Glycogen synthase kinase 3B in bovine oocytes and granulosa cells: possible involvement in meiosis during *in vitro* maturation

Svetlana Uzbekova, Mohamad Salhab, Christine Perreau, Pascal Mermillod and Joëlle Dupont

Physiologie de la Reproduction et des Comportements, UMR6175 INRA, CNRS, Université de Tours, Haras Nationaux, 37380 Nouzilly, France

Correspondence should be addressed to S Uzbekova; Email: svetlana.uzbekova@tours.inra.fr

Abstract

Glycogen synthase kinase 3 (GSK3) regulates cellular metabolism and cell cycle via different signalling pathways. In response to insulin and growth factors GSK3 is serine-phosphorylated and inactivated. We analysed GSK3B expression and activation in bovine cumulus cells (CC) and oocytes at different meiotic stages *in vitro* in parallel with MAP kinases ERK (MAPK3/MAPK1) and p38 (MAPK14). GSK3B localised to cytoplasm in granulosa cells and in oocytes throughout folliculogenesis. In mature metaphase-II (MII) oocytes, GSK3B was concentrated to the region of midzone between the oocyte and the first polar body, as well as active phospho-Thr Aurora A kinase (AURKA). During *in vitro* maturation (IVM), in oocytes, phospho-Ser⁹-GSK3B level increased as well as phospho-MAPK3/MAPK1, while phospho-MAPK14 decreased. In CC, phospho-MAPK14 increased upon germinal vesicle breakdown (GVBD)/metaphase-I (MI) and then decreased during transition to MII. Administration of inhibitors of GSK3 activity (lithium chloride or 2',3',5'-6-bromoindirubin-3'-oxime) rapidly increased phospho-Ser⁹-GSK3B, and led to transient decrease of phospho-MAPK3/MAPK1 and to durable enhancing of phospho-MAPK14 in granulosa primary cell culture. GSK3 inhibitors during IVM diminished cumulus expansion and delayed meiotic progression. In cumulus, phospho-MAPK14 level was significantly higher in the presence of inhibitors, comparing with control, through the time of MI/MII transition. In oocytes, phospho-GSK3B was increased and phospho-MAPK3/MAPK1 was decreased before GVBD and oocytes were mainly arrested at MI. Therefore, GSK3B might regulate oocyte meiosis, notably MI/MII transition being the part of MAPK3/1 and MAPK14 pathways in oocytes and CC. GSK3B might be also involved in the local activation of AURKA that controls this transition.

Reproduction (2009) **138** 235–246

Introduction

Glycogen synthase kinase 3 (GSK3) is a highly evolutionary conserved serine–threonine kinase, which was described first as a key enzyme in glycogen metabolism and was then found to be a multifunctional enzyme. GSK3 phosphorylates multiple substrates and is involved in regulation of protein synthesis, cell proliferation and differentiation, microtubule dynamics and apoptosis, being a part of Wnt/ β -catenin, NF- κ B and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB) signalling pathways (for review see (Ali *et al.* 2001)). GSK3 has two protein isoforms, 51 kDa GSK3- α (GSK3A) and 46 kDa GSK3- β (GSK3B), which are encoded by different genes, highly similar in their kinase domains (Woodgett 1990). GSK3A and GSK3B are variably expressed in different tissues in humans and mice (Lau *et al.* 1999, Yao *et al.* 2002). In mammals, GSK3 is constitutively phosphorylated at Tyrosine^{279/216} residues and dephosphorylation on these sites is accompanied by kinase inactivation. In contrast,

phosphorylation of serine²¹ and serine⁹ residues inactivates GSK3A and GSK3B respectively. This phosphorylation occurs in response to insulin, amino acids or polypeptide growth factors like epidermal growth factor (EGF) either by PKB (also named AKT) in PI3K/AKT pathway (Cohen *et al.* 1997), or by p70 S6 kinase in MTOR signalling or by MAPK-activated protein kinase 1 in classical MAPK cascade respectively (Cohen & Frame 2001). Nucleotide polymorphisms in two components of the insulin signalling pathway, genes *GSK3B* and *AKT2*, were reported to be associated with polycystic ovary syndrome in women, characterised by different metabolic and reproductive disorders including hyperandrogenaemia and anovulation often associated with insulin resistance (Goodarzi *et al.* 2007, 2008). Cystic ovarian follicles are also a major cause of reproductive failure in high-lactating dairy cattle (Vanholder *et al.* 2006), which could also affect the oocyte quality (Bartolome *et al.* 2002). In vertebrates, GSK3 was shown to be involved in regulation of meiosis progression in frog oocytes

