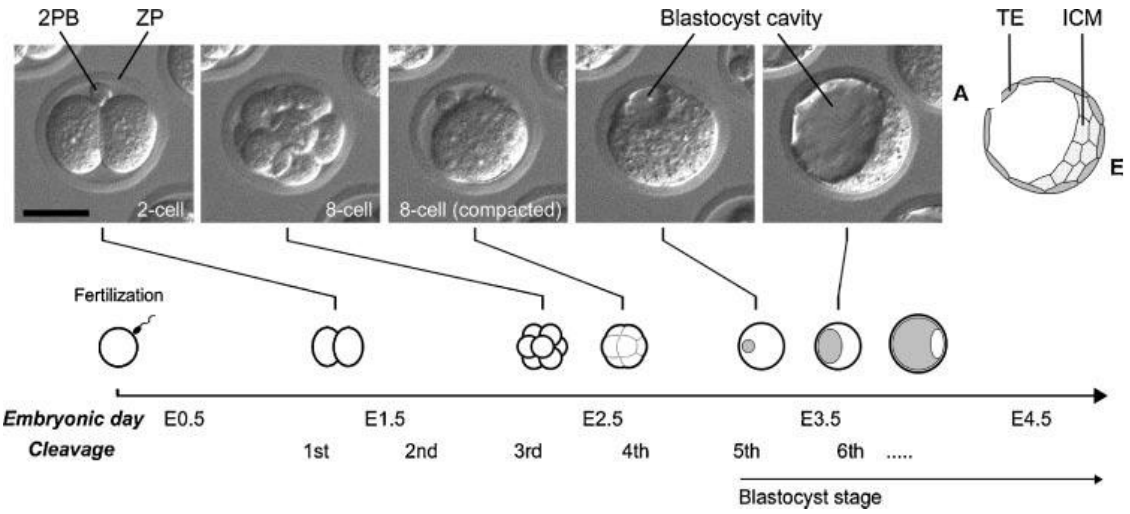

Figure 6: The *in vitro* development of a mouse embryo from fertilization to blastocyst formation. 2PB: second polar body, ZP: zona pellucida, TE: trophectoderm, ICM: inner cell mass. Scale bar = 50 mm. Adapted from Marikawa and Alarcón, 2009.

While oocytes and pre-implantation embryos are attempting to perform these delicate transformations in the presence of imperfect culture conditions, many things can go wrong. When mouse embryos matured *in vivo* are compared to counterparts cultured in various media, many gene expression abnormalities are found (Fig 7). The vast majority of these changes result in down-regulation of expression, but in varying degrees (Ho *et al.*,

1995). These modifications, while small, are not insignificant. Many of the genes involved play roles in cell proliferation, protein synthesis, apoptosis, and trafficking; consequently, they are very important in the earliest stages of development. Small changes in the expression patterns of these genes, aquaporin 8 (Aqp8) which is involved in transport of water and solute carrier 7 and 15 (slc7, slc15) which are involved in the transport of molecules across membranes, and can negatively impact embryo viability. Embryos grown in different culture media show different gene expression alterations, but none appear to approximate conditions *in vivo* (Rinaudo and Schultz, 2004). While this research has provided insight into changes induced by the micro-environment, little progress has been reported to mitigate the perturbations.

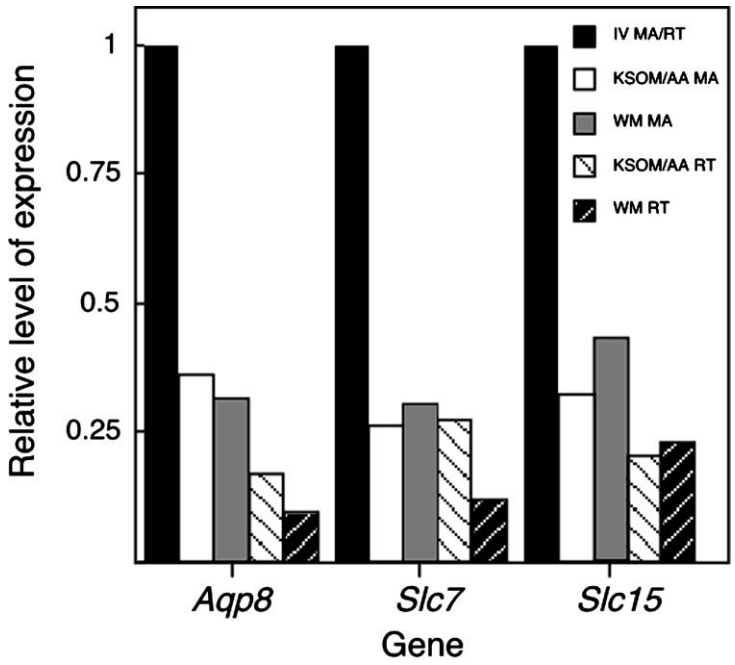

Figure 7: Three genes exhibiting decreased expression in embryos cultured in multiple media, assayed via multiple methods to confirm results. Aqp8 – aquaporin 8, Slc7 – solute carrier family 7, Slc15 – solute carrier family 15. IV: *in vivo*, KSOM/AA: KSOM/AA medium, WM: Whitten's medium, MA: microarray analysis, RT: rtPCR analysis. Rinaudo and Schultz, 2004.

While the Aurora Kinase family is fairly well characterized in somatic cells of many species, little is known about these proteins in gametes, and even less is known about their role during meiosis and early embryo development. When oocytes and embryos are matured *in vitro*, and it is common to see a high percentage of these embryos arrest cell division and cease to develop, rendering them non-viable. These losses are problematic in human fertility clinics as well as research laboratories focused on mammalian oocytes and

embryos (Rizos *et al*., 2008). Previous attempts have been made to increase efficiency of *in vitro* maturation of mouse oocytes in a strain dependent manner. It was shown that the most efficient agent to reactivate meiosis after the metaphase II checkpoint in oocytes is strain dependent and the timing of completion of meiosis also depends heavily upon the strain of mouse. This demonstrates oocytes are heavily affected by treatment and culture conditions and subtle differences in protocol may result in significant differences in efficiency (Ibáñez *et al*., 2005).

Manipulation of oocytes for somatic cell nuclear transfer (SCNT) requires the cell cycle of the oocyte to continue despite the addition of a somatic nucleus. Embryos reconstructed through SCNT are often observed to have cell cycle defects or developmental arrest. Yoo *et al.* were unable to produce reconstructed SCNT rat embryos that developed beyond the 2-cell stage despite several variations of the SCNT protocol. Further investigation revealed abnormal microtubule distribution and organization in the reconstructed embryos which likely did not allow for further development due to the inability to assemble a proper spindle (Yoo *et al*., 2007). Similarly, nonhuman primate SCNT embryos showed increased competency with enucleation just prior to the metaphase II arrest, but reconstructed embryos remained inferior to *in vivo* fertilized controls. This was attributed to spindle defects that resulted in aneuploid embryos among other factors, including improper reprogramming of the chromatin (Simerly *et al*., 2004).

Drastic changes to the SCNT protocol, including chemically induced enucleation, have been attempted. Demecolcine, a microtubule depolymerization agent, can be used to disrupt the metaphase spindle in a metaphase II stage oocyte. This yields chromatin that remains in a single cluster and ultimately completely extrudes from the oocyte in the second polar body. The resultant cytoplast can then be used for nuclear transfer to reconstruct a cloned embryo. Although this procedure eliminates the first mechanical manipulation of the oocyte, its efficiency remains relatively low at 23-70%. In the oocytes that do not complete extrusion of the second polar body, a protrusion of the plasma membrane is often observed, but the completion of cytokinesis never occurs (Fig 8, Ibáñez *et al*., 2003). Interestingly, the protrusion of the plasma membrane observed in these reconstructed embryos mimics the phenotype often observed in somatic cells deficient of Aurora Kinase B. In light of these findings, factors that control progression of the cell cycle and microtubule organization, such as the Aurora Kinase family, are of great interest for their possible implications in the inefficiency of SCNT.

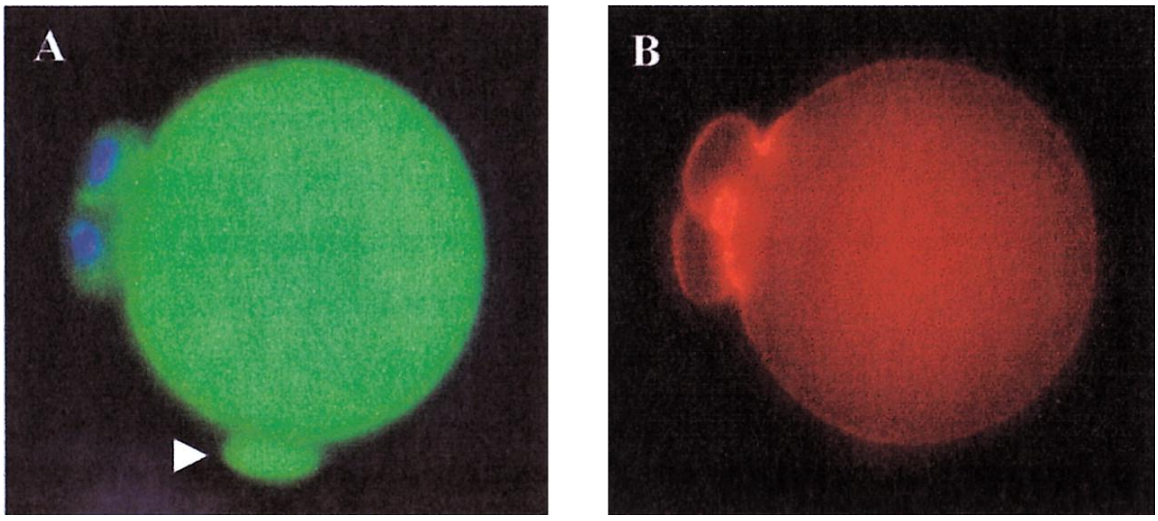

Figure 8: A Demecolcine treated oocyte at 135 minutes post activation. A, Tubulin is stained in green, chromatin in blue. The first polar body is marked with the arrowhead. There are two partially extruded second polar bodies, each with chromatin. B, Actin is stained in red. The second polar body has not completely extruded as evidenced by the absence of actin contact from either side of the plasma membrane. Adapted from Ibáñez et al., 2003.

The possibility remains that some of the defects that result in the low efficiency of *in vitro* maturation of oocytes is due to aberrant expression or localization of the Aurora Kinase proteins. Therefore, elucidation of the role of the Aurora Kinases in *in vivo* matured mammalian oocytes and early embryos is of great importance due to the cell cycle defects that are commonly observed in *in vitro* cultured and manipulated cells. The similarities of phenotypes observed in cells depleted of Aurora Kinase proteins and in manipulated or IVM oocytes make the Aurora Kinase proteins especially attractive to study. Understanding the correct expression and localization of the Aurora Kinase proteins in oocytes and pre-implantation embryos will allow for further investigation of the Aurora Kinases in manipulated or *in vitro* cultured samples.

Thesis Objectives

The studies described in this thesis were designed to reveal the localization and function of Aurora Kinase B and C in mouse oocytes and early embryos. *In vitro* matured oocytes and embryos were used to study the proteins of interest through the progression and completion of meiosis as well as during the initial mitotic divisions of blastomeres of preimplantation stage embryos.

It was hypothesized that:

1. Aurora Kinase B and C would be expressed and localized in oocytes and embryos similarly to the localization previously reported for somatic cells. Likewise, presence of mRNA transcript for these genes would mimic the pattern established in somatic cells.
2. Upon loss of function of Aurora Kinase B or C there would be cell cycle defects similar to those previously observed in somatic cells depleted of either protein.

Several approaches were employed to answer these questions. Localization of the protein was attempted through the use of immunocytochemistry with primary antibodies targeted to the proteins of interest and secondary antibodies with fluorescence tags. Presence and relative quantification of the proteins was determined with Western blotting. The presence and relative quantification of mRNA gene transcripts was determined using reverse transcriptase PCR (rtPCR) and quantitative rtRCR respectively. The function of Aurora Kinase B and C in mouse oocytes and embryos was determined with a combination of specific chemical inhibitors and siRNA constructs designed to target individual mRNA transcripts.

Chapter 2: Aurora Kinase B

Materials & Methods

Oocyte/Embryo Collection & Culture

CF-1 female mice (8-12 weeks, Charles River, MA) were superovulated with 5 IU eCG (Calbiochem #367222) intraperitoneally followed 48h later with 5 IU hCG (Calbiochem #230734) intraperitoneally. Germinal vesicle stage oocytes were collected from ovarian follicles 46h post-eCG injection. Ovulated MII oocytes were collected from oviducts 14-16h post hCG injection. Embryos were produced by mating superovulated females with CF-1 males overnight. Embryos were then harvested from the oviducts into FHM medium (Millipore #MR-024-D, see Appendix A) 14-16h post-hCG injection. Oocytes or embryos surrounded by cumulus cell masses were briefly treated with hyaluronidase (Sigma #H-3884) and gently pipetted to remove the cumulus cells and washed 3 times in FHM medium. Oocytes were cultured in medium for four hours with 10mM strontium chloride ($SrCl_2$, Sigma #S-0390) to parthenogenetically active them as necessary (Ibanez *et al*., 2005). Samples were randomly assigned to groups for either immediate processing or further culture in KSOM medium (Millipore #MR-106-D, see Appendix A). Culture was performed in humidified chambers at 37°C, 5% CO_2 in air for various lengths of time.

Immunocytochemistry

Mouse oocyte and embryo samples were fixed in several different solutions: 1) 1% paraformaldehyde and 0.15% triton X-100 (see Appendix A) for 30m at 37°C, 2) MTSB-XF solution (see Appendix A) for 30m at 37°C, or 3) ice cold methanol for 5m on an ice block. Regardless of fixation method all samples were subsequently stored at 4°C in immunocytochemistry blocking solution (see Appendix A) until being further processed. Microscopy with probes for Aurora Kinase B (AurB), microtubules (Tub), and chromatin was performed. Aurora Kinase B was detected with primary polyclonal rabbit anti-AurB antibody (Abcam #ab2254, 1:500 dilution). The primary AurB probe was followed by a secondary Alexa fluor 594 labeled goat anti-rabbit antibody (Invitrogen #A11072, 1:400 dilution). A mixture of primary monoclonal anti-α-tubulin and anti-β-tubulin antibodies (Sigma-Aldrich Co #T-9026 and #T-4026 respectively, 1:1000 dilution) followed by a secondary Alexa fluor 488 labeled goat anti-mouse antibody (Invitrogen #A21121, 1:400 dilution) was used to label microtubules. Chromatin was labeled with 10µg/ml of Hoechst 33258 (Molecular Probes #H-1398). Labeled eggs were then mounted onto slides in a mounting solution of 50% glycerol in PBS with sodium azide and covered with a glass coverslip. Samples were analyzed using a Zeiss Axiovert 200M inverted microscope coupled to a Roper CoolSnapFx high resolution

CCD camera. Images were captured and rendered in real time using Axiovision software. Mouse embryonic fibroblasts were used as positive controls.

