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# REGULATORS OF MEIOTIC CELL DIVISION OF THE GROWING OOCYTES

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## ABSTRACT

The growth of vertebrate oocyte stops temporarily at the late diplotene stage of 1st meiotic cell division and stays at that stage until puberty. Before ovulation, the arrest is resumed and enters the 2nd meiotic phase and stops again at the metaphase until fertilization. The arrest is mediated by the constant flow of cAMP, which is generated by activated adenylyl cyclase. This state is manifested by the presence of the intact nuclear envelope (NE) or germinal vesicle (GV). When the flow of cAMP is reduced, the arrest resumes followed by the breakdown of nuclear envelop/germinal vesicle (NEBD/GVBD). Studies reveal that various control factors regulate the entire process. Cyclin dependent kinases (CDKs), A kinase anchoring protein (AKAP) and the CDC2/SPDY complex have been shown to play vital roles in that mechanism. The connexin proteins between granulosa cells and oocyte remain opened during arrest and close after the LH surge to resume the arrested meiosis. Recent data indicate the correlation of a newly discovered protein Aurora Kinase A (AURKA) in the regulation of the cell cycle events of the oocyte. Here, we tried to present a synopsis of the major regulatory factors that control the entire morphological cycle of a growing oocyte, *in vivo.*

## KEY WORDS

Meiosis, AURKA, SPDY, AKAP1, CDC25A, NEBD

## INTRODUCTION

The formation of oocytes starts soon after fertilization. The primordial germ cells begin to travel from the yolk sac to the gonads, which are the site for their proliferation. The total numbers of germ cells remain almost 7 million whereas it started to proliferate only from a few thousands. These germ cells are converted to oocytes when they pass via the phases of meiotic cell division after birth. The primitive follicles, the primordial follicles are composed of oogenic cells from the germ lineage surrounded by follicular cells from the somatic lineage. It is the time when the oocyte is arrested in the late diplotene phase of the first meiotic prophase until puberty. This phase is marked by the state of immature oocytes with a prominent nucleus called germinal vesicle or nuclear envelope, which contains decondensed transcriptionally active chromatin[1]. At puberty, 4 to 10 follicles begin to develop, although only 1 to 2 is actually released. The oocyte is surrounded by a zona pellucida, membrana granulosa and theca cell layer. At puberty, just before ovulation, each oocyte finishes its first meiotic division by producing a secondary oocyte and a polar body. In response to a stimulus, meiosis is resumed and manifested by the breakdown of

the nuclear envelope or germinal vesicle (NEBD/GVBD). It is arrested in the metaphase of the second meiotic cell division, at which point it is released from the ovary in ovulation. It never finishes the meiotic cycle until it is encountered by the stimuli of a sperm during fertilization[2].

## THE KEY FACTORS

### *(i) cAMP and cGMP in the maintenance of Meiotic Arrest*

High levels of cyclic AMP determine the meiotic arrest of the fully-grown oocytes. cAMP is formed within the oocytes by the activation of adenylate cyclase, which is also activated by a G-protein coupled receptor. The GPCR, which plays key roles in the oocytes, is GPR3 (mouse) and GPR12 (rat)[3]. The GPR12 has been isolated recently in the human ovary[4]. Another GPCR, GPR6 has been identified in human brain[4] that play crucial role in signal transduction.

Alteration of any one of these signaling proteins can hamper the persistence of meiotic arrest of the oocytes. The unavailability of human oocytes to study their intrinsic characteristics has now a day been overcome by the implementation of *in vitro fertilization* (IVF)[4]. It has been reported that the phenomena are almost similar with that of the rodents[4]. The role of cAMP in maintaining meiotic arrest has been proved in various studies where it was shown that the human oocytes in prophase I stage released from their follicles matured spontaneously[5,6,7] and this could be reversibly inhibited by incubating oocytes in the presence of phosphodiesterase inhibitors[6,8]. It was also shown that the human oocytes contain the same cell cycle regulatory proteins with that of the other mammalian species[9,10]. But the difference lies in their respective length of cycle as while the human oocytes acquire their meiotic competence during the menstrual cycle (about 28±2 days), in case of the rodents these are on a relatively short estrous cycle (4±2 days).