Figure 1 GSK3B mRNA and protein detection. (A) Detection by RT-PCR of GSK3B transcripts in cattle immature (0 h) and mature (24 h IVM) oocytes, cumulus cells (CC) and hypothalamic region of brain (Br). Control β -actin (AKTB) transcripts were detected in the same cDNA samples. (B) Total GSK3B protein (46 kDa) and phospho-Ser^{21/9} GSK3A/B detection by western blot. Total proteins were extracted from oocytes and cumulus cells before or after 24 h IVM and from brain biopsy, and then subjected to SDS-PAGE and immunoblot. In total, 40 immature and mature oocytes and cumulus cells from 10 COCs were loaded per lane. (C) Immunofluorescence of GSK3B in the cumulus-oocyte complex (green) showed that GSK3B was less detected in oocyte as compared with cumulus cells, CC (a). Phospho-Ser⁹ GSK3B was less detected in germinal vesicle immature oocytes (GV) compared with oocytes that over-passed the GVBD as demonstrated by simultaneous phospho-Ser⁹ GSK3B (green) and Lamin A/C nuclear membrane (red) staining (b). In metaphase-II oocytes, GSK3B (c) and phospho-Thr AURKA (d) localised to a contractile ring between the ooplasm and polar body (encompassed by white arrows). White-dotted line rectangles encompass the regions, which are magnified below. Chromatin is blue-coloured by DAPI. Bars: 50 µm.

already passed through GVBD after IVM (Fig. 1C, b). In mature oocytes, GSK3B was particularly concentrated closely to a polar body/oocyte border, that might be a contractile ring, bundling microtubule-based structure (Fig. 1C, c). The active form of Aurora A kinase, phospho-Thr AURKA, was also localised to this structure (Fig. 1C, d), as we already reported (Uzbekova *et al.* 2008). No significant fluorescence was observed in oocyte and CC, if rabbit IgG replaced the first antibodies.

We then analysed the localisation of GSK3B and its serine-phosphorylated form throughout folliculogenesis by 3,3'-diaminobenzidine (DAB)-immunostaining on the paraffin-embedded sections of bovine ovaries, containing the follicles of different stages from

primordial to antral (Fig. 2). GSK3B was not detected in interstitial tissue, but was clearly immunostained in primordial and primary follicles both in oocytes and in a monolayer of surrounding granulosa cells (Fig. 2A). In secondary and antral follicles, GSK3B was detected in oocytes, in granulosa and in theca cell layers (Fig. 2D and G). In large antral follicles, GSK3B was detected in CC and much less in oocyte cytoplasm (Fig. 2J). No significant immunoreactivity was detected in control adjacent sections stained with rabbit IgG (Fig. 2B, E, H and K). In secondary preantral and antral follicles, phospho-Ser⁹ GSK3B was detected in granulosa cells and in CC. Nevertheless, oocytes also showed a slight immunoreactivity (Fig. 2C, F, I and L).

Figure 2 Localisation of GSK3B in bovine ovary. Analysis of total GSK3B protein expression (A, D, G and J) and its Ser⁹-phosphorylated form, pSer-GSK3B (C, F, I and L) during folliculogenesis was performed by DAB-immunoperoxidase staining on paraffin-embedded ovary sections (immuno-specific staining is brown and cell nuclei are violet). GSK3B was detected in oocytes and surrounded follicular cells in different follicles: primary (A), secondary pre-antral (D), tertiary small antral (G) and in COCs from large antral follicles (J). Serine-phosphorylated GSK3B was detected mainly in granulosa and cumulus cells (C, F, I and L) and more faintly in oocytes. Control immunostaining was performed by using rabbit IgG instead of primary antibodies on adjacent sections (B, E, H and K). Cumulus–oocyte complex (COC), oocyte (Oo), cumulus cells (CC), antrum (An), granulosa (Gr), theca cells (Th) and interstitial tissue (Int) are designated. Bars: 50 µm.