Western Blotting

For protein expression analysis by western blotting Mouse oocytes or embryos for were lysed using a mammalian cell lysis kit (Sigma-Aldrich Co #MCL-1). Cells were added to the solution, vortexed vigorously, and stored at -20°C. Prior separating proteins by 1-D PAGE, samples were briefly thawed at room temperature, mixed 50:50 with Laemmli sample buffer (BioRad #161-0737) containing 3% β-mercaptoethanol. Samples were then boiled in a 100°C heat block for at least 5m and loaded onto a 12% polyacrylamide gel (VWR #81002-028). Rainbow Molecular Weight Markers (Amersham Biosciences #RPN800E) were used to determine protein separation and estimate molecular weight. Gels were run at 150V, 100mA until marker bands were appropriately separated. Transfer of the protein to ECL+ membrane (Amersham Biosciences #RPN68D) was performed using a wet apparatus with 40V of current for 90m and membranes were then stored in Western blocking solution (see Appendix A) at 4°C until analyzed.

Membranes were washed 3x with TBS-T solution and then probed with AurB polyclonal antibody (Abcam #ab2254, 1:1500) in TBS-T for 1h at room temperature. Membranes were washed as previously described, and then probed with anti-rabbit-HRP secondary antibody using the Visualizer Western blotting kit (Upstate #64-202). Membranes were washed again and chemiluminescent detection was performed using the Visualizer Western blotting kit as described and ECL+ film (Amersham Biosciences #28-9068-35).

Histology

Samples of mouse ovary tissue were fixed in 10% formalin solution (Newcomer Supply #1090) and paraffin embedded and processed by the Worcester Polytechnic Institute histology core facility. 5µm sections were mounted on slides and de-paraffinized via xylene and ethanol baths followed by rehydration in water. Sections were then stored overnight at RT in blocking solution. Protein localization was done using a primary polyclonal Aurora Kinase B antibody (Abcam #ab2254, 1:500) for 60m at 37°C in a humidified chamber, washed, and a secondary Alexa fluor 594 labeled goat anti-rabbit antibody (Invitrogen #A11072, 1:400 dilution) for 60m at 37°C in a humidified chamber. Chromatin was labeled with 10µg/ml of Hoechst 33258 (Molecular Probes # H-1398) at RT for 10m. Samples were washed in PBS/PVP between staining steps. Samples were sealed with a coverslip and analyzed using a Zeiss Axiovert 200M inverted microscope coupled to a Roper CoolSnapFx

high resolution CCD camera. Images were captured and rendered in real time using Zeiss Axiovision software. Testis samples were used as a positive control.

Reverse Transcriptase PCR

Total RNA was extracted from mouse oocyte and embryo samples for reverse transcriptase PCR (rtPCR) with the Stratagene Absolutely RNA Nanoprep kit (Stratagene #400753). cDNA was produced from extracted RNA through the use of the SuperScript First Strand Synthesis System (Invitrogen #12371-019) and PCR was performed using intron spanning primers specific to Aurora Kinase B and β-actin. Several primer pairs were evaluated and the primer pair which yielded the most robust reaction was utilized in subsequent analyses. Aurora Kinase B primers used were: cctgaaacatcccaacatcc and ctccctgcagacctaacagc. β-actin primers used were: tgttaccaactgggacgaca and ggggtgttgaaggtctcaaa. PCR cycle conditions were: 95°C 30 sec, 55°C 45 sec, 72°C 1 min, and 30 cycles were run. Reaction products were run out on 2% agarose gels with Ethidium Bromide. All PCR products were of the expected size: β-actin = 165bp, Aurora Kinase B = 245bp. Images were aquired using a Kodak Image Station 400MM and densitometry was performed using Adobe Photoshop. Aurora Kinase B products were normalized to β-actin.

Quantitative Real Time Reverse Transcriptase PCR (q-rtPCR)

Oocyte and embryo samples (n=5/group) were lysed, treated with DNAse, and subjected to reverse transcription using the TaqMan Gene Expression Cells to CT kit (Ambion #4399002) as described. Samples were stored at -20°C until being processed further. Quantitative real time reverse transcriptase PCR was performed using an Applied Biosystems 7500 Real-Time PCR System, TaqMan probes specific to Aurora Kinase B (Applied Biosystems #4331182- Mm01718140_m1), master mix solution provided in the Cells to CT kit, and 2.5µL reverse transcriptase product. All experimental samples were run in triplicate. Control reactions were performed with TaqMan probes specific to β-actin (Applied Biosystems #4331182-Mm00607939_s1), no reverse transcriptase product, and RNA only template in duplicate on every reaction plate. Data was collected and analyzed using the Applied Biosystems 7500 System software and Microsoft Excel.

Loss of Function Experiments

Mouse embryos were collected from oviducts at the fertilized 1-cell stage and cultured as previously described for approximately 24h. Once divided to the 2-cell stage, 1 blastomere of the 2-cell embryo was microinjected with a pool of three to five siRNA constructs specific to Aurora Kinase B (Santa Cruz Biotechnology, Inc. #sc-43532). Approximately 65pL of RNAi solution was injected into each blastomere and solution

concentrations of 57µM, 40µM, and 20µM were used. Injections were conducted using an Eppendorf PipetteMan microinjection system attached to a Zeiss Axiovert 200M inverted microscope. During the microinjection procedure embryos were held in FHM media droplets under oil in glass bottom dishes and transferred back to culture conditions as soon as possible. Control and experimental microinjections were performed in alternating order to mitigate any effect treatment timing may have. Controls were produced via microinjection of an inert pool of siRNA (Santa Cruz Biotechnology, Inc. #sc-37007) and embryos introduced to the condition changes necessary for microinjection, but not treated. Embryos were cultured (37°C, 5% CO_2 in air) for 24h or 48h following microinjection and then processed for immunocytochemistry or q-rtPCR.

Results

Localization of Intracellular Aurora Kinase B protein in Oocytes and Embryos

Mouse oocytes and embryos were subjected to immunocytochemistry to determine what extent Aurora Kinase B protein was present or absent in the samples. If the protein was detectable, then localization characteristics were established. Oocytes and embryos prior to the 8-cell stage were found to have signal from the Aurora Kinase B probes displayed as homogeneous punctate staining throughout the cytoplasm (Fig 9b and c). From the 8-cell stage to blastocyst, the most developmentally advanced stage studied, Aurora Kinase B protein was observed on the midbody as cells complete the last steps of cytokinesis (Fig 9d-f). As the midbody is narrowed further with increasing separation of the daughter cells Aurora Kinase B was localized to a decreasing focal point at the point of contact between the cells prior to excision. This was comparable to the localization observed in mouse embryonic fibroblast control cells (Fig 9a). These data suggest that prior to the 8-cell stage, Aurora Kinase B does not have a specific localization within the cell during any portion of the cell cycle. Similar results were obtained with three different fixation techniques, although the samples subjected to MeOH fixation were difficult to image as the native morphology was severely distorted.

Quantification of Aurora Kinase B protein in Oocytes and Embryos

In conjunction with the immunocytochemisty studies Western blotting was performed in parallel experiments to determine if Aurora Kinase B was present in the oocyte and embryo samples. Relative amounts of protein were determined from samples subjected to Western blotting. Oocytes and embryos were collected in groups ranging from 10 to over

250 cells per sample. Mouse embryonic fibroblasts were used as positive controls. Initially a band of inappropriate size was detected when the blots were probed for Aurora Kinase B. This band was proven to be remnant bovine serum albumin from the medium that the oocyte and embryos were collected and cultured in prior to lysis. Vigorous washing of the samples in PBS prior to lysing eliminated the band during subsequent analyses. Despite the large numbers of cells lysed and extremely sensitive detection systems used, no Aurora Kinase B protein was found in the samples analyzed (Fig 10). In contrast, α/β-tubulin was easily detected in both experimental and control samples, which is most likely due to its high abundance. This control validated the lysis, blotting, and detection techniques and confirmed that Aurora Kinase B was likely absent or present at extremely low abundance.

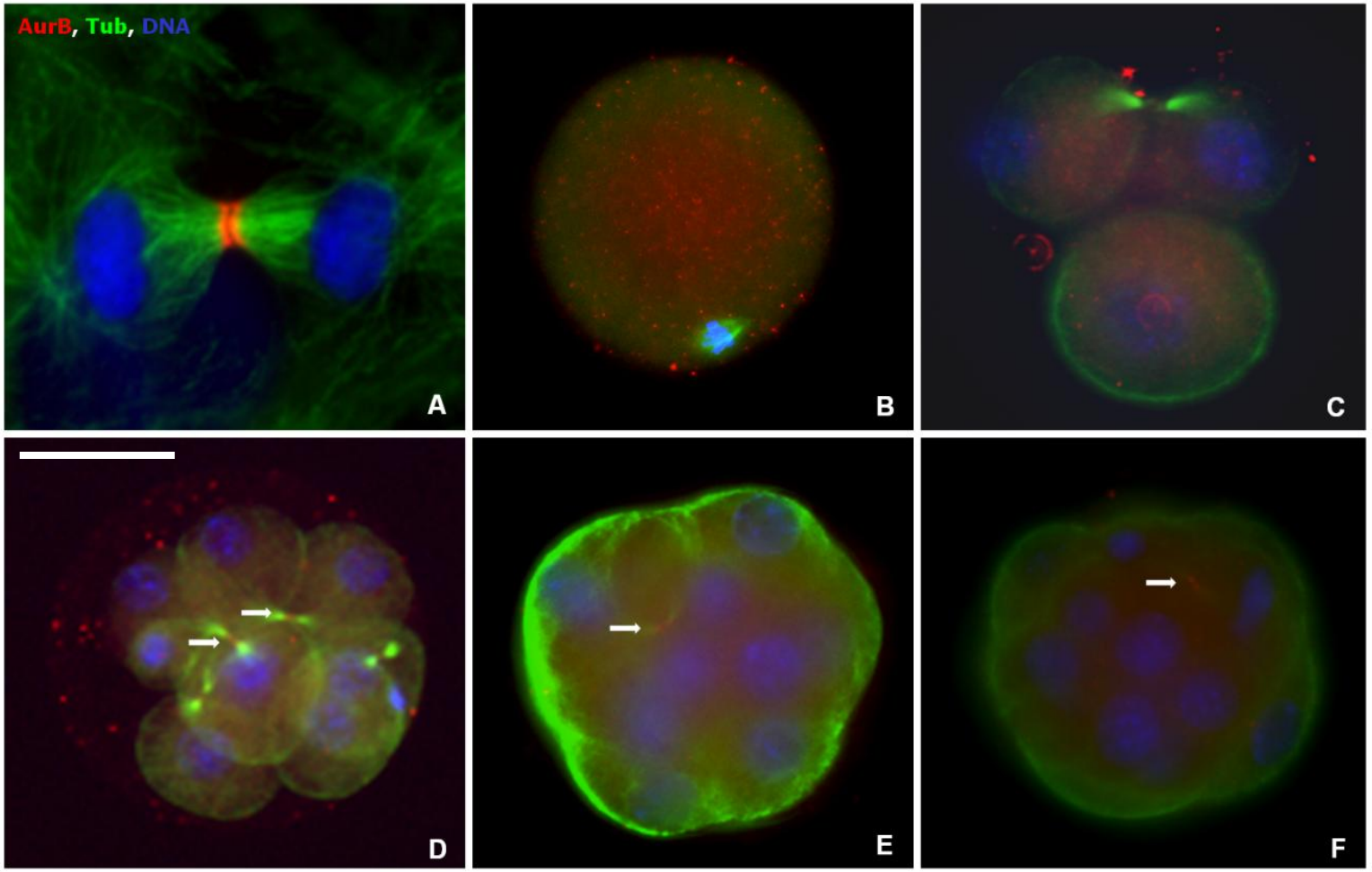

Figure 9: Immunocytochemical localization of Aurora Kinase B, tubulin, and chromatin in of mouse oocytes, embryos, and mouse embryonic fibroblasts (MEF, control) fixed in PFA solution. A: MEF cells. B: A metaphase II stage mouse oocyte. C: A 3-cell stage mouse embryo. D: An 8-cell mouse embryo.with Aurora Kinase B at several midbodies (arrows). E and F – Compacted morula stage mouse embryos with Aurora Kinase B at midbodies (arrows). Scale bar represents ~40µm.

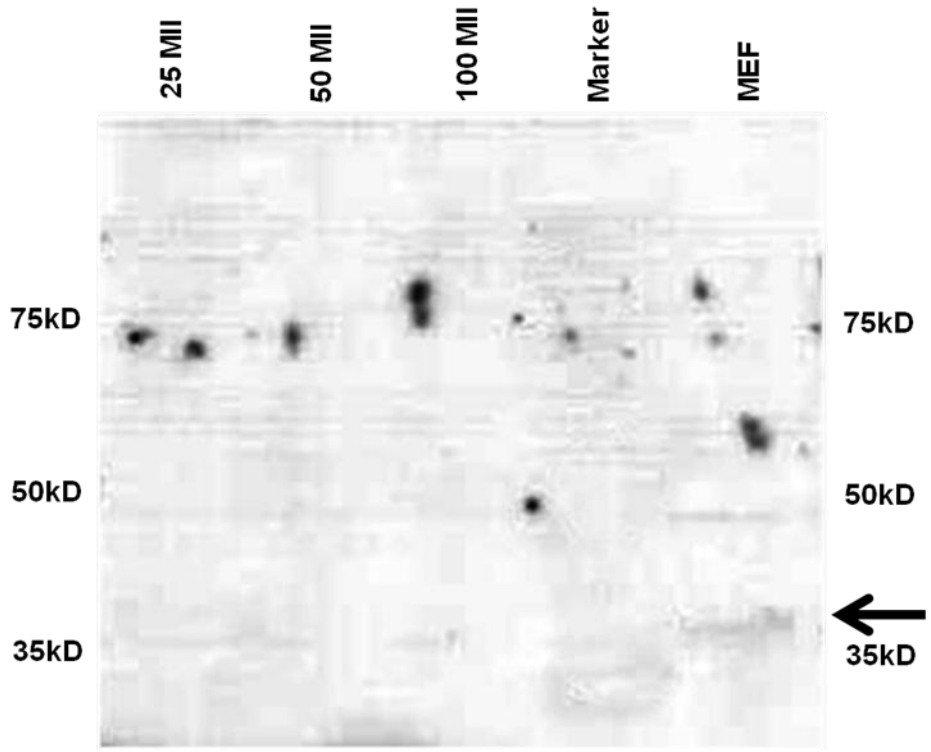

Figure 10: Western blot of MII stage mouse oocytes and mouse embryonic fibroblasts (MEF) probed for Aurora Kinase B. There is a band visible of appropriate size for Aurora Kinase B (~37kD, arrow) in the MEF lane, yet no corresponding bands apparent in any of the oocyte sample lanes. The higher molecular weight band also visible in the MEF lane is residual tubulin signal from a previous probing.