It has been found that the signaling pathway via a Gs-coupled receptor is same in human with that of the rodents. DiLuigi *et al*. (2008) have been successful to isolate RNAs encoding GPR3 and AC3 in human oocytes[4]. Though GPR12 was shown to express by the human ovary, it is not expressed by the oocytes. The team was also able to show the expression of the Gαs by the human oocytes. Still it was not detected whether or not GPR3 protein could be expressed by the human oocytes due to the difficulties of getting antibodies to bind with the proteins. It was well established that the presence of phosphodiesterase inhibitor could block the breakdown of germinal vesicle and allow the oocyte to be arrested in prophase[11,12]. DiLuigi *et al*. (2008) showed that blocking Gαs by an antibody caused an isolated oocyte to resume meiosis when co-cultured with a PDE3A-specific inhibitor cilostamide[4]. All these indicate that cAMP is responsible for the maintenance of meiotic arrest by the activation of Gαs. All these have produced an indication to the concept that the rates of cAMP production or degradation are the key controller of the cell cycle regulation of oocytes. Gpr3 RNA has been shown to localize highly in oocytes than that of the somatic cells and most of the oocytes in antral follicle undergo spontaneous meiosis if the Gpr3 gene is knocked-out[10,13]. Ledent *et al*. (2005) showed that preantral follicles remained arrested in prophase I in Gpr3 knockout mice[13]. But when the antral spaces began to form, the oocyte resumed meiosis independently with an increase in LH. They concluded that females with disrupted Gpr3 gene are subfertile. Mehlmann *et al*. (2004) showed that spontaneous resumption of meiosis in $Gpr3^{-/-}$ females could be reversed with an injection containing Gpr3 RNA into the preantral follicle-enclosed oocytes[10]. Therefore, the evidences of the presence of GPR3 in the maintenance of meiotic arrest are strongly established.