Phosphorylation of GSK3B, MAPK3/MAPK1 and MAPK14 in oocytes and CC during IVM

Once extracted from the antral follicles and transferred into IVM culture medium for 24 h, bovine oocytes inside of the COC passed through the sequential stages of nuclear maturation and finally stopped at MII stage. In our conditions, the recovered oocytes were in meiotic prophase and retained the intact GV for at least 6 h. Initially GVBD occurred after ~6–7 h of IVM and the MI stage was achieved at 10–12 h of IVM. At 22–24 h, most of the oocytes were mature and arrested at MII. We analysed GSK3B, MAPK3/MAPK1 and MAPK14 expression by immunoblot in both oocyte and CC at different time points of IVM (Fig. 3). In oocytes, progressive GSK3B phosphorylation at Ser⁹ occurred throughout IVM and reached a maximum at MII stage, being more than 12 times increased as compared with immature oocytes (Fig. 3, left). A similar evolution was observed for phospho-MAPK3/MAPK1 level, which also significantly increased. In contrast, phospho-MAPK14 decreased drastically in mature oocytes as compared with immature ones.

In CC, GSK3B was already Ser-phosphorylated before IVM, phospho-GSK3B increased 1.5 times at the beginning of the culture and then maintained slightly variable phosphorylation level (Fig. 3, right). We previously reported a similar pattern of MAPK3/MAPK1 phosphorylation in CC during IVM (Tosca *et al.* 2007), i.e. slight increase from the beginning of maturation and the maintenance at that level. In contrast, phospho-MAPK14 level increased more than three times in CC between 1 and 10 h of IVM and then dropped to the initial level after 24 h of culturing.

Thus, we showed that GSK3B, MAPK14 and MAPK3/MAPK1 were present in oocyte and surrounding CC and their phosphorylation levels were modulated during IVM.

Effect of GSK3 inhibitors on bovine oocyte nuclear maturation in vitro

To look for a potential function of GSK3B in oocyte, we first used LiCl₂ as a direct inhibitor of GSK3 kinase activity. Final concentration of 20 mM LiCl₂ previously shown to be non-toxic for mouse oocytes and embryos

Figure 3 Immunoblot detection of GSK3B, MAPK3/1 and MAPK14 total proteins and their phosphorylation forms in oocytes and cumulus cells before IVM (0 h) or after 3, 6, 10 and 24 h of IVM. Denuded oocytes (30 oocytes per lane) or CC from ten COCs were subjected to immunoblot by using successively antibodies, detecting phosphorylated or total proteins on the same membrane. Representative blots from at least three experiments are shown. Signals were quantified and ratios of phosphoprotein kinase/total protein kinase are represented as histograms (mean ratio $\pm$ S.E.M.; the level in immature oocytes was considered as one). Different letters designated a significant difference ($P < 0.05$).

(Acedo *et al.* 2007b), was used in these studies. The addition of 20 mM of LiCl_2 to bovine COC during IVM led to reduced expansion of cumulus after 24 h of IVM as compared with control (Fig. 4A, left column). All lithium-treated oocytes accomplished GVBD, as was confirmed by lamin A/C immunostaining (not shown), but were then arrested in MI-like stage (Fig. 4A). GSK3B distribution after IVM differed between the oocytes matured with or without LiCl_2 : in control matured oocytes at MII stage, GSK3B was particularly concentrated in the region of polar body (which could be the midbody or contractile ring), while no particular concentration was observed in MI-arrested lithium-treated oocytes. Chromosome alignment was disturbed in MI figures observed in lithium-treated oocytes and the spindle was asymmetric (Fig. 4B).