Localization of Intracellular Aurora Kinase B protein in Ovary and Testis Tissue

Histology was performed on mouse ovarian tissue to determine if Aurora Kinase B was present in oocytes within follicles prior to ovulation or in the cells directly surrounding the developing follicle. The ovarian samples analyzed displayed subtle staining in the red channel, which is indicative of Aurora Kinase B presence. The signal was observed primarily in the follicular cells surrounding developing oocytes (Fig 11). The staining within oocytes appeared very similar to the diffuse punctate pattern observed in ovulated oocytes. Unfortunately, a significant fluorescent background signal was observed in the green channel, indicating that the samples had high levels of autofluorescence. Much of the signal observed in the red channel also co-localized with the autofluorescence observed in the green channel. This raises the possibility that the observed red signal may not be due to Aurora Kinase B protein presence, but rather a result of autofluorescence. Testicular tissue was used as a control and it displayed much of the same autofluorescence that was detected

in ovarian tissue (Fig 9). Controls stained with the secondary antibody only did not hsow any evidence of non-specific binding. Accordingly, it is impossible to draw solid conclusions about the presence or absence of Aurora Kinase B in this tissue. Alternative methods of fixation and/or localization that do not rely upon fluorescent probes may be worth investigating in the future.

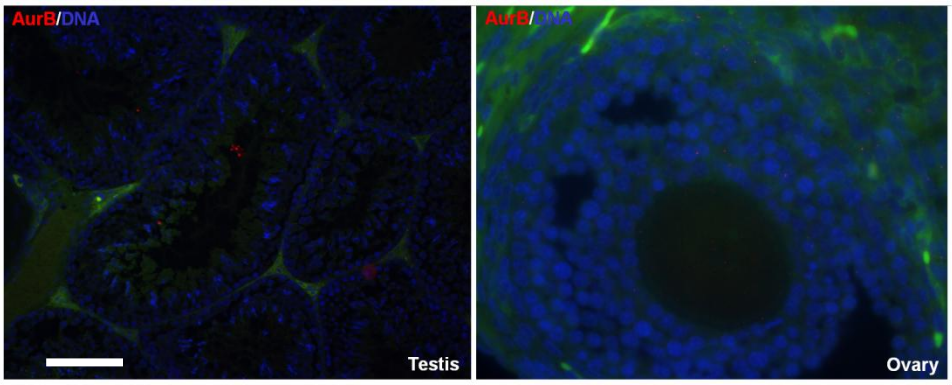

Figure 11: Fixed sections of mouse ovary and testis stained for Aurora Kinase B (red) and chromatin (blue). Green signal represents autofluorescence. Scale bar represents ~40µm.

Identification of Aurora Kinase B mRNA in Oocytes and Embryos

Non-quantitative reverse transcriptase PCR reactions were performed on oocyte and embryo lysate samples to determine that Aurora Kinase B mRNA was present. Oocytes (germinal vesicle to MII, and MII to $SrCl_2$ activated stage) were analyzed as well as embryos from the one-cell to four-cell stages. Aurora Kinase B mRNA was detected in all of the stages of oocytes and embryos tested (Fig 12a). All samples were analyzed for the presence of β-actin as a control for the assay (Fig 12b). Densitometry analysis was used to normalize Aurora Kinase B bands to β-actin bands (Fig 12c). FHM medium was also analyzed as a negative control as the cells lysed for rtPCR may have retained some residual medium, which would introduce it to the reaction.

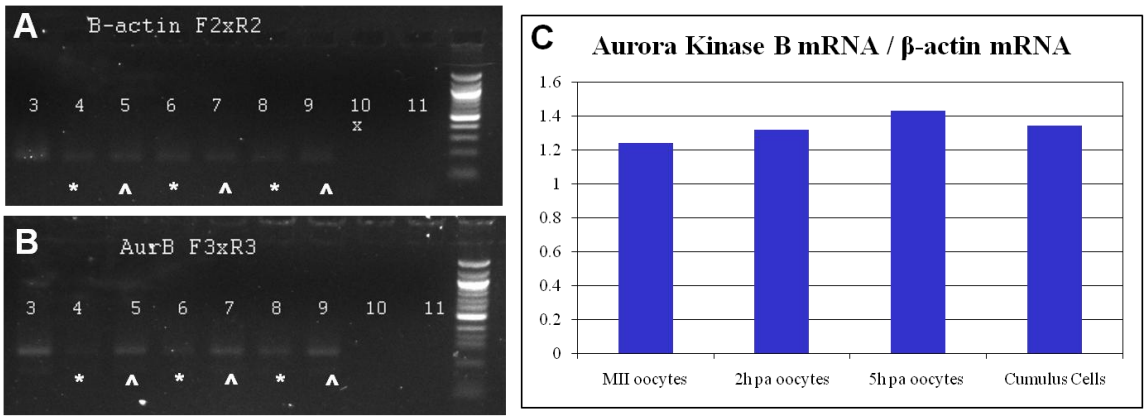

Figure 12: Presence of Aurora Kinase B and β-actin mRNA in mouse oocytes. A: β-actin reaction product on agarose gel. B: Aurora Kinase B reaction product on agarose gel. Lanes are as follows: 3, Cumulus Cells; 4, 10 MII oocytes; 5, 20 MII oocytes; 6, 10 oocytes 2h post activation; 7, 20 oocytes 2h post activation; 8, 10 oocytes 5h post activation; 9, 20 oocytes 5h post activation; 10, blank; 11, Negative Control Water. C: Aurora Kinase B rtPCR reaction product normalized to β-actin rtPCR product through densitometry analysis. n=10 oocytes/sample. pa = post-activation.

Quantification of Aurora Kinase B mRNA in Oocytes and Embryos

Once rtPCR experiments demonstrated the presence of Aurora Kinase B mRNA in mouse oocytes and embryos, q-rtPCR analysis was carried out on oocyte and early embryo samples to quantitate changes in the levels of Aurora Kinase B mRNA as development progressed. The results of the previously described rtPCR experiments were further supported by this analysis as Aurora Kinase B mRNA was again found in all samples analyzed (Fig 13A). Ct values obtained for Aurora Kinase B and β-actin were inverted by subtracting the raw Ct value from 40, which is the maximum possible Ct value when 40 cycles are run (Inverted Ct = 40-Ct). Each individual sample contained 5 oocytes or embryos and there were 3-6 samples for each developmental stage except the 4-cell stage which had 1 sample. Aurora Kinase B mRNA levels stayed at a relatively steady state from germinal vesicle (GV) stage through fertilization (1-cell). This is the expected result as there is little to no transcription thought to occur in oocytes and early embryos until the zygotic genome is activated at about the 2-cell stage. Between the 1-cell and 2-cell stage there was a decrease in the Aurora Kinase B mRNA level. This is likely due to the degradation of maternal transcripts prior to the synthesis of new transcripts by the zygotic genome. Embryos that have developed to the morula or blastocyst stage have relatively little Aurora Kinase B mRNA when compared to the earlier developmental stages. At these later stages the pace of the embryonic cell cycle slows considerably in blastomeres when compared to earlier stage embryos. Therefore, it is not surprising to see a decrease in mRNA levels associated with cell

division. Cumulus cells were used as a control to ensure consistency between separate analyses using 96-well plates. Cumulus cells were found to have a low level of Aurora Kinase B mRNA which was expected as they are known to be not actively proliferating (G_0 arrest).

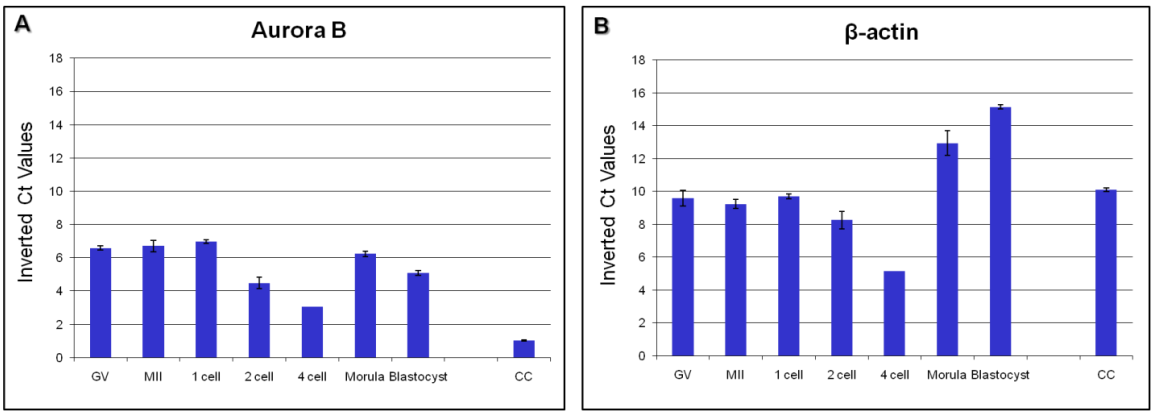

Figure 13: Levels of Aurora Kinase B mRNA and β-actin in mouse oocytes and embryos. A: Inverted Aurora Kinase B Ct values, B: Inverted β-actin Ct values. Error bars represent standard error of the mean.

Analysis of Loss of Function of Aurora Kinase B mRNA in Embryos

In an attempt to better understand the role of Aurora Kinase B in early mouse embryos, one blastomere of a 2-cell embryo was microinjected with a solution of inhibitory siRNA constructs specific to Aurora Kinase B. This resulted in each 2-cell embryo containing an experimental blastomere devoid of active Aurora Kinase B mRNA and an untreated control blastomere. The siRNA solution was a pool of three to five individual constructs, resulting in an injection cocktail that was expected to yield high efficiency knockdown of the target. To further control for any artifact induced by the microinjection process, a control solution consisting of a pool of inert siRNA constructs was microinjected into 1 blastomere of 2-cell control embryos. A range of concentrations of siRNA solution was used (57 µM, 40µM, and 20µM) to determine if there was a difference in knock-down effect with differing dosages. Embryos were observed for development and processed for further analysis at 48h post-microinjection. This time point gave sufficient time for the siRNA to have an effect. The data indicates that the knockdown of Aurora Kinase B was successful in these embryos (Fig 15) and that the resultant effect observed is similar to the effect of knocking down Aurora Kinase B in somatic cells.

The embryos injected with either 57 µM or 40µM Aurora Kinase B siRNA solution appeared to be severely and similarly affected by the treatment. At 48h post-microinjection a majority of embryos subjected to Aurora Kinase B knockdown treatment appeared to be at a 3-cell stage (Fig 14a, c, e, and g). Two of the cells within the embryo were approximately

equal in size while the third cell was markedly larger. Upon further analysis it was found that the large cell of these embryos often contained two distinct nuclei. This data implies that there was an attempt at cell division, and karyokinesis progressed while cytokinesis was disrupted. Altered progression of cytokinesis is commonly observed in somatic cells in which Aurora Kinase B has been inhibited. Control embryos microinjected with the inert siRNA solution appeared to have continued a normal pace of development and consisted of many cells which were well compacted and in some cases starting to form early blastocysts (Fig14b, d, f, and h).

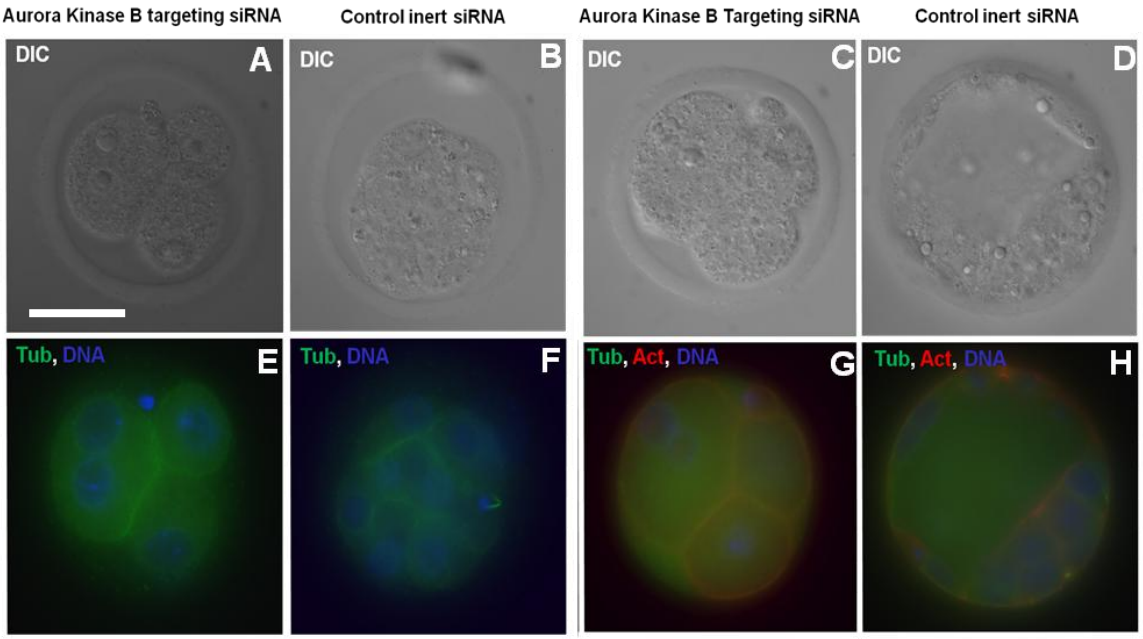

Figure 14: Mouse embryos microinjected at the two-cell stage with 40µM Aurora Kinase B targeting siRNA. Tub: Tubulin, Act: Actin. Embryos were cultured for 48h post-microinjection before being fixed with PFA and processed for immunohistochemistry. DIC imaging shows that the experimental embryos have developed to the 3-cell stage (A and C) and have one large multinucleate cell (E and G). One of the control embryos (B) is well compacted while the other (D) has entered the early blastocyst stage. Immunohistochemistry reveals that the control embryos (F and H) consist of many cells of similar size. Scale bar represents ~40µm.