**Figure1**
***Immunofluorescence localization of Gαs in a mouse ovary***

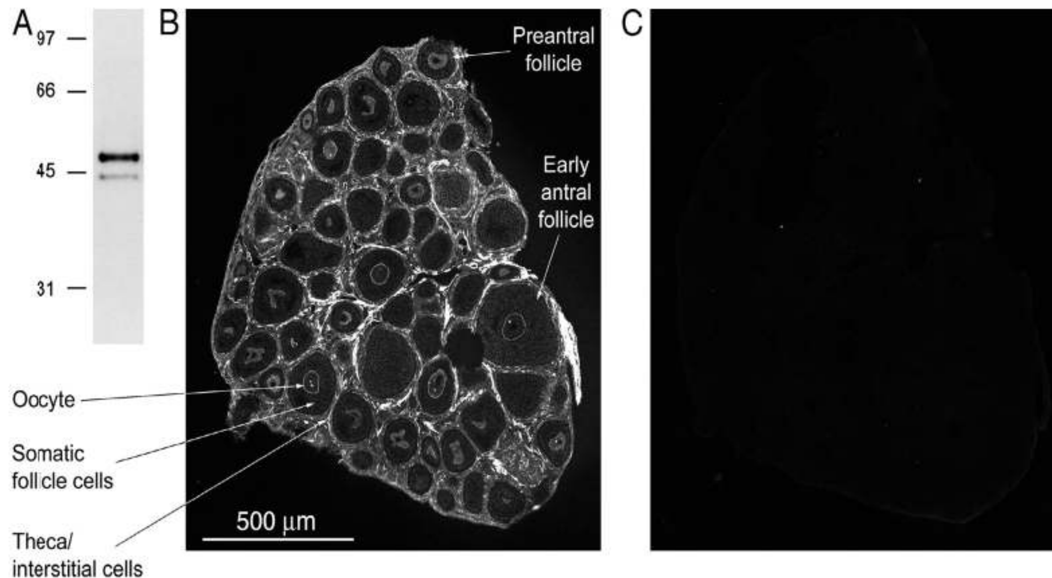



***Figure 1:*** *Immunofluorescence localization of Gαs in a mouse ovary. (A) Immunoblot demonstrating Gαs antibody specificity. 1 µg of ovary lysate (Gpr3$^{-/-}$) was loaded on the gel; both the 52- and 45-kD splice variants of Gαs (Robishaw et al., 1986) were seen. (B) A section of a Gpr3$^{-/-}$ ovary labeled with an antibody against Gαs. This ovary, which was obtained from a mouse that had not been injected with eCG to stimulate antral follicle formation, contained mostly preantral follicles and some early antral follicles. Follicles with 120–190-µm diameter were classified as preantral, whereas those with ≥200-µm diameter were classified as early antral (with multiple small antral spaces) or antral. These categories were not completely distinct because some follicles with <200-µm diameter showed a formation of antral spaces. (C) A control section labeled with nonimmune IgG. Confocal microscope settings and bars were the same for B and C. [Reproduced with permission from: Freudzon et al. (2005): Regulation of meiotic prophase arrest in mouse oocytes by GPR3, a constitutive activator of the $G_s$G protein. J Cell Biol.* ***171(2)****: 255-265. © Freudzon et al., 2005. Originally published in J. Cell Biol. doi:10.1083/jcb.200506194]*

### *ii) cGMP and Meiotic Arrest*

It has recently been postulated that the cyclic guanosine mono-phosphate (cGMP) plays crucial role in the maintenance of meiotic arrest. It has been found that the primary cAMP phosphodiesterase present within the mouse oocyte is PDE3A[14] and its action could be inhibited by cGMP[15]. It was documented that the cGMP enters the oocyte from the surrounding somatic cells through the gap junctions and maintain the arrest by inhibiting the cAMP phosphodiesterase, PDE3A[7]. When cGMP was directly injected into the oocytes, the resumption was observed to delay[16] and when soluble guanylyl cyclase[17] or ionosine monophosphate dehydrogenase (enzyme needed for cGMP production)[18,19] was inhibited, the oocytes surrounded by somatic cells entered the phase of meiotic resumption. All these data prove that the cGMP is produced by the somatic cells around the oocytes and this cGMP controls the meiotic arrest. Jaffe *et al*. (2009) showed that the cGMP is produced primarily in the granulose cells and enters the oocyte where by inhibiting the PDE3A activity, it causes the maintenance of arrest[20].

### *(iii) Calcium Ions and Meiotic Resumption*

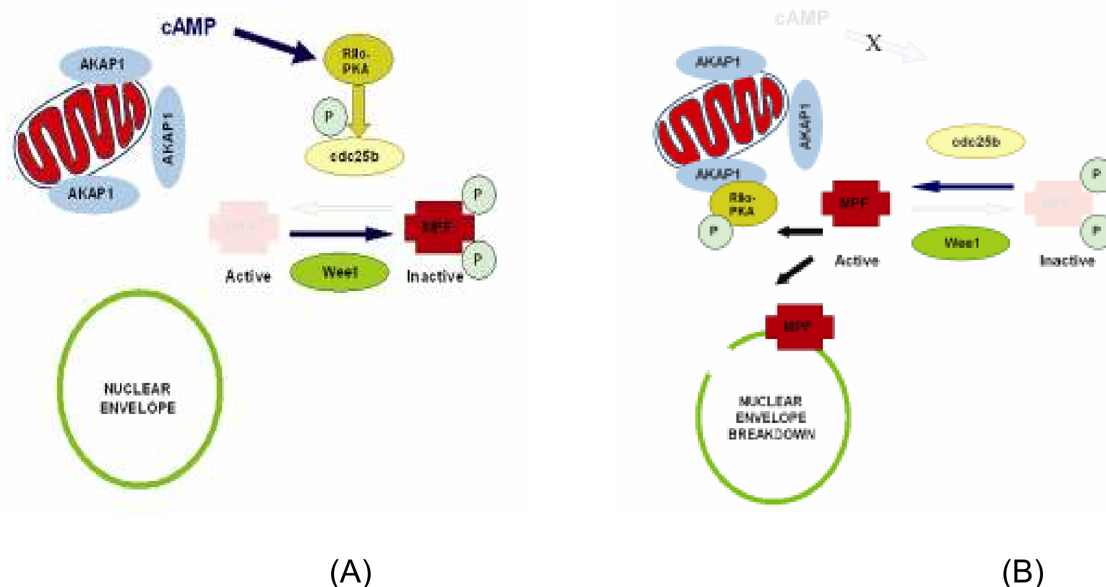
The role of calcium ions has been shown to be very important in the resumption of meiosis in growing oocyte[21]. The intracellular elevation of