In control IVM, 89.7% attained MII and only 10.3% did not progress beyond MI, while in the lithium-treated group 85.9% of oocytes were blocked in the first metaphase (Fig. 4C). When BIO inhibitor was added, a dose-dependent decrease of oocyte nuclear maturation level was observed, being reduced more than twice at 50 μM concentration. Moreover, the cumulus expansion was also significantly reduced at a concentration higher than 10 μM (data not shown).

Thus, we showed that addition of GSK3 inhibitors LiCl_2 or BIO during IVM reduced cumulus expansion and delayed oocyte maturation by blocking MI/MII transition in bovine oocyte.

Effect of LiCl_2 on cell survival and progesterone secretion by granulosa cells and COC in vitro

In order to explain the possible reasons of meiotic arrest of oocytes in COC cultured with LiCl_2 , we first looked for the dose-dependent effect of LiCl_2 on cell survival and proliferation level on primary culture of granulosa cells in the presence of increasing concentrations of LiCl_2 . At 20 mM no impact was observed in granulosa cells' proliferation level as compared with untreated cells (Fig. 5A). Nevertheless, significant decrease in progesterone secretion by granulosa cells was observed from a concentration of 10 mM and higher. At 20 mM, progesterone secretion was three times less than in control (Fig. 5B). This decrease was observed both in basal and FSH- or insulin-like growth factor-I (IGF-I)-supplemented medium (Fig. 5C). COC also secreted progesterone during IVM, and its level increased mostly between 10 and 24 h of maturation (Fig. 5D, line-curve graph). Similar to granulosa cells, in the presence of lithium COC secreted two-fold less progesterone than in control IVM conditions.

Effect of GSK3 inhibitors on the phosphorylation of GSK3B, MAPK3/MAPK1 and MAPK14 in bovine granulosa cells, CC and oocyte in vitro

After LiCl_2 addition in granulosa cell culture we observed very rapid (already 1 min after lithium administration) increase of phospho-Ser⁹-GSK3B level (Fig. 6A). In order to test what signalling pathways have been affected by lithium and could be involved in oocyte meiotic arrest, we then quantified the phosphorylation of MAPK3/MAPK1, AKT, AMPK and MAPK14. Neither AMPK nor AKT phosphorylation were affected by LiCl_2 both in granulosa cells during the first 60 min of treatment and in CC after 24 h IVM (data not shown). Phospho-MAPK3/MAPK1 level significantly decreased in granulosa cells 10 min after lithium administration and then remained stable during 60 min of observation. In contrast, phospho-MAPK14 level significantly and constantly increased at the same time points. In the presence of BIO inhibitor, phospho-Ser⁹-GSK3B level was progressively increasing during 60 min. At this time point, dose-dependent decrease of phospho-MAPK3/1 and increase of phospho-MAPK14 was also observed (Fig. 6B).

In CC from lithium-treated or control COC, no differences in phospho-GSK3B and phospho-MAPK3/1 levels were observed at 1, 3, 6, 9, 18 or 24 h of IVM (data not shown). However, in the presence of either LiCl_2 or BIO, phospho-MAPK14 level in CC was stably higher than in control during IVM (Fig. 7A). In oocytes at 6 h of IVM, we observed two-time higher level of phospho-GSK3B and eight-time lower level of phospho-MAPK3/1 in lithium-treated group (Fig. 7B). These effects were already visible at 3 h of IVM; however, at the end of maturation (at 24 h) no more differences were observed (data not shown).