Analysis of these embryo samples by q-rtPCR revealed that Aurora Kinase B mRNA was diminished in the experimental treated group when compared to the control microinjected group (Fig 15). Calculations were performed on q-rtPCR data as described above in the previous section. While the degree of knockdown is not complete, this is to be expected given that one of the two blastomeres of the experimental embryo is not treated

with siRNA and therefore has a full compliment of Aurora Kinase B mRNA. β-actin levels in experimental and control embryos were comparable which suggests that global mRNA reduction is not the reason for the decrease in Aurora Kinase B mRNA.

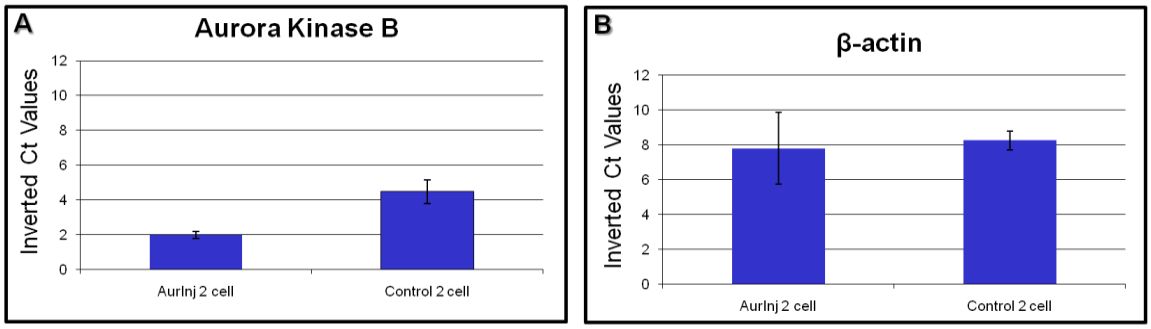

Figure 15: Levels of Aurora Kinase B and β-actin mRNA in mouse oocytes and embryos microinjected with 40µM Aurora Kinase B targeting siRNA. A: Inverted Aurora Kinase B Ct values, B: Inverted β-actin Ct values. n=3, each sample contains 5 embryos. Error bars represent standard error of the mean.

Embryos injected with 20µM Aurora Kinase B siRNA solution appeared to be similarly affected, but to a lesser degree than the embryos treated with higher concentrations. Experimental embryos and controls from this group both display development well past the two-cell stage. However, upon analysis by immunohistochemistry, the experimental embryos appeared to have less total cells and were comprised of cells of widely varied sizes (Fig 16a and c). Control embryos were comprised of many cells comparable in size to the smallest cells found in experimental embryos (Fig 16b and d). This suggests that the blastomere microinjected with 20µM Aurora Kinase B siRNA was able to undergo mitosis several times, but at a reduced pace.

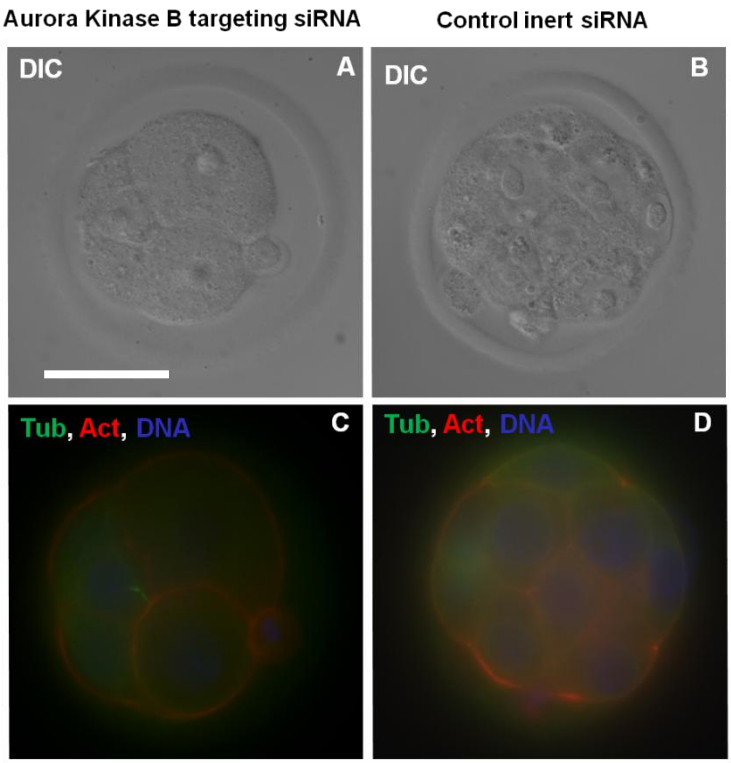

Figure 16: Mouse embryos microinjected at the two-cell stage with 20µM Aurora Kinase B targeting siRNA. Tub: Tubulin, Act: Actin. Embryos were cultured for 48h post-microinjection before being fixed with PFA and processed for immunohistochemistry. DIC imaging shows that the experimental embryo has developed well past the 2-cell stage (A) but, immunohistochemistry analysis (C) reveals that the cells are of varied sizes. The control embryo (B) is well compacted and contains many cells. Immunohistochemistry shows that the many cells of the control embryo (D) are uniform. Scale bar represents ~40µm.

Chapter 3: Aurora Kinase C

Materials & Methods

Oocyte/Embryo Collection & Culture

Methods were as previously described in Chapter 2.

Immunocytochemistry

Methods were as previously described in Chapter 2 with the exception that either a primary polyclonal anti-AurC antibody (Abgent #AP7000d, 1:100 dilution) or a custom produced polyclonal rabbit AurC antibody (Genemed Synthesis, 1:100) was used in these analyses.

Reverse Transcriptase PCR

Methods were as previously described in Chapter 2. PCR was performed using primers specific to Aurora Kinase C and β-actin. Aurora Kinase C primers used were: ggctggagtcagagcgttac and tccgggttttcctacctctt, and β-actin primers used were: tgttaccaactgggacgaca and ggggtgttgaaggtctcaaa.

Western Blotting

Methods were as previously described in Chapter 2 with the exception that an AurC polyclonal (Abgent #AP7000d, 1:400 dilution) was used as the primary antibody.

Histology

Methods were as previously described in Chapter 2 with the exception that a primary polyclonal rabbit Aurora Kinase C custom antibody (Genemed Synthesis, 1:100) was used.

Results

Localization of Intracellular Aurora Kinase C in Oocytes and Embryos

Mouse oocytes and early embryos (1-, 2-, 4-, 8-cell, morula, blastocyst stages) were collected and processed using immunocytochemistry methods to assess the presence or absence and the localization of Aurora Kinase C. Oocytes ranged from germinal vesicle (GV) stage to parthenogenetically activated oocytes cultured to the point of second polar body extrusion. Despite employing three different fixation methods, ice cold methanol, microtubule stabilizing buffer (MTSB), and 1% paraformaldehyde (PFA), and the use of two different Aurora Kinase C polyclonal antibodies, there was no detectable staining specific to Aurora Kinase C in any of the oocyte, embryo, or control mouse embryo fibroblast (MEF)

samples. The oocyte samples fixed in MTSB and PFA displayed a diffuse punctate staining pattern throughout the cytoplasm using both of the Aurora Kinase C antibodies. While this pattern was consistent throughout all oocyte samples analyzed, the staining pattern does not appear to be concentrated to a specific area of the cell or the spindle itself (Fig 17A). Embryos fixed with and probed using the same methods did not appear to exhibit the punctate pattern that was obvious in the cytoplasm of oocytes, and did not display Aurora Kinase C protein localization to the spindle (Fig 17B). Oocyte and embryo samples fixed in ice cold methanol were not able to be analyzed. Much of the structure of the cell was destroyed by this harsh fixation technique rendering the samples impossible to analyze. Nuclei and microtubules were clearly localized as expected, revealing uniform stained nuclei and spindle apparatus respectively. These results validated the immunocytochemistry method used. Based upon the previous data showing upregulation of all Aurora Kinase family members in cancer cells, a mouse cancer cell line may prove useful as a positive control.

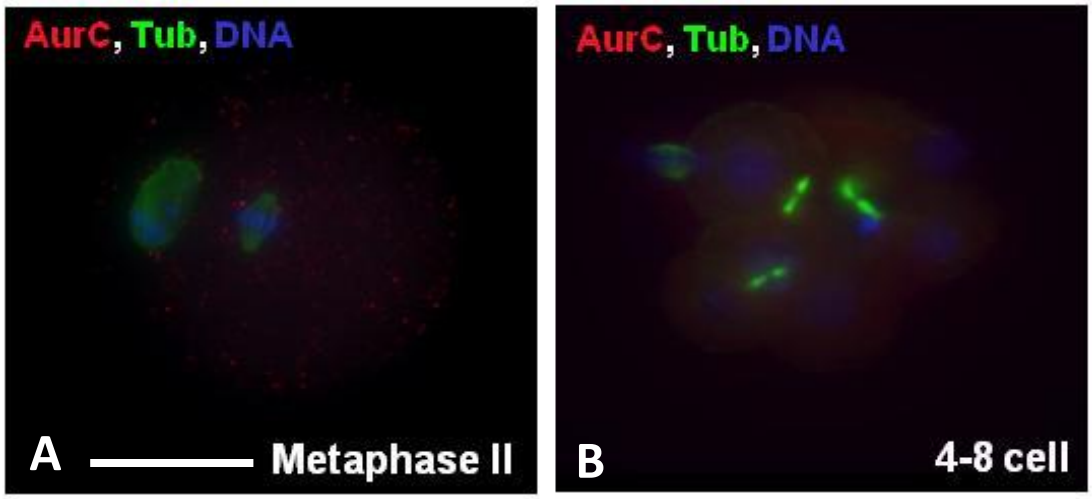

Figure 17: Immunocytochemical localization of Aurora Kinase C (AurC, red), tubulin (Tub, green), and chromatin (DNA, blue) in mouse oocytes and early embryos. Samples were fixed in PFA and probed with a custom prepared rabbit polyclonal Aurora Kinase C antibody. Panel A shows diffuse punctate red staining is visible throughout the cytoplasm of the metaphase II oocyte, but does not appear to be concentrated on the spindle. In panel B, Aurora Kinase C protein is not evident in the cytoplasm or on the spindles of blastomeres of the cleavage stage embryo. Scale bar represents ~40µm.

Quantification of Intracellular Aurora Kinase B Protein in Oocytes and Embryos

Mouse oocytes and embryos of various stages were subjected to Western blotting concurrently with the immunocytochemistry studies to further validate the presence or

absence of Aurora Kinase C in these samples. Samples were processed as described previously in Chapter 2. Several bands were detected when membranes were probed for Aurora Kinase C, but were of inappropriate size (Fig 18a). Bands were determined to be remnant bovine serum albumin from the oocyte and embryo medium. Extensive washing of the cells in PBS/PVP prior to lysis eliminated these bands. No protein bands were detected in any stage oocyte or embryo when samples were subjected to the washing procedure. Increasing the numbers of cells in the lysate samples (n > 250 oocytes) did not yield positive results despite the use of an extremely sensitive chemiluminescent detection method. Probing for α/β tubulin protein was successful in these samples, with a band apparent at the expected molecular weight (Fig 18b). Therefore, similar to the results from immunocytochemistry analysis, there was no Aurora Kinase C protein detected in the lysed oocyte or embryo samples. However, it is impossible to conclude that the protein is not present in the oocyte and embryo samples due to the lack of a positive control. Again, the inclusion of a mouse cancer cell line would be helpful to validate the sensitivity and specificity of the Aurora Kinase C antibodies that were used.

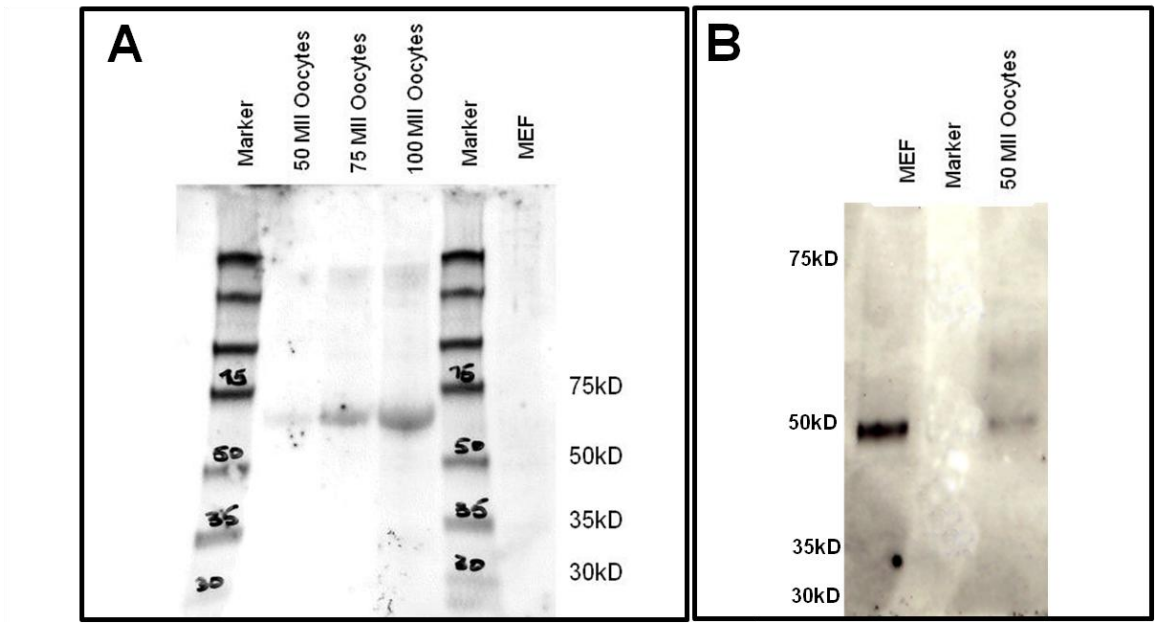

Figure 18: Western blot of MII stage mouse oocytes and mouse embryonic fibroblasts probed for A – Aurora Kinase C and B – Tubulin. A – A band of inappropriate size is seen at approximately 70kD, but no bands are detected at ~33kD, the predicted size for Aurora Kinase C. Blot probed with 1:400 diluted commercially available Aurora Kinase C polyclonal primary antibody and 1:10,000 diluted anti-rabbit HRP secondary antibody. Mouse Embryonic Fibroblasts (MEF) were used as controls. B – A band of appropriate size is observed at approximately 50kD, the predicted size for Tubulin, in both MEF and oocyte lanes. Blot probed with 1:500 diluted α/β tubulin monoclonal primary antibody and 1:10,000 diluted anti-mouse HRP secondary antibody. Mouse Embryonic Fibroblasts (MEF) were used as controls.