calcium ions is partially responsible for the maturation of oocytes in many ways. The intracellular $Ca^{2+}$ store in the endoplasmic reticulum by the SERCA pumps pulling the calcium ions via the gap junctions between the oocyte and granulose cells or by the membrane bound calcium channels with the help of mitochondria[2]. During oocyte maturation, these ions are released into the oocytes due to activation of IP3 receptors the cytoplasmic concentration of which increases during maturation phase[22,23]. The correlation between the calcium ions and the breakdown of the nuclear envelope (NEBD) has been proved in many studies. If calcium chelators are introduced, the nuclear envelope breakdown is hindered up to the first metaphase[24]. *In vitro* maturation of oocyte does not take place in the absence of intracellular elevation of calcium ions[25].

***(iv) A Kinase Anchoring Proteins and Protein Kinase-A work together in maturation process.***

The cAMP, which is produced during the arrest mechanism works by activating protein kinase-A (PKA). After the LH surge, there are the decreased activities of both cAMP and PKA that synergistically lead to the resumption of meiotic arrest. The substrate level phosphorylation and intracellular localization of the protein kinase A is mediated by A-kinase anchoring proteins (AKAPs)[26]. AKAP1 is found in the oocytes of the preantral and the antral follicles of the ovary and immunocytochemical study revealed the localization of AKAP1 with the mitochondria of oocytes rather than the cumulus granulosa cells[27]. AKAP1 has been shown to derive from the gene *Akap1*[27]. If the *Akap1* gene is knocked out, the females lost their capabilities of being pregnant. Newhall *et al*. (2006) showed that after superovulation with gonadotropins and *in vivo* mating, most of the oocytes remained arrested in the GV stage or degenerated indicating that the progression towards the metaphase II (MII) were stopped and therefore infertile[27]. The association of the cAMP with the type II PKA (RIIα-PKA) maintains the arrested state. After the LH surge, there is a decrease of cAMP signal in the oocyte that leads to the movement of RIIα-PKA from the cytosolic phase to the mitochondrial outlet tending the anchoring of RIIα-PKA with the AKAP1[27]. This helps in the activation of an M-phase promoting factor (MPF), a well-known central regulator of the meiotic and mitotic events. CDC2 (also known as cyclin-dependent kinase1/ CDK1) complexes with cyclin B to form the MPF[28]. Nuclear envelope breakdown, chromosome condensation and formation of metaphase spindles are associated with the MPF[28].

**Figure 2**
***Model of AKAP1 activity showing the localization of type II PKA (RIIα-PKA)***




(A) (B)

***Figure 2:*** *Model of AKAP1 activity showing the localization of type II PKA (RIIα-PKA). The association of the cdk1 and cdc25b, a phosphatase that activates cdk1 generates MPF. Wee1 is a tyrosine kinase that phosphorylates and deactivates cdk1. AKAP1s remain associated with the mitochondria. The maturation involves the attachment of phosphorylated type II PKA with the AKAP1, which by virtue tends to the breakdown of the nuclear envelope. (A) Inactivation and (B) Activation of CDK1 molecule.*