Figure 4 Effect of LiCl_2 and GSK3 inhibitor BIO on maturation of bovine oocytes *in vitro*. (A) COCs expansion (first column, bar 500 μm), stage of oocyte chromatin (blue DAPI-staining) and GSK3B immunofluorescence (green) were analysed before (0 h) and after 24 h IVM (second column, bars 50 μm). White-dotted line rectangles encompassed regions that are magnified on the right pictures. Region of particular GSK3B concentration near the polar body (PB) in control mature oocyte is encompassed by white arrow. (B) Spindle detection by TUBA immunofluorescence (red) in metaphase-I control and LiCl_2 -treated oocytes; chromosomes (blue) are misaligned in the presence of lithium. Bars 10 μm . (C) Histogram representing the percentage of mature oocytes after 24 h of IVM in control or in GSK3 inhibitor-treated oocytes. 20 mM LiCl_2 (right graph) or different concentration of BIO (left graph) was used. Different letters designated a significant difference ($P < 0.05$).

Taking together, GSK3 inhibitors LiCl_2 and BIO increased phospho-Ser⁹-GSK3B and phospho-MAPK14 levels, decreased MAPK3/MAPK1 phosphorylation in bovine granulosa and CC *in vitro*, and delayed oocyte maturation by blocking transition from MI to MII (Fig. 8).

Discussion

GSK3B expression in bovine oocytes and surrounding follicular cells

We showed that GSK3B is present in bovine oocytes and in surrounding follicular cells throughout meiosis from primary follicles up to ovulation stage. Between two GSK3 isoforms, GSK3B was the most abundant in bovine oocytes and in CC. Effectively, the expression of GSK3A and GSK3B varied in different tissues of mammals with a poor correlation between mRNA and protein levels,

reflecting the differential regulation of transcription and translation for these highly similar kinases (Woodgett 1990, Lau *et al.* 1999). GSK3A mRNA was not detected in mice oocytes and embryos, whereas both GSK3A and GSK3B proteins were evidenced and differentially phosphorylated (Wang *et al.* 2003, Acevedo *et al.* 2007b). GSK3 is involved in the regulation of transcription by phosphorylation or destabilisation of transcriptional factors, maintaining them in an inactive state under basal conditions (Frame & Cohen 2001). Transcriptional factors c-Myc, c-Jun and CREB, known substrates of GSK3, are also expressed in mammalian oocytes (Li *et al.* 1994, Vigneault *et al.* 2004, Baatout *et al.* 2007). In bovine follicles, GSK3B is expressed continuously during oogenesis and meiosis progression, thus transcription activity might be partially regulated via GSK3B pathway in bovine oocyte. GSK3 is also known

Figure 5 Effect of different concentrations of LiCl₂ on (A) thymidine incorporation and on (B) progesterone secretion by primary culture of bovine granulosa cells *in vitro*. (C) Effect of 20 mM LiCl₂ on progesterone secretion by granulosa cells in basal culture medium and in response to FSH or IGF-1. (D) Time course of progesterone secretion by COCs during IVM (curve line) and final progesterone concentrations at 24 h IVM in control and LiCl₂ supplemented medium (histogram). Different letters and asterisks designated a significant difference ($P < 0.05$).

to regulate protein synthesis: when GSK3B is Ser-phosphorylated in response to insulin, eukaryotic protein synthesis initiation factor 2B is activated and contributes to an increased rate of protein synthesis (Welsh *et al.* 1998). In bovine oocytes, protein neosynthesis is indispensable for meiosis resumption and progression. A significant increase in overall protein synthesis has been reported in cattle oocytes at 6–10 h after beginning of IVM (Tomek *et al.* 2002), and numerous proteins were translated *de novo* (Coenen *et al.* 2004). Coincidentally, GSK3B was shown to be progressively inactivated, i.e. phosphorylated at Serine⁹

during IVM and was localised in the cytoplasm. Therefore, GSK3B might also participate in the regulation of protein synthesis in oocytes. Recent data showed that in mouse oocyte, soluble kit ligand (KL), a protein expressed in granulosa cells and indispensable for follicular and oocyte growth, could inactivate GSK3 via KL-Kit-PI3K-AKT pathway (Liu *et al.* 2007). Expression of KL was reported in bovine ovarian surface epithelium, stroma and granulosa cells (Parrott *et al.* 2000). We detected GSK3B in granulosa cells from primary follicular stage and through further follicular growth. Thus, GSK3B may participate in the regulation of early follicular development and oocyte growth.