Localization of Intracellular Aurora Kinase C Protein in Ovary and Testis Tissue

Histology was performed on mouse ovary tissue to determine if Aurora Kinase C was present in early oocytes prior to ovulation or in the follicular cells surrounding the oocytes *in situ*. The ovary samples that were analyzed (Fig 19A) showed red fluorescence that was indicative of Aurora Kinase C protein localization. The staining pattern was found primarily in the follicular cells surrounding the early oocytes, but occasional diffuse staining was observed in the oocyte cytoplasm. Signal was detected in the green channel despite the absence of a green fluorochrome. The red and green fluorescence appeared to co-localize. Accordingly, it was concluded that the ovarian tissue displayed a high level of auto-fluorescence. Control testis samples (Fig 19B) displayed similar auto-fluorescence in both green and red channels further supporting the auto-fluorescence conclusion. Further experiments with non-fluorescent detection methods or fluorochromes that do not interact

with autofluorescence in these samples may prove more useful for localizing Aurora Kinase C in germ cells *in situ*.

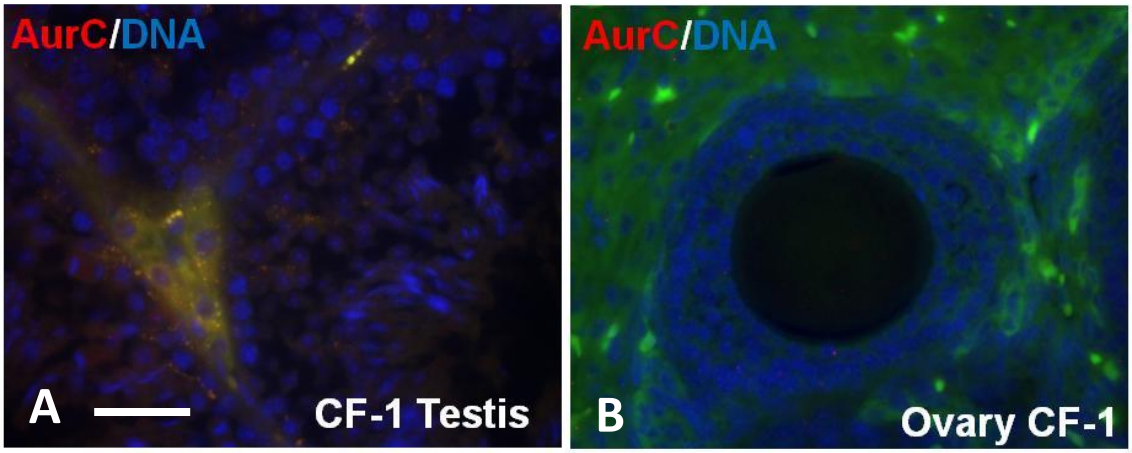

Figure 19: Fixed sections of mouse ovary and testis stained for Aurora Kinase C (red) and chromatin (blue). The custom polyclonal Aurora Kinase C rabbit antibody is used. No Green fluorochromes were not used and demonstrate a high level of auto-fluorescence in these samples. Scale bar represents ~40µm.

Identification of Aurora Kinase C mRNA in Oocytes and Embryos

Given that Aurora Kinase C protein was not able to be detected in any of the samples examined via immunocytochemistry or Western blotting, reverse transcription PCR was used to determine if there was Aurora Kinase C mRNA transcript present in oocytes and early embryos. The presence of the transcript would confirm the possibility of the presence of the protein. This would validate further attempts to identify the protein in these samples. As expected, Aurora Kinase C transcript was found in all oocyte and embryo samples tested(Fig 20A). All samples were also analyzed for the presence of β-actin as a control for the assay (Fig 20B). Densitometry analysis was used to normalize Aurora Kinase C bands to β-actin bands (Fig 20C). FHM medium was also analyzed as a negative control as some of it may have been introduced to the reactions with the cells.

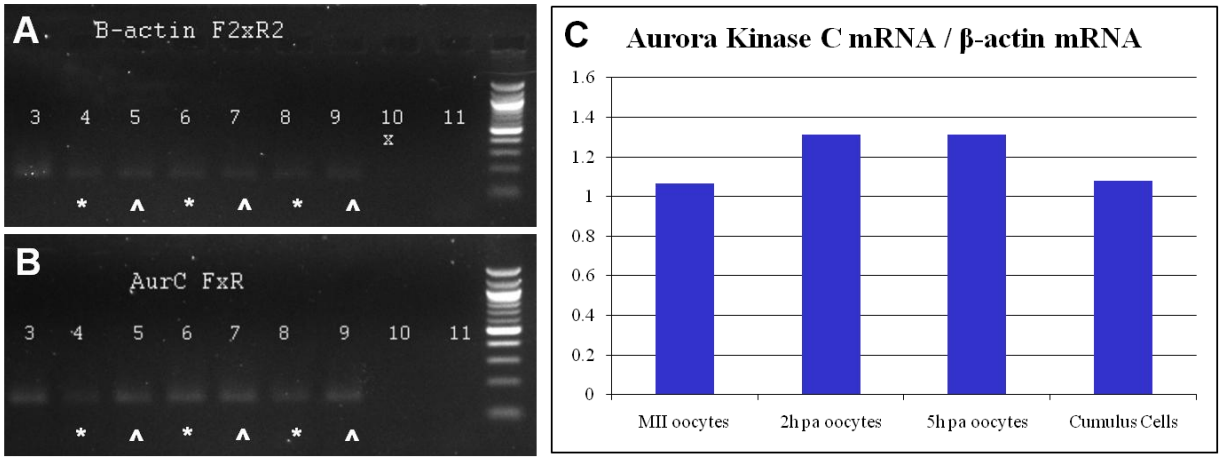

Figure 20: Presence of Aurora Kinase C and β-actin mRNA in mouse oocytes. A: β-actin reaction product on agarose gel. B: Aurora Kinase C reaction product on agarose gel. Lanes are as follows: 3, Cumulus Cells; 4, 10 MII oocytes; 5, 20 MII oocytes; 6, 10 oocytes 2h post activation; 7, 20 oocytes 2h post activation; 8, 10 oocytes 5h post activation; 9, 20 oocytes 5h post activation; 10, blank; 11, Negative Control Water. C: Aurora Kinase C rtPCR reaction product normalized to β-actin rtPCR product through densitometry analysis. n=10 oocytes/sample.

Chapter 4: Simultaneous Inhibition of Multiple Aurora Kinases

Materials and Methods

Oocyte Collection & Culture

Methods were as previously described in Chapter 2.

Hesperadin Treatment

Oocytes were harvested at telophase I or metaphase II stages and treated with 250nM Hesperadin, an inhibitor of all Aurora Kinase proteins (Hauf *et al*., 2003), in the culture medium. Control samples with no treatment or treatment with an inert compound were cultured simultaneously with the experimental group. Telophase I oocytes were collected from ovaries 10h post-hCG injection and cultured for 6h and then activated with 10mM $SrCl_2$ in the culture medium. Oocytes collected and treated at the metaphase II stage (16h post-eCG injection) were immediately cultured with 10mM $SrCl_2$ in the culture medium. Samples were then fixed in 1% paraformaldehyde and 0.15% triton X-100 (see Appendix A) for 30m at 37°C and processed for immunofluorescence as described below. Oocytes collected at the telophase I stage were fixed at 6h and 10h post-harvest. Oocytes collected at the metaphase II stage were fixed at 4h post-harvest.

ZM447439 Treatment

Oocytes were harvested from stimulated ovaries at the GV or metaphase II stages and treated with 20µM ZM447439 (Tocris bioscience #154252) dissolved in DMSO in the culture medium. At this concentration ZM447439 is reported to inhibit Aurora Kinase B, but not Aurora Kinase A (Mortlock *et al*., 2007). The effect upon Aurora Kinase C is unknown. Control samples with no treatment or DMSO only treatment were cultured simultaneously with the experimental group. GV samples were cultured for 20h post-harvest. MII samples were activated with 10mM $SrCl_2$ in the culture medium and matured for 4h post-harvest. Samples were then fixed and processed for immunofluorescence as described below.

Immunocytochemistry

Mouse oocyte and embryo samples were fixed in 1% paraformaldehyde and 0.15% triton X-100 for 30m at 37°C. Samples were subsequently stored at 4°C in a blocking solution (see appendix A) until being further processed. Immunolocalization was performed using antibodies for Aurora Kinase B (AurB), Histone H3 (phosphorylated on serine 10, phospho H3), and microtubules (Tub). Chromatin was labeled using Hoechst 33258 (Molecular Probes #H-1398) as described previously. Aurora Kinase B was detected with primary polyclonal

anti-AurB antibody (Abcam #ab2254, 1:500 dilution). Phosphorylated Histone H3 was detected with a polyclonal rabbit anti-Ser10 phospho H3 antibody (Upstate #06-570, 1:200 dilution). The primary probes, AurB or phospho H3, were followed by a secondary Alexa fluor 594 labeled goat anti-rabbit antibody (Invitrogen #A11072, 1:400 dilution). A mixture of primary monoclonal anti-α-tubulin and anti-β-tubulin antibodies (Sigma-Aldrich Co #T-9026 and #T-4026 respectively, 1:1000 dilution) followed by a secondary Alexa fluor 488 labeled goat anti-mouse antibody (Invitrogen #A21121, 1:400 dilution) was used to label microtubules. F-actin was labeled using of 8U/mL rhodamine phalloidin (Molecular Probes #R-415). Labeled eggs were then mounted onto slides and analyzed using a Zeiss Axiovert 200M inverted microscope coupled to a Roper CoolSnapFx high resolution CCD camera. Images were captured and rendered in real time using Axiovision software.

Results

Hesperadin Treatment

The compound Hesperadin is known to inhibit the activity of all three of the Aurora Kinase proteins (Hauf *et al*., 2003). Mouse oocytes were treated with Hesperadin to determine the extent and nature of functional loss of the Aurora Kinase proteins as oocytes underwent the process of meiosis. Phosphorylation status of Histone H3 on Serine 10 was monitored as an indicator of Aurora Kinase B activity. Although roles for individual Aurora Kinases were not distinguishable, the Hesperadin studies did reveal the broad impact of Aurora Kinase inhibition during progression of meiosis in mouse oocytes.

Metaphase II (MII) oocytes were treated with Hesperadin to ascertain the effect of Aurora Kinase inhibition in the completion of M-phase. Analysis of the treated and control samples revealed that Hesperadin treatment had little effect on these oocytes. At 4h post-treatment and $SrCl_2$ activation the Hesperadin treated MII oocytes appeared to proceed through M -phase without delay or defect. The extrusion of the second polar body occurred on time (Fig 21A-C) and the chromosomes appeared to segregate normally (Fig 21G-H). Histone H3 phosphorylation was not detected in any of the samples analyzed most likely due to the stage of the cell cycle (Fig 21D-F). Analysis of the oocytes gross morphology and meiosis I completion through immunocytochemistry did not reveal any differences between experimental and control oocytes. This suggested that either the Aurora Kinase proteins did not have a role in the completion of meiosis in mouse oocytes or that the timing of the treatment did not allow it to have an effect. Given that at the MII stage the spindle is already

assembled and the chromosomes have aligned, forming the metaphase plate, it is possible that the Hesperadin compound was unable to inhibit the Aurora Kinase proteins from participating in the completion of M-phase of the cell cycle.

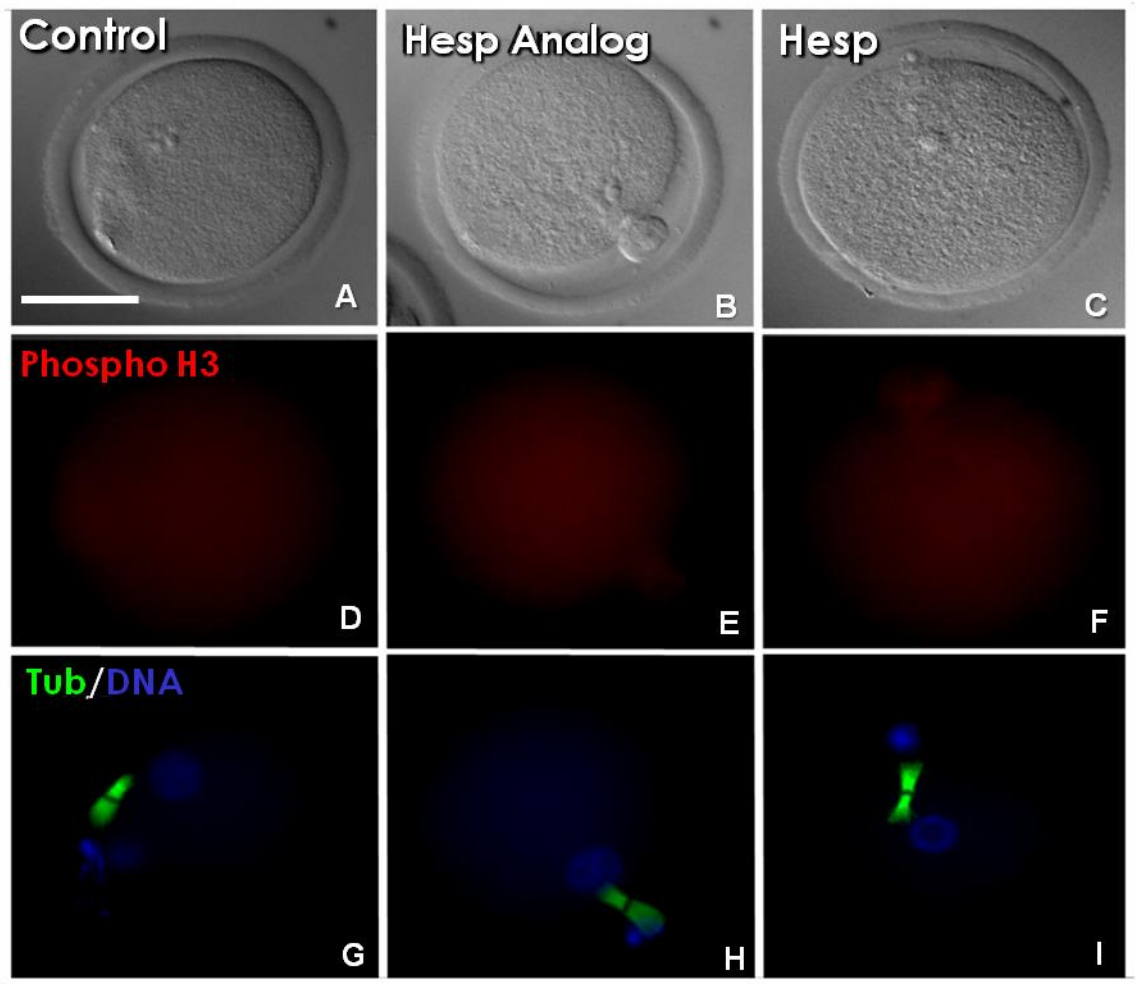

Figure 21: Mouse oocytes treated with Hesperadin (Hesp) at the MII stage and controls fixed at 4h post-treatment. A, D, G – Controls cultured in medium. B, E, H – Controls cultured in inert compound and vehicle in medium. C, F, I – Hesperadin treated samples. There is no discernable difference between control oocytes (untreated), inert Hesperadin analog treated oocytes, and Hesperadin treated oocytes in gross morphology (A-C) or protein staining patterns (phospho H3 D-F and Tubulins (Tub)/chromatin (DNA) G-H). Scale bar represents ~40µm.