### *(v) CDC25A phosphatase regulates the oocyte maturation process*

Two distinct kinases have been found in the vertebrates as well as other animals like flies, which inhibit meiosis resumption by reversible manner, WEE1 and MYT1 kinases. The major inhibitory effect is inserted by MYT1, which is found in the membranes of Golgi apparatus and endoplasmic reticulum. It phosphorylates the threonine and tyrosine molecules present in the $14^{th}$ and $15^{th}$ position of CDK1. WEE1 is a soluble and predominantly nuclear protein that phosphorylates only the tyrosine residue[29]. The resumption of meiosis is controlled by the activity of the formation of an M-phase promoting factor, which is a product of the CDK1-CDC25B complex[30,31,32]. Thus this is necessary to activate CDK1. The CDC25 phosphatase dephosphorylates the threonine and tyrosine molecules leading to the activation of cdk1. CDC25b phosphatase plays a crucial role in the activation of CDK1. *Cdc25b* knockout oocytes failed to undergo cdk1 activation and NEBD, but did so following the microinjection of *Cdc25b* mRNA[33]. *Cdc25a* mRNA is expressed by mouse oocytes[34] and if the gene is knocked out, mice show embryonic lethality[35]. Okazaki *et al*. (1996) showed that microinjection of *Xenopus Cdc25a* mRNA into the *Xenopus* oocytes resumed meiosis more potently than that of the *Cdc25c* mRNA[36]. CDC25A regulates both the G1/S and G2/M associated CDK activities in somatic cells[37,38]. It has been observed that the association of protein kinase B (PKB/AKT) facilitates the activation of CDK1 molecule during the resumption of meiosis. It is a signaling protein molecule that contains a binding domain called the pleckstrin- homology (PH) domain that interacts with various cell-cycle regulators. The serine and threonine residues present in the 473 and 308 positions respectively, are the phosphorylation sites of

PKB. During the meiotic resumption, the former site of phosphorylation (i.e., Ser473) is associated with the nuclear envelope and centrosome while the latter site of phosphorylation (i.e., Thr308) is associated with the centrosome only. The activated PKB in turn phosphorylates MYT1. This causes the withdrawal of the inhibitory signal from the CDK1, which then progresses towards its activation[39].

***(vi) Maturation is dependent on association of CDC2/SPDY with Endoplasmic Reticulum Exit Sites (ERES)***

The distribution and integrity of the Golgi bodies and endoplasmic reticulum have been shown to be associated with the cytoplasmic maturation of oocytes[40]. In somatic cells, Golgi bodies undergo fragmentation during the onset of prophase and starts reforming at the telophase[41]. Depending on this cytoarchitecture, two views have been proposed[41,42]. The first view states that association of Golgi fragments with the metaphase spindles helps equal distribution of Golgi apparatus into the two offspring cells[43,44,45]. Another hypothesis is based on the dynamic pattern of Golgi apparatus. Lippincott *et al*. (2006) showed that the Golgi proteins continue cycling through the endoplasmic reticulum[46]. The specific area of the endoplasmic reticulum from where the coat protein II (COPII) vesicles originate is called the endoplasmic reticulum exit sites (ERES). SEC23 is one of the major components of COPII, which is involved in the formation of transport vesicles in the ERES. It is necessary for the translocation of the vesicles from endoplasmic reticulum to Golgi apparatus. During the early phase of mitosis the vesicle formation and membrane trafficking are inhibited. It may be the reason behind the decreased association of SEC23 with ERES. Therefore, the Golgi proteins are trapped in the endoplasmic reticulum. When telophase appears, the ER export block is withdrawn and the formation of Golgi vesicles from ERES resumes. This entrains the equal distribution of Golgi components into daughter cells together with the endoplasmic reticulum and Golgi apparatus[47,48]. The vesicular transport of Golgi proteins from ER to Golgi apparatus in mitotic cell division is regulated by the CDC2 kinase dependent disassembly of Golgi and endoplasmic reticulum exit sites (ERES), as CDC2 kinase directly phosphorylates Golgi protein GM130 and p47 respectively[45,49]. Speedy (SPDY) and cyclin B has been found to be the important controlling factors behind the activity of CDC2 during oocytes maturation[50,51]. Haeften *et al*. (2009) showed that after the release of inhibitory influences from the oocytes, the meiotic regulator CDC2 translocates to a novel domain, which comprises a cluster of ERES[28]. They also showed that the coordinated function of CDC2 and SPDY regulate storage of structural Golgi elements at the endoplasmic reticulum exit sites (ERES) cluster. The preordain phase of breakdown of nuclear envelope (NEBD) are not only manifested by the change in the chromatin configuration, rather this includes the synchronized local events within the cytosol of the oocyte that may control the secretory events during mitotic division.