We detected GSK3B into cytoplasm but not in the nucleus of immature growing and fully grown oocytes, like in mice (Wang *et al.* 2003). Ser⁹-phosphorylated inactive GSK3B was detected in mice MII oocytes (Acevedo *et al.* 2007b), and we found that this form was more abundant in mature bovine oocytes compared with immature ones. Similarly, in *Xenopus*, GSK3B was active in GV oocytes and inactivated during maturation in response to progesterone (Fisher *et al.* 1999).

In bovine mature oocytes, GSK3B was particularly concentrated to a region separating the polar body from oocyte, what could be a part of a midbody or contractile ring. No particular concentration of pSer⁹-GSK3B was detected at that site; therefore, GSK3B could be active at the contractile ring/midbody. Aurora kinases AURKB, AURKC and active Thr-phosphorylated form of AURKA were also detected at this particular site in mature oocytes (Uzbekova *et al.* 2008). In *Xenopus* oocytes GSK3 was reported to phosphorylate Aurora-A at Serine³⁴⁹, inhibiting its kinase activity and in turn stimulating progesterone-triggered oocyte maturation (Sarkissian *et al.* 2004). Indeed, this phosphorylation of Aurora-A at Serine³⁴⁹ was found to be indispensable for meiosis

Figure 6 (A) Time-course effects of LiCl₂ on phosphorylation of GSK3B, MAPK3/MAPK1 and MAPK14 in bovine granulosa cells *in vitro*. Different concentrations of LiCl₂ were added to primary granulosa cells (48 h culturing) and cells were lysed after 1, 5, 10, 30 or 60 min after beginning of treatment. Quantified values from western blot signals (three replicates) were presented on histograms as a ratio phosphorylated protein kinase/total protein kinase. Different letters designated a significant difference ($P < 0.05$). (B) Time-course effect of 10 μM BIO on GSK3B phosphorylation (left picture) and dose-dependent effect of BIO on MAPK3/MAPK1 and MAPK14 phosphorylation in granulosa cells *in vitro*. Extracted proteins were separated by SDS-PAGE and immunoblots with different antibodies were performed on the same samples. Representative western blots from two independent experiments are shown.

Figure 7 (A) Effect of 20 mM LiCl₂ or 10 μM BIO on MAPK14 phosphorylation in cumulus cells during IVM. Proteins were extracted from 10 COCs after 6, 9, 18 and 24 h of IVM culture and analysed by western blot. (B) Effects of 20 mM LiCl₂ on phosphorylation of GSK3B and MAPK3/MAPK1 in oocytes after 6 h IVM. Different letters designated a significant difference ($P < 0.05$).

progression in *Xenopus* oocytes (Pascreau *et al.* 2008). In human HeLa cells, it was recently shown that GSK3B finely regulated synthesis and activity of AURKA through binding and phosphorylation of Aurora-A-interacting protein (AIP) during mitosis (Fumoto *et al.* 2008). At the same time, GSK3B, AIP and AURKA were co-localised at the spindle poles, and when AIP was depleted, AURKA remained permanently active and caused a pro-metaphase arrest (Fumoto *et al.* 2008). We detected active phospho-Thr AURKA in cytoplasm of bovine immature oocytes and at the contractile ring of midbody in mature MII-arrested oocytes (Uzbekova *et al.* 2008). Similar pattern for GSK3B was observed in the present work. AURKA inactivation induced aberrant chromosome distribution and inhibition of polar body extrusion in bovine and mice oocytes (Swain *et al.* 2008, Uzbekova *et al.* 2008). GSK3 inhibition also led to chromosome misalignment and thus, meiosis could not be completed (Wang *et al.* 2003, and present study). Thus, GSK3B might be involved in the regulation of AURKA activity in mammalian oocyte, although mechanisms of GSK3B effects on AURKA remain to be clarified.