In order to determine if the lack of observable aberrant phenotype in the MII Hesperadin treated oocytes was due to the timing of the treatment in the cell cycle, telophase I (TI) stage oocytes were treated with Hesperadin. This would allow the compound to be present earlier when the meiosis II spindle was assembled. Analysis of TI oocytes

revealed that Hesperadin treatment resulted in aberrant completion of meiosis. At 6h post-treatment the oocytes appeared morphologically normal when observed by vital phase contrast microscopy (Fig 22C). However, immunocytochemical analysis revealed that the MII spindle was abnormally shaped and lacked any distinct poles (Fig 22I). The chromatin was condensed and associated with the mictotubules, but not in an organized fashion. Histone H3 was not phosphorylated which indicated inhibition of Aurora Kinase B (Fig 22F). Control oocytes displayed a normal MII morphology with bi-polar spindles, metaphase plates, and phosphorylated of Histone H3 (Fig 22A-B, D-E, G-H).

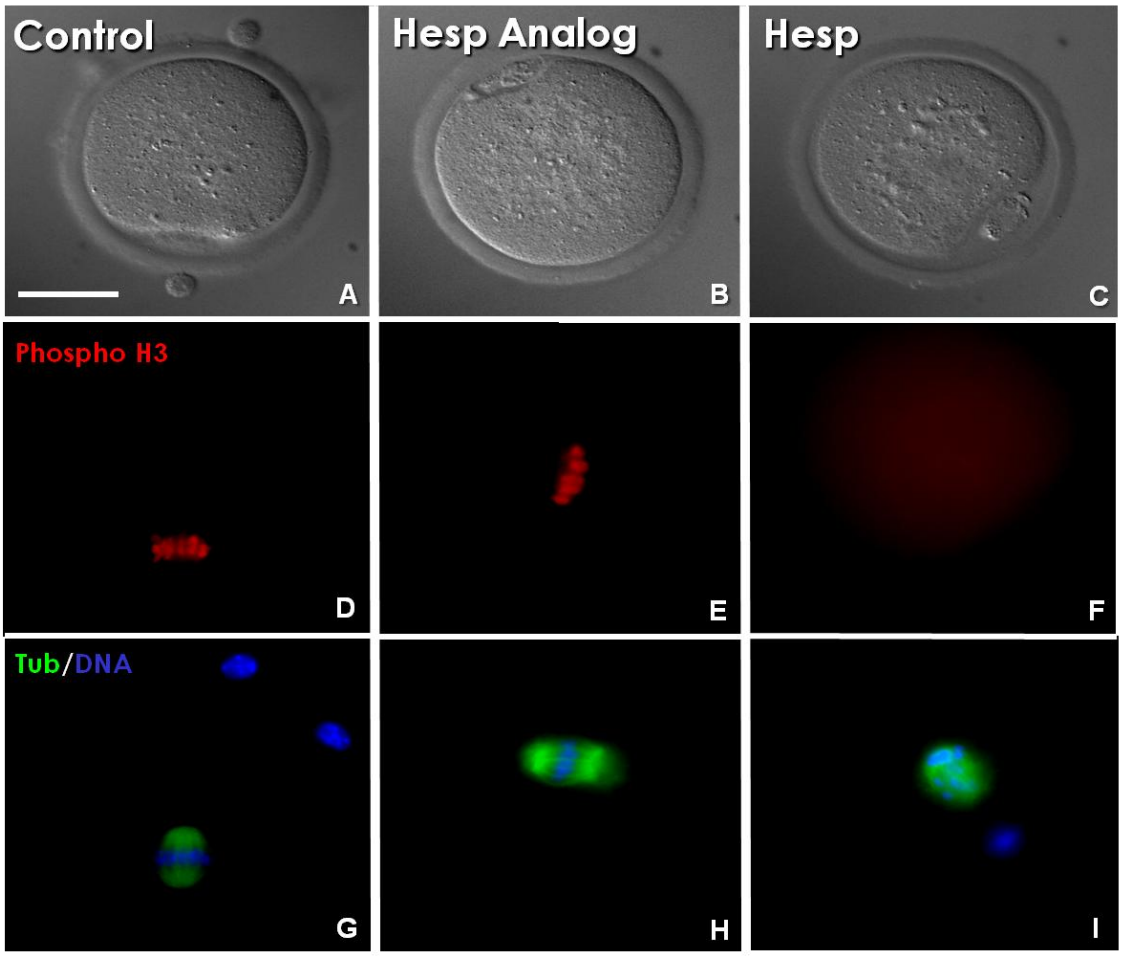

Figure 22: Mouse oocytes treated with Hesperadin (Hesp) at the TI stage and controls fixed at 6h post-treatment. A, D, G – Controls cultured in medium. B, E, H – Controls cultured in inert compound and vehicle in medium. C, F, I – Hesperadin treated samples. Control oocytes and inert Hesperadin analog treated oocytes display a typical MII stage gross morphology (A & B) with bi-polar spindles and chromatin aligned on a metaphase plate (G & H). Serine 10 of Histone H3 is phosphorylated (D & E). Hesperadin treated oocytes display an abnormal phenotype despite appearing to have a similar gross morphology to control oocytes (C). Microtubules are disorganized without discernable spindle poles, chromatin is not aligned (I), and there is absence of Serine 10 phosphorylation on Histone H3 (F). Scale bar represents ~40µm.

Oocytes that were exposed to Hesperadin at the TI stage were further affected at 10h post-treatment. Non-treated $SrCl_2$ activated control oocytes completed M-phase as revealed by extruding a second polar body. Although experimental and control oocytes were not phenotypically distinguished from each other when viewed by bright field microscopy (Fig 23A-C), immunocytochemistry exposed severe defects in meiosis completion in Hesperadin treated oocytes. Polymerized microtubules were arranged into a complex

network throughout the cytoplasm rather than forming a midbody as was observed in control oocytes (Fig 23I). There was no evidence of extrusion of the second polar body and the chromatin appeared to be in one large cluster suggesting that neither spindle formation nor chromosome alignment has occurred. Control oocytes displayed a normal interphase morphology with elongated, disintegrating spindles and de-condensed chromatin (Fig 23G-H). Histone H3 was not phosphorylated on Serine 10 in experimental or control samples at this time point most likely due to the stage of cell cycle (Fig 23D-F).

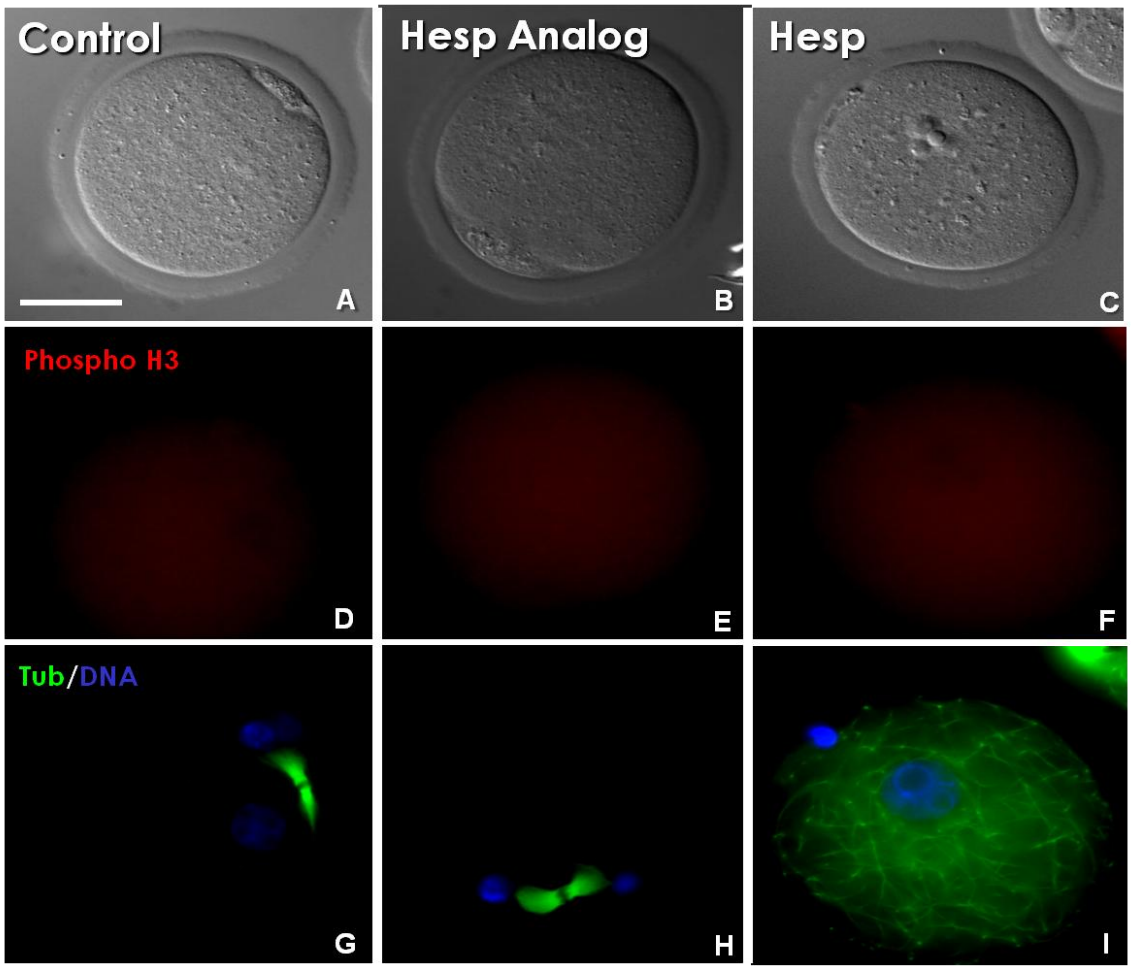

Figure 23: Mouse oocytes treated with Hesperadin (Hesp) at the TI stage and controls fixed at 10h post-treatment. A, D, G – Controls cultured in medium. B, E, H – Controls cultured in inert compound and vehicle in medium. C, F, I – Hesperadin treated samples. Control oocytes (untreated) and inert Hesperadin analog treated oocytes display a typical interphase morphology. Whereas, hesperadin treated oocytes display an abnormal phenotype. Scale bar represents ~40µm.

ZM447439 Treatment

The compound ZM447439 (ZM) is known to inhibit Aurora Kinase B, but not thought to affect Aurora Kinase A when used at 20µM concentration (Mortlock *et al*., 2007). The effect upon Aurora Kinase C at this concentration is not known. Experiments conducted with ZM allow the further dissection of the role of Aurora Kinase proteins in the completion of meiosis in mouse oocytes. Staining for tubulin, actin, and chromatin was chosen because it allowed for the study of spindle formation, chromatin separation, and polar body extrusion in the oocytes.

Metaphase II (MII) oocytes were treated with 20µM ZM compound to elucidate the role of Aurora Kinases B (and/or C) in the completion of meiosis after fertilization or activation. Analysis of the treated samples at 4h post-activation and treatment revealed oocytes that had not properly completed meiosis (Fig 24B). It was apparent that the tubulin spindle formed prior to the MII stage had been disrupted and tubulin protein was now organized in to a complex matrix throughout the cytoplasm. There was no evidence of extrusion of polar body II and the chromatin appeared to be concentrated in one area. This indicated that ZM was able to disrupt an assembled spindle and that both karyokinesis and cytokinesis had failed in the treated oocytes. Control oocytes completed meiosis as normal (Fig 24A). Polar body II was extruded along with approximately half of the chromatin. The remnant of the tubulin spindle was elongated and had formed a midbody between the oocyte and second polar body.

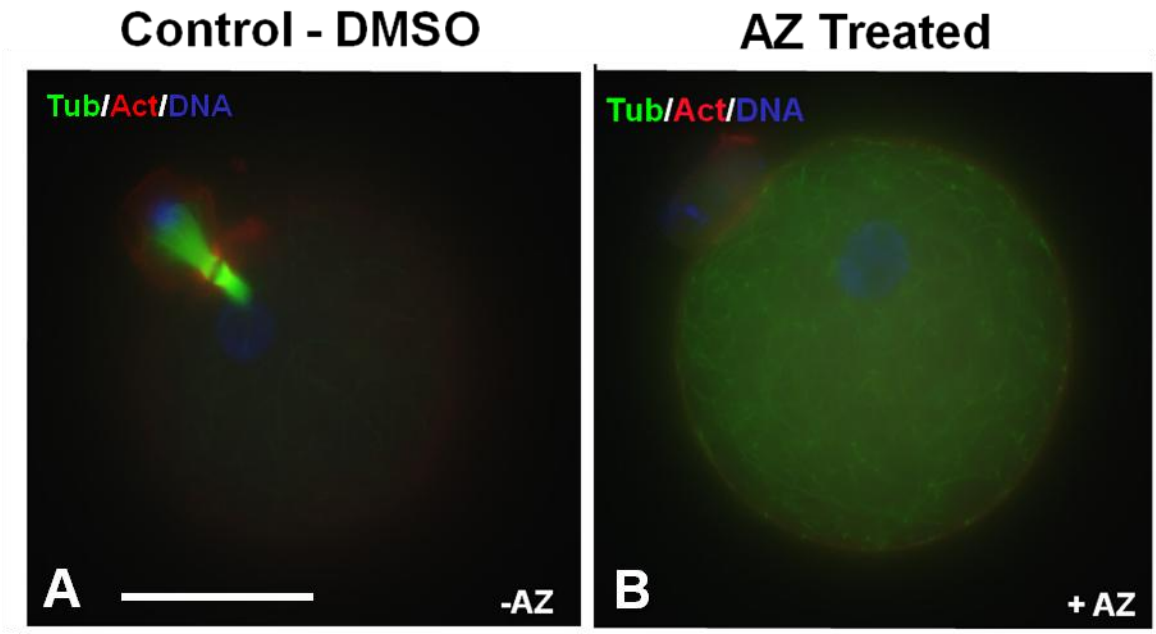

Figure 24: Mouse oocytes activated and treated with ZM447439 at the MII stage and controls treated with DMSO fixed at 4h post-treatment and activation. A – Control oocytes and display a typical interphase morphology. B – ZM447439 treated oocytes display an abnormal phenotype. Scale bar represents ~40µm.