***(vii) Luteinizing hormone-induced closure of connexin 43 (Cx43) gap junctions mediates oocyte maturation***

Somatic cells remain interconnected by tight and gap junctions. The gap junctions play crucial roles in the maintenance of cellular exchange of various substances. The major kinds of gap junctions are connexin proteins the types of which differ in the somatic cells and the oocyte. The major connexin protein in the somatic cells is the connexin 43 (Cx43 or Gja1)[52,53,54]. Cx45 and Cx37 (Gja4) are also present to some extent[1,53,55,56], but their supporting roles are of less importance compared with that of the Cx43[54]. Cx37 is expressed by the mouse oocytes and the

oocyte-cumulus interaction is mediated through Cx37[1]. The cumulus cells which are adjacent to the to the oocyte also express the Cx37 and they apparently extend to the zona pellucida[1,56]. This specific subtype is important for the maintenance of oocyte's microenvironment, as the Cx37 gene knockout mice did not express the Cx37 proteins resulting the destabilized environment of the oocyte[57]. The major signaling route of LH-induced closure of gap junctions is still to be elucidated but it is evident that LH mediates a transformational change in the distribution pattern of the connexins between the somatic cells[58], the rapid phosphorylation of Cx43[59,60], and closure of gap junctions between granulose cells[60,61]. Using gap junction tracers- Alexa Fluor 350, Alexa Fluor 488 and mouse gap junction antibody (AntiCx43 antibody), Jaffe *et al*. (2008) have shown that LH causes the MAP-Kinase dependent phosphorylation of the serine residues at 255, 262, 279/282 positions of Cx43. This in turn results in the decreased permeability of the gap junctions[62]. The major pathway by which the stimulus reaches to the oocyte is still under investigation but it is thought that the signal may pass through the Cx37 gap junction protein, which is present between the oocyte and the cumulus oophorus, as in the presence of the gap junction inhibitor glycerrhetinic acid, oocyte within dbcAMP arrested cumulus complex fails to resume meiosis in response to EGF[63]. Thus, the major communicating pathway of LH-induced meiosis resumption has been established to be by the Cx43 phosphorylation[62].