During IVM, the level of phosphoSer⁹-GSK3B in CC increased rapidly in response to components like insulin and EGF contained in IVM culture medium. In oocytes, GSK3B Ser⁹-phosphorylation significantly increased from GVBD stage, in parallel with the increasing of progesterone secretion after 10 h of IVM, which is the period of MI/MII transition. Variations of GSK3B activity in oocytes and progesterone secretion by correspondent COC were negatively correlated during lithium treatment: in the presence of LiCl₂ progesterone synthesis decreased; however, GSK3B activity was diminished. In human endometrial-derived epithelial cell line, progesterone induced a dose-dependent increase in

phospho-GSK3B, which could be blocked by competitive progesterone receptor antagonist RU486 (Salameh *et al.* 2006). This suggests that progesterone might regulate the expression of GSK3. In *Xenopus* oocytes, progesterone and insulin stimulated egg's maturation by inhibiting GSK-3 kinase activity (Sarkissian *et al.* 2004). Therefore, intra-follicular pools of GSK3B in bovine might also be regulated by progesterone.

Possible mechanism of meiotic arrest mediated by GSK3 inhibitors in bovine COC during IVM

When IVM was performed in the presence of lithium or BIO, oocytes could not progress beyond MI stage, which occurred after 9–12 h as expected. Chromosomes were misaligned in lithium-treated MI-like oocytes although spindle was present. In mice early embryos treated with lithium or with another GSK3 inhibitor alsterpaullone, chromatin segregation abnormalities were also observed during the first cleavages (Acevedo *et al.* 2007b). Recently, GSK3B has been found to be involved in the stabilisation of meiotic spindle, and it was localised to spindle poles and to kinetochore region in mature mice oocytes (Baluch & Capco 2008). MAPK14 is also considered as a component of the spindle assembly checkpoint in cell cycle. Addition of activated recombinant p38 to *Xenopus* cell-free extracts or in early embryo caused arrest in M phase (Takenaka *et al.* 1998).

Figure 8 Hypothetical schema of involvement of GSK3B in oocyte transition from metaphase-I to metaphase-II concluded from the experiences with GSK3 inhibitors. Phospho-Ser⁹-GSK3B, phospho-MAPK3/1 and phospho-MAPK14 are present in COC at metaphase-I. During the period of 10–24 h of IVM, cumulus is largely expanded, progesterone secretion increased and level of phospho-MAPK14 diminished in CC and in oocyte. In oocyte, remains of active GSK3B might participate in phosphorylation of AURKA, which is involved in regulation of chromosome segregation and MI/MII transition. Oocyte thus, reached correctly metaphase-II stage. In the presence of GSK3 inhibitors, cumulus was not expanded and progesterone secretion diminished. GSK3 inhibitors led to the additional Ser⁹-phosphorylation of GSK3B, decreased phospho-MAPK3/1 level and maintained phospho-MAPK14 at a higher level, which could hamper the normal meiosis progression. When inhibitors inactivated GSK3B, local phosphorylation of AURKA might not occur, spindle was not correctly formed and oocyte was blocked at metaphase-I.

In our experiments, we observed an increase of phospho-GSK3B and phospho-MAPK14 and a decrease of MAPK3/MAPK1 phosphorylation in granulosa cells in response to lithium or BIO. During IVM, these effects were associated with the decrease of progesterone secretion and with the arrest of oocytes at MI-like stage as resumed in Fig. 8. Both LiCl₂ and BIO induced a stable phosphorylation of MAPK14 in bovine CC during IVM. During IVM, MAPK14 was progressively phosphorylated in bovine CC with a maximum at 6–10 h, corresponding to GVDB-MI transition. Then, phospho-MAPK14 level in cumulus decreased drastically during MI/MII transition. During this period (10–24 h of IVM), cumulus expanded and gap junctions closed between CC and oocyte. Specific inhibition of p38/MAPK14 phosphorylation by SB203580 during IVM led to a drastic decrease of cumulus expansion in porcine COC and to the arrest of oocytes at GV stage (Villa-Diaz & Miyano 2004). Lithium and BIO also inhibited the expansion in bovine COC during IVM. We directly showed by immunoblot that MAPK14 was phosphorylated in immature oocytes, whereas phosphorylation mostly disappeared after IVM. Similarly, in sea star immature oocytes, MAPK14 orthologue was Tyr-phosphorylated and dephosphorylation occurred during maturation (Morrison *et al.* 2000). In mice, p38 γ isoform was shown to be phosphorylated since GVBD (Perdiguero *et al.* 2003). In pig oocytes, after observation of ATF-2, a p38 substrate, it was supposed that p38 activation occurred after GVBD (Villa-Diaz & Miyano 2004). In fact, different isoforms of p38 MAPK exist and their activation is tissue specific (Zarubin & Han 2005). Thus, MAPK14 might participate in meiosis progression by modulating its activity.