Germinal vesicle (GV) stage oocytes were also treated with 20µM ZM compound to determine the role of Aurora Kinase B and/or C in the earliest stages of meiosis in mouse oocytes. At 20h post-treatment the ZM treated GV oocytes appeared to have arrested and displayed a morphology much like that of a newly harvested GV stage oocyte (Fig 25B). There was no evidence of germinal vesicle break down or DNA condensation. Tubulin was distributed throughout the cytoplasm without any indication of spindle formation. Control oocytes treated with DMSO in the medium and cultured for 20h post-treatment show a typical metaphase II morphology (Fig 25A). The tubulin is organized into a bi-polar spindle and all chromatin is condensed and aligned at the metaphase plate. The first polar body extruded successfully along with chromatin. The control oocytes appear to have progressed normally through M-phase to the MII checkpoint and arrested.

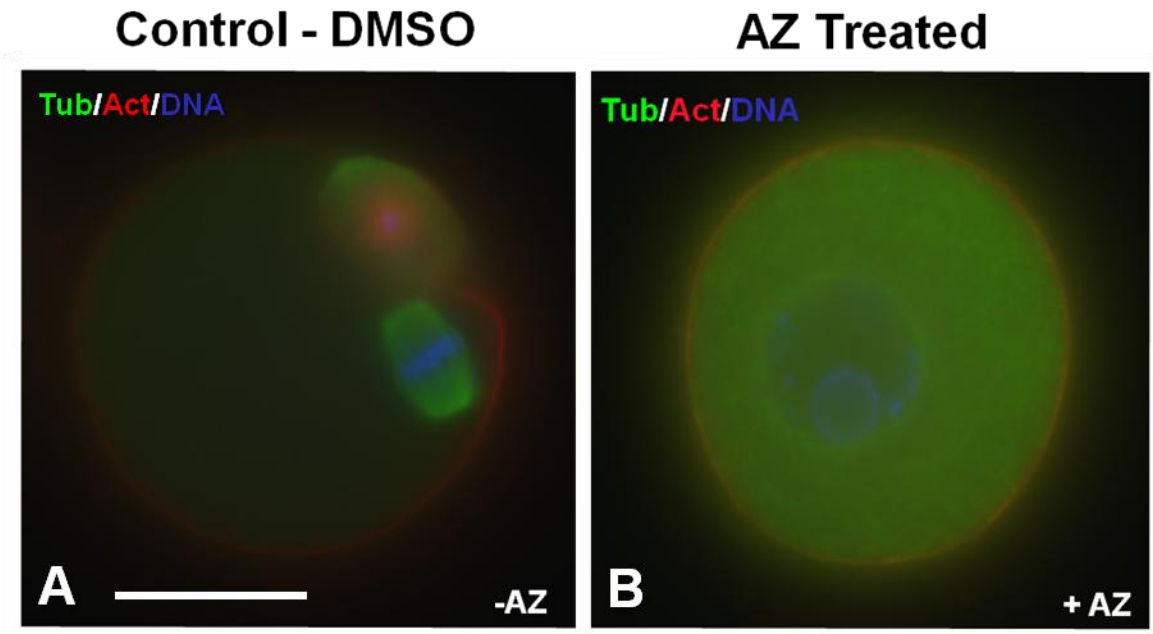

Figure 25: Mouse oocytes treated with ZM447439 at the GV stage and control treated with DMSO fixed at 20h post-treatment. A – Control oocytes and display a typical MII morphology. Tubulin has formed a bi-polar spindle and the chromatin is condensed and aligned on the metaphase plate. B – ZM447439 treated oocytes display an abnormal phenotype that appears to be much like a GV oocyte without any progression into meiosis. Scale bar represents ~40µm.

Chapter 5: Discussion and Conclusions

The purpose of these studies was to elucidate the localization and function of several of the Aurora Kinase family proteins in during late oogenesis and early embryonic development in the mouse. An understanding of the roles of these key regulatory kinases would be useful for several *in vitro* applications including maturation of oocytes, culture of early embryos, and oocytes manipulated for somatic cell nuclear transfer. Results of these studies have established that the localization pattern of Aurora Kinase B in mouse oocytes does not mimic the pattern observed in somatic cells. For example, Aurora Kinase B was not present in the oocytes or embryos until the 4-cell embryos cleaved to 8-cells. The timing of the appearance coincides with the activation of the embryonic genome in mouse embryos. In contrast, the localization and expression of Aurora Kinase C proved more difficult to uncover. The apparent low abundance of Aurora Kinase C in somatic cells proved to be a major obstacle for studies in mouse oocytes and embryos. However, a functional role for Aurora Kinase proteins B (and/or C) was demonstrated in mouse oocytes and embryos. The inhibition of Aurora Kinase B mRNA with specific targeting siRNAs revealed a role for the gene product in the first cleavage events of embryos. Broad, simultaneous drug inhibition of Aurora Kinases also demonstrated the functional requirement of the Aurora Kinase family during late oogenesis. Tubulin assembly, chromatin organization, and cytokinesis were all affected leading to failure of proper cell division. When considered as a whole, this work reveals new information about the Aurora Kinase family in mouse oocytes and embryos. In particular, the absence of Aurora Kinase B in meiosis II oocytes and the functional roles of Aurora Kinase B and C in oogenesis and early embryogenesis are both valuable pieces of information. Understanding the endogenous expression patterns of the Aurora Kinase mRNAs and proteins in mouse oocytes and early embryos elucidates important controls of the cell cycle in these cells. Further knowledge of the properties of proteins in control of the cell cycle in oocytes and embryos will allow for future work on a wide variety of topics such as somatic cell nuclear transfer, assisted reproductive techniques, and enhanced agricultural breeding.

Aurora Kinase B

Protein Expression and Localization

The experimental studies designed to illustrate the characteristics of Aurora Kinase B showed unexpected results, but these results were confirmed through the use of several different techniques. The working hypothesis was that Aurora Kinase B in mouse oocytes and embryos would function much like what is observed in somatic cells. This hypothesis

was first challenged when immunocytochemical analysis showed that Aurora Kinase B was not found to be localized to the meiotic spindle in metaphase II stage mouse oocytes despite clear evidence of control staining of mouse embryonic fibroblasts that displayed typical Aurora Kinase B localization. Aurora Kinase B protein was found co-localized with centromeres at metaphase, remained at the spindle midzone during anaphase and early telophase, and was on the narrowing midbody during late telophase. These findings were further supported by analysis of protein expression using Western blotting methods. The apparent absence of Aurora Kinase B protein in mouse oocyte samples demonstrated that not only is the protein not localized, it is not detectable based on the high resolution methods used. This also showed that the diffuse punctate staining pattern observed in the mouse oocytes is not likely Aurora Kinase B given that it is not detected in any quantity or form on Western blots.

Aurora Kinase B protein was not detected in early embryo samples. From the 1-cell fertilized stage to approximately the 4-cell stage Aurora Kinase B protein was undetectable. As the 4-cell embryos cleaved to the 8-cell stage Aurora Kinase B appeared and was localized on the midbody between the dividing cells as is seen in the control mouse embryonic fibroblast cell samples. Given the timing of appearance of Aurora Kinase B protein, one explanation is that the protein is produced as a result of the activation of the zygotic genome. In mice, zygotic gene activation is initiated at approximately the 2-cell stage. Prior to activation, it is generally accepted that all of the proteins used by the embryo are of maternal origin since the embryonic genome is silent (Bettegowda & Smith, 2007). The appearance of Aurora Kinase B protein post-zygotic genome activation indicates that the protein is not necessary for proper cell cycling in the early cleavage stage embryo.

This data is contrary to what has been previously reported for other chromosome passenger complex proteins in mouse oocytes and early embryos. Survivin protein, another member of the chromosomal passenger complex, has been found in mouse oocytes currently undergoing meiosis (Sun *et al*., 2009). Localization of survivin protein in mouse oocytes was found to be similar to the pattern observed in somatic cells. Survivin was observed to be co-localized with centromeres during germinal vesicle breakdown in early meiosis, and then remained at the spindle midzone as the chromatin migrated to the poles of the spindle. Finally, survivin protein was observed at the spindle midbody just before the completion of cytokinesis, or excision, as is observed in somatic cells (Sun *et al*., 2009). Aurora Kinse B has also been found present and localized in mouse oocytes, but only in meiosis I stage oocytes (Shuda et al., 2009). These data, along with the Aurora Kinase B immunocytochemistry data discussed above above, indicate that it is not the entirety of the chromosomal passenger complex that is absent in mouse oocytes, but that the complex

consists of different components in these cells. Other members of the chromosomal passenger complex have not been studied in mouse oocytes, and future analyses of proteins such as INCENP and Borealin may provide further insight into the makeup and function of the chromosomal passenger complex in mouse oocytes performing meiosis.

mRNA Expression and Relative Quantitation

Although this work established that Aurora Kinase B protein is not detectable in the mouse oocyte or the earliest embryos through complimentary immunohistochemistry and Western blotting analysis, further studies were warranted to determine if Aurora Kinase B mRNA was present, but not translated, or if it was also not expressed. Reverse transcriptase PCR and quantitative-rtPCR were both employed to uncover the details of Aurora Kinase B mRNA in mouse oocytes and embryos. Both approaches showed that Aurora Kinase B mRNA is indeed present in both oocytes and embryos. This suggests that Aurora Kinase B protein is absent due to a transcriptional control rather than absence of transcript. Details of the mechanism and proteins associated with this regulation are currently unknown and further studies with inhibitors of transcription may be useful to address this question.

When compared to β-actin, Aurora Kinase B mRNA is found in very low abundance in oocytes and embryos assayed. Aurora Kinase B mRNA levels remain unchanged form GV stage through fertilization to the 1-cell stage. There is an evident decrease in Aurora Kinase B mRNA at the 2-cell stage which is most likely attributed to the degradation of maternal transcripts. This pattern of mRNA quantification is commonly observed in mouse oocytes subjected to qrt-PCR analysis. SEBOX, a regulator of transcription, is observed in a similar expression level outline. A slight decrease of SEBOX mRNA is observed between the 1- and 2-cell stages and then the mRNA becomes nearly undetectable at the 4-cell stage (Kim *et al*., 2008). Peroxiredoxin II mRNA also follows a comparable expression profile. The peroxiredoxin family has been found to play roles in eliminating reactive oxygen species, intracellular signal transduction, and control of cell proliferation (Kang *et al*., 1998). In mouse oocytes and early embryos peroxiredoxin II mRNA is found present fro the germinal vesicle stage through the 2-cell stage. From the 4-cell stage forward the mRNA is not able to be detected (Wang *et al*., 2010).

Aurora Kinase B transcript levels recover at the morula and blastocyst stages after the activation of the embryonic genome. β-actin transcript levels mimic Aurora Kinase B in the oocyte and early embryo stages, but after the activation of the zygotic genome there is a marked increase in level of β-actin mRNA in the embryos. The comparative low levels of Aurora Kinase B transcript in the later stage embryos may be due to the slowing of the cell cycle in the embryonic cells as well as the tendency toward asynchrony of the cell cycles in

the many embryonic cells. There are other reports in the literature of mitotic cell cycle control proteins being upregulated after the activation of the zygotic genome, namely retinoblastoma. This protein controls the mitotic cell cycle by inhibiting the cell from progressing from G1 to S phase. The mRNA transcript of this gene is found in oocytes, decreases at zygotic genome activation, and is again propagated from the 8-cell stage onward (Xie et al., 2005). This closely mimics the pattern observed for Aurora Kinase B mRNA in mouse oocytes and early embryos.

Loss of Function

The results observed in the mouse embryos treated with various concentrations of Aurora Kinase B targeting siRNAs appeared in a dose dependant manner. Blastomeres subjected to higher concentrations of Aurora Kinase B inhibitory RNAi (57μM and 40μM) appeared to have immediate difficulty completing cytokinesis. In addition, the entire embryo ceased development shortly after one cell division. Reducing the concentration of the RNAi solution to 20μM mitigated the resultant effects, but did not completely suppress the phenotype. Embryos were able to continue development and perform compaction and early blastocyst formation. While the experimental embryos progressed through several developmental stages, the cells comprising these embryos were not comparable to control embryos. Similarly to the embryos treated with more concentrate RNAi solution, cytokinesis defects were evident.

Embryos depleted of Aurora Kinase B mRNA through the use of RNAi knockdown showed striking differences when compared to control embryos. Interestingly, the experimental embryos displaying an abnormal phenotype were at developmental stages prior to when Aurora Kinase B protein is detected. Given that in these stages of embryos there is no detectable Aurora Kinase B protein, these results suggest that the observed effect may be due to an off-target effect of the siRNA, a function of the Aurora Kinase B mRNA that is lost upon its degradation, or that Aurora Kinase B protein is present and unable to be detected with the methods employed. The likelihood of an off-target effect of the siRNA is small. In particular, the quantification of β-actin mRNA in the treated and control embryos did not show any differences. While this is one transcript, amongst thousands of mRNAs, in the mouse embryo, its stable quantification between experimental and control embryos demonstrates that there is no global decrease in the mRNA quantity in the treated embryos. The siRNA used was a pool of 3 constructs commercially designed to specifically target Aurora Kinase B. The use of more than one construct at the same time diminishes the outcome of a possible off-target effect of one of the constructs. Microarray analysis of the treated and control embryos would allow for the comparison of many genes to better

determine the specificity of the siRNA pool used. If Aurora Kinase B protein is present in the embryos, but not detected, the use of different antibodies, alteration of staining technique, or more sensitive detection methods may reveal the presence and localization of the protein.