**Figure 3**
***Model of Oocyte and its surrounding granulose cells***

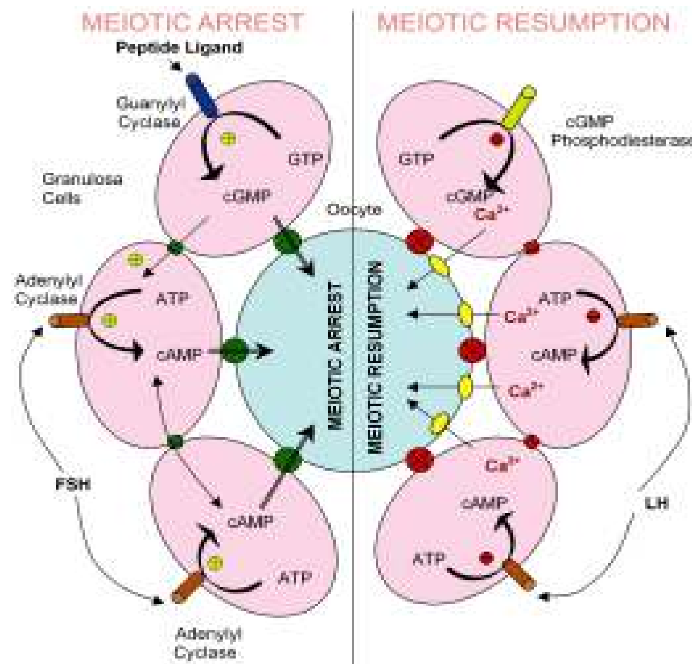



*__Figure 3:__ The left half of the figure indicates the factors regulating the arrest phase and the right side indicates the stage of maturation. The granulose cells are interconnected with gap junction protein connexin 43 (Cx43) that remain open (Green) during arrest phase and close (Red) after LH surge. Connexin 37 (Cx37) is the gap junction protein that is present between the granulose cells and the oocyte, which also show same opening (Green) and closing (Red) pattern with that of the Cx43. During the arrest, the production of cAMP is high and this can pass into the oocyte from the surrounding cells. This production is inhibited after LH surge and due to the closure of the gap junctions, cAMP cannot pass into the oocyte. During the maturation phase, calcium ions enter into the oocyte from the granulose cells through calcium ion channels (Yellow) and this facilitates the breakdown of the nuclear envelope.*

### *(viii) Role of Aurora Kinase A in the progression of meiosis*

The regulators behind the cell cycle events in oocytes have been studied extensively. A recent finding showed the involvement of Aurora Kinases in the regulation of the cell cycle events. Aurora kinases are centrosome-localized serine/threonine protein kinases that have three subtypes- Aurora A (AURKA), Aurora B (AURKB) and Aurora C (AURKC)[27]. Each exhibits a specific sub-cellular domain and function during the various events of cell cycle[37,64]. Zhang *et al*. (2003), Sun *et al*. (2005) and Uzbekova *et al*. (2007) reported the presence of Aurora kinase A (AURKA) in the mammalian oocyte[26,66] and Solc *et al*. (2008) have shown that the AURKA is present in immature (NE), maturing (NEBD, MI) and mature (MI and MII) oocytes[29]. There are two specific locations in the AURKA, the phosphorylations of which facilitate the activation process. Phosphorylation of the threonine and serine residues present at the 288 and 342 respectively, are dependent on the LIM-domain protein Ajuba and p21-activated protein kinase (Pak1)[48]. Solc *et al*. (2008) reported that AURKA is a crucial regulator of meiosis progression[29]. The activation of AURKA happens before the breakdown of nuclear envelope and this activation is protein kinase B/AKT independent. After the activation, AURKA remains localized in the microtubule organizing centers (MTOCs) and spindles[29]. It has been observed that AURKA facilitates the MTOC maturation. The MTOC maturation involves recruitment of various phosphorylated compounds that help in turn in assembling of mitotic spindle. Thus the breakdown of the nuclear envelope and the completion of the cell cycle is facilitated by the involvement of the AURKA in such a fairly manner.

## CONCLUSION

The oocytes, which are selected for use by the female, undergo a spontaneous on-off cycle so that the duration of the reproductive cycle remains normal. The cyclical switching of arrest and resumption of the meiotic cell division is perfectly mechanized by the molecular regulators that act in a very fairly manner. The main purpose of the arrest is to provide sufficient time to the oocyte so that it can grow up and the cellular organelles are enough matured to assist fertilization, which is the ultimate destiny. The cyclic AMP provides the suitable environment for temporary cessation of the gonadal growth. As the receptors of FSH present in the granulose cells are stimulated, they start synthesizing cAMP by activating adenylyl cyclase. It comes into the oocyte through connexin protein gap junctions and provides barriers to all kinds of maturation promoting factors, which could initiate the resumption phase. As the oocyte progresses to be ejected out from the Graaffian follicles by means of ovulation, there is a potential increase in the level of Luteinizing hormone. Just before ovulation there is an LH surge, which facilitates the ovulation process. The LH surge in turn destabilizes the interconnected environment of the granulosa cells and oocyte by closing the gap junctions. These include a fall in the cAMP concentration in the oocyte, thus the inhibitory signals by cAMP on the maturation promoting factors are withdrawn and various cyclin dependent kinases (CDK) as well as other stimulatory factors are activated. The CDK1, which is the precursor of the maturation promoting factors (MPF), begins to be activated and the associations of the other synergistic facilitators stimulate the breakdown of the nuclear envelope (NEBD) of the oocyte. The formation of the spindle during the cell division is facilitated by a specific kinase known as Aurora kinase A, that enhance the potency of the cell to divide. The associated functions of other regulatory factors thus maintain the proper timings of the cell cycle.

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