It is well known that RNAs can have an additional function as well as their role in gene expression. It has been previously shown that coding RNAs have structural functions in the assembly of the microtubule spindle and chromatin organization in oocytes and embryos of *Spisula* (surf clam), *Xenopus,* and *Drosophila*. *Spisula* embryos were found to have centrosome-associated RNAs which are thought to participate in the assembly of the mitotic sprindle. In *Xenopus* oocytes microtubule-associated RNAs were found and inhibition of translation did not alter their localization (Kloc, 2009). If the Aurora Kinase B mRNA is playing a role similar to any of these examples, its depletion via the siRNA constructs may yield a phenotype much like what was observed.

In addition to the activities of coding RNAs, many RNAs that were previously thought to be non-functional or untranslated, are now being found to be a regulatory mechanism (Keene, 2010). There is evidence that there may be specific microRNAs (miRNAs) in pluripotent cells, such as those in an embryo, that are not found in differentiated cells. Many of these miRNAs have been implicated in differentiation of different cell types and the proliferation of cells from the pluripotent original cell (Foshay and Gallicano, 2007). If the siRNA pool used to target Aurora Kinase B had an off-target effect upon one of these miRNAs it would not be evident in somatic cells, but would cause distinct defects in the development of embryos. This is a possible cause for the phenotype observed in cells microinjected with Aurora Kinase B targeting siRNA. To further investigate this prospect, microarray analysis of miRNAs in the embryo could be conducted.

While this preliminary data suggests that Aurora Kinase B may play a pivotal role in the cell division of early mouse embryos, there are several deficiencies which must be remedied before conclusions may be drawn. It is assumed that the larger cells observed in the experimental microinjected embryos are the blastomeres that were treated with Aurora Kinase B siRNA solution. Without a tracking system for the microinjected cell, it is impossible to know which cells are derived from treated or untreated blastomeres. Once this obstacle is remedied, further studies can be conducted and significant sample numbers can be collected for each category at several time points.

Aurora Kinase C

The studies focused upon Aurora Kinase C protein proved far less fruitful than those concentrated on Aurora Kinase B. Few conclusions can be drawn from the many experiments

performed due to the fact that a reliable positive control was unable to be found. Somatic cells have a very low level of Aurora Kinase C making them a poor positive control. The low levels of the protein make it difficult to reliably detect with a variety of methods. Given the known upregulation of all Aurora Kinase proteins in many types of tumors, it may prove useful to investigate them as a positive control. A mouse tumor cell line would be ideal to ensure that the probes used detect the protein in the species being studied. Given the interesting results found when studying Aurora Kinase B in mouse oocytes and embryos, Aurora Kinase C investigations may unveil exciting new information about cell cycle mechanisms and controls in these cells. The absence of Aurora Kinase B in meiosis II oocytes and early embryos along with the successful progression of the cell cycle in these cells may indicate that another protein, such as Aurora Kinase C, could be compensating. Aurora Kinase C has been shown to be able to compensate for the loss of Aurora Kinase B in HeLa cells (Sasai *et al*., 2004). It is possible that this scenario is occurring in oocytes and early embryos as well. Additional antibodies targeted to different regions of the protein or transgenic animals with fluorescently tagged Aurora Kinase C protein may help to unveil the properties of this protein in mouse oocytes and early embryos.

Inhibition of Multiple Aurora Kinases

In order to further study the effects of the loss of the Aurora Kinase proteins in mouse oocytes chemical compounds were used to simultaneously inhibit multiple members of the family. Oocytes treated with Hesperadin or ZM447439 showed marked defects catastrophic to development. While it is impossible to know exactly which of the Aurora Kinase family members is responsible for the observed effects, it is possible to compare the results with previous studies targeting a single protein. Yao et al. (2004) showed that inhibition of Aurora Kinase A protein in mouse oocytes through the use of monoclonal antibodies produced a variety spindle defects. Cytokinesis defects were not noted in these oocytes. When Hesperadin was used with telophase I oocytes spindle defects were observed as well as the failure of cytokinesis. Clearly, a protein in addition to Aurora Kinase A is being affected in these oocytes. The results of Shuda *et al*. show that Aurora Kinase B protein is involved in meiosis I, but not meiosis II (Shuda *et al*., 2004). Therefore, the defect observed with broad Aurora Kinase inhibitors in meiosis II can not be attributed to Aurora Kinase B inhibition. These previous data, coupled with the findings of Shuda *et al*. and our own results, indicating that Aurora Kinase B protein is not present during meiosis II, one can theorize that Aurora Kinase C may be the inhibited protein.

Similar effects were observed in oocytes that were treated with ZM447439, which is specific to Aurora Kinase B and perhaps affects Aurora Kinase C. Aurora Kinase A is not

affected by this inhibitor (Mortlock *et al*., 2007). The meiotic spindle failed to organize in these oocytes. Even with ample time to develop, meiosis did not continue in these oocytes and ultimately there was no cytokinesis or karyokinesis evident. Given that Aurora Kinase A is not affected in these studies, the spindle assembly failure must be attributed to another inhibited protein. It has been previously shown that Aurora Kinase C protein is responsible for proper chromatin segregation and cytokinesis during meiosis in developing sperm cells (Kimmins *et al*., 2007). It is possible, although not yet proven, that Aurora Kinase C may play this same role during meiosis in developing oocytes. The data of Shuda *et al*. explain that Aurora Kinase B inhibition may be the responsible for the observed phenotype. Unfortunately, Shuda *et al*. were not able to confirm the presence of Aurora Kinase B with immunocytochemistry or Western blotting, but rather relied upon the microinjection of a fluorescently tagged mRNA to indicate protein localization (Shuda *et al*. 20079). Given the difficulties had identifying the native protein with several methods, it must be considered that the native protein may not be present in the oocytes. The microinjected fluorescently tagged mRNA could have been translated in the treated oocytes, giving the impression that the protein localized to the spindle. In summary, the failure of the oocytes treated with ZM447439 to enter GVBD and progress through M phase may be due to the inhibition of Aurora Kinase B or C protein.

Conclusions

The results of these studies suggest a possible role for Aurora Kinase C in the maturation of mouse oocytes and early embryo development, and illustrate the importance of future studies of this protein during oogenesis. Aurora Kinase C may prove to be a very important player in the orchestration of a successful cell cycle in mouse oocytes and early embryos given its previously demonstrated ability to compensate for the lack Aurora Kinase B (Sasai *et al*., 2004) and its role in spermatogenesis (Kimmins *et al*., 2007). This also demonstrates the importance of an Aurora Kinase C positive control. Our data show that there are distinct differences in the members of the chromosomal passenger complex in mouse oocytes and mouse embryonic fibroblast somatic cells. These findings represent a significant advancement in the understanding of cell cycle control in developing oocytes and the differences in meiosis and mitosis. This insight becomes especially important when somatic cells and oocytes are combined through the process of somatic cell nuclear transfer. Typically, a somatic nucleus is inserted into a cytoplast produced from a meiosis II oocyte. As this reconstructed embryo divides the somatic chromatin must reprogram to develop the embryonic organism. In somatic cells the chromosomal passenger complex includes Aurora Kinase B protein, but the embryonic chromosomal passenger complex in oocytes at this

stage does not. This reprogramming may not occur efficiently and therefore impact the ability of the developing embryo to properly undergo cell division. Future experiments including the study of the chromosomal passenger complex in somatic cell nuclear transfer reconstructed embryos will further elucidate the members and status of this complex in manipulated embryos.

Further experimentation to discover the role of Aurora Kinase B mRNA in early embryo samples is also an interesting topic. It was shown via several different methods that Aurora Kinase B protein is absent in embryos prior to zygotic genome activation. Despite this finding, the depletion of Aurora Kinase B mRNA in one blastomere of a 2-cell embryo produced a profound effect on early embryos. Future experiments to uncover the finctions and interactions of this mRNA may also yield important information about the control of the cell cycle of early embryos. Microarray analysis of the Aurora Kinase B mRNA depleted embryos would allow for the simultaneous study of many transcripts. This may show transcripts that the siRNA is targeting as well as transcripts that are affected by the loss of Aurora Kinase B mRNA. These proposed studies would expand upon the results of the current investigations as well as increase overall understanding of cell cycle mechanisms and control in mouse oocytes and embryos.

Appendix A

Solutions

Stabilization Buffer (SB)

Composition:

100mM PIPES
5mM $MgCl_2$
2.5.mM EGTA

Preparation: 10mL 1x SB

0.3024g PIPES (Sigma #P-6757, MW 302.4)
0.0102g $MgCl_2 \cdot 6H_2O$ (Sigma #M-2393, MW 203.3)
0.0095g EGTA (Sigma #E-4378, MW 380.4)

Microtubule Stabilization Buffer – Extraction Fixative (MTSB-XF)

This fixative is designed to diminish changes in microtubule structure as a result of fixation while effectively fixing and extracting the cells.

Composition:

100mM PIPES
5mM $MgCl_2$
2.mM EGTA
1mM DTT (Sigma #D-0632, FW 154.2)
1µM Taxol
0.01% Aprotinin
50% Deuterium Oxide
3.7% Formaldehyde
0.1% Triton X-100

Preparation: 1mL MTSB-XF

200µL 5x Stabilization buffer
50µL Aprotinin 0.2%
50µL DTT 20mM
50µL Taxol 20µM
500µL Deuterium oxide 99.9%
50µL Triton X-100 2%
100µL Formaldehyde 37%

Store at 4°C. Solution expires 3 weeks from date of preparation.

Immunocytochemistry Blocking Solution

Composition:

0.2% Sodium azide
1% (w/v) Bovine serum albumin, Fraction V (BSA, Sigma #A-6793)
0.2% (w/v) Powdered milk (non-fat dry milk, Carnation brand)
2% (v/v) Normal goat serum (heat-inactivated, Sigma #S-6898)
0.1M Glycine (Sigma #G-7126, MW 75.1)
0.01% Triton X-100

Preparation: 50mL Immunocytochemistry Blocking Solution

49mL Dulbeccos'a Phosphate-Buffered Saline
0.098g Sodium azide
0.1g Powdered milk
1mL Normal goat serum
0.5g Bovine serum albumin fraction V
500µL Deuterium oxide 99.9%
50µL Triton X-100 2%
100µL Formaldehyde 37%

Store at 4°C. Solution expires 3 weeks from date of preparation.

Western Blotting Blocking Solution

Tris-Buffered Saline (TBS, 10x)

Composition:

250mL 1M Tris-HCl pH 8.0 (Sigma #T5941)
80g NaCl (Sigma #9625)

Dilute to 1x with distilled H_2O

Tris-Buffered Saline with 0.1% Tween-20 (TBS-T, 1x)

Composition:

100mL 1x TBS
100µL Tween-20 (Fisher #BP337)

Western Blotting Blocking Solution

Composition:

50mL TBS-T
2.5g Powdered milk

Store at 4°C.

FHM HEPES Buffered Medium with Phenol Red and amino acids (Millipore #MR-024-D)

Working pH range 7.2 - 7.4

Composition: (mg/L)

Inorganic Salts	
$CaCl_2$-$2H_2O$	251.00
KCL	186.00
$KH2PO_4$	47.60
$MgSO_4$ (anhyd)	24.10
$MgSO_4$-$7H_2O$	---
NaCl	5550.00
$NaHCO_3$	336.00

Amino Acids	
L-Glutamine	146.00

Antibiotics	
Penicillin G NaSalt (u/L)	100,000.00
Streptomycin Sulfate	50.00

Other Components	
BSA	1000.00
EDTA	3.80
D-Glucose	36.00
HEPES	4760.00
Hyaluronidase	---
Ca Lactate	---
Na Lactate 60% (ml/L)	1.86
Lactate NaSalt (ml/L)	---
Sodium Pyruvate	22.00
Phenol Red	10.00

KSOM Medium with amino acids (Millipore #MR-106-D)

Working pH range 7.2 - 7.4

Composition: (mg/L)

Inorganic Salts		**Other Components**	
$CaCl_2$-$2H_2O$	250.00	EDTA	3.72
KCL	186.38	D-Glucose	36.03
KH_2PO_4	47.99	Sodium Lactate	1121.00
$MgSO_4$	---	Lactate NaSalt (ml/L)	---
$MgSO_4$ $7H_2O$	49.30	Sodium Pyruvate	22.00
NaCl	5551.80	BSA	1000.00
$NaHCO_3$	2100.25	Phenol Red	----

Amino Acids			
L-Arginine	63.20	L-Serine	5.26
L-Cystine	12.02	L-Threonine	23.82
L-Cystine-2HCL	---	L-Tryptophan	5.11
L-Glutamine	146.15	L-Tyrosine	18.12
Glycine	3.75	L-Tyrosine NaH_2O	---
L-Histidine	---	L-Valine	23.42
L-Histidine.HCl.H_2O	20.96	L-Alanine	4.45
L-Isoleucine	26.23	L-Asparagine	---
L-Leucine	26.24	L-Asparagine-H_2O	7.50
L-Lysine	---	L-Aspartic Acid	6.66
L-Lysine.HCl	36.52	L-Glutamic Acid	7.36
L-Methionine	7.46	L-Proline	5.76
L-Phenylalanine	16.52		

Antibiotics	
Pen G Na Salt (units)	100,000.00
Strep Sulfate	50.00

Appendix B

Raw Ct Values

Raw Ct values are provided for Aurora Kinase B and β-actin. Raw CT Values were obtained as described in Chapter 2. Inverted CT values were calculated as: Inverted Ct = 40-Raw Ct.

Aurora Kinase B			
	Raw Ct	**Inverted Ct**	**Std Error**
GV	33.409	6.591	0.110
MII	33.294	6.706	0.335
1 Cell	33.039	6.961	0.112
2 Cell	35.514	4.486	0.341
4 Cell	36.941	3.060	
Morula	33.769	6.231	0.169
Blastocyst	34.911	5.089	0.138
CC	38.969	1.031	0.033

β-actin			
	Raw Ct	**Inverted Ct**	**Std Error**
GV	30.417	9.583	0.468
MII	30.762	9.238	0.262
1 Cell	30.293	9.707	0.153
2 Cell	31.750	8.250	0.536
4 Cell	34.847	5.153	
Morula	27.061	12.939	0.754
Blastocyst	24.860	15.140	0.129
CC	29.908	10.092	0.106

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