LiCl₂ was shown to mimic insulin induced stimulation of glucose transport, and this effect was associated with a significant enhancement of GSK3B and MAPK14 phosphorylation in muscle adipocytes (MacAulay *et al.* 2003). Lithium also enhanced basal and insulin-stimulated MAPK14 phosphorylation in insulin-sensitive rat skeletal muscle (Harrell *et al.* 2007). Our data demonstrated, that in the presence of lithium or BIO, MAPK14 phosphorylation in COC was maintained at a higher level at the time of MI/MII transition compared with control IVM. We hypothesised that MAPK14 could be involved in the regulation of meiosis progression and is co-regulated with GSK3B. Recently, direct phosphorylation of GSK3B at serine³⁸⁹ by p38/MAPK14 has been reported, and this is a tissue-specific alternative pathway for GSK3B inactivation in mammalian cells (Thornton *et al.* 2008). In our experiments, inactivation of GSK3B by lithium correlated in oocytes with a transient decrease of MAPK3/MAPK1 activity preceding GVBD (at 3–6 h of IVM). Nevertheless, this might explain the delay in meiosis progression and the block of MI/MII transition. When MAPK3/MAPK1 activity was blocked by specific MEK1/2 inhibitor U0126 from the beginning of IVM, bovine oocytes progress through

GVBD, but could not reach the correct MI stage (Tosca *et al.* 2007); the same effect was reported in mice (Lee *et al.* 2007). In contrast, when mouse oocytes were treated by U0126 after GVBD, although the phosphorylation of MAPK3/MAPK1 was drastically reduced, the polar body extrusion was normal, but the organisation of metaphase plate and chromosome segregation were affected (Lee *et al.* 2007). Thus, in bovine oocytes, LiCl₂ led to the inhibition of MAPK3/MAPK1 activation prior to GVBD, which in turn contributed to the arrest at first meiotic division.

In conclusion, spatio-temporal pattern of GSK3B expression and activation in bovine oocytes and surrounding granulosa cells together with a functional analysis of GSK3B by modification of its activity by using specific GSK3 inhibitors, pointed to several possible functions of this kinase in oocyte meiosis. First, GSK3B is probably involved in follicle and oocyte growth during folliculogenesis. Second, during the first meiotic division, GSK3B could act as a regulator of chromosome segregation and correct MI/MII transition by interacting with AURKA. As in other tissues, GSK3B takes part in different signalling pathways; nevertheless in COC, GSK3B–MAPK14 interactions seem to contribute to cumulus expansion and transition of oocyte from MI to MII.

Materials and Methods

Ethics

All procedures were approved by the Agricultural and Scientific Research Government Committees in accordance with the guidelines for Care and Use of Agricultural Animals in Agricultural Research and Teaching (approval A37801).

Materials

All substances for *in vitro* culture of bovine oocytes and granulosa cells and molecular grade LiCl₂ (8 M LiCl₂) were purchased from Sigma.

BIO was from Calbiochem (VWR, Meudon, France). Purified ovine FSH-20 (oFSH; lot no.AFP-7028D, 4453 IU/mg, FSH activity=175 times activity of (oFSH-S1)) used for culture treatment was a gift from NIDDK, National Hormone Pituitary Program, Bethesda, MD, USA.

Samples collection

Cattle ovaries were collected from a slaughterhouse. COCs were aspirated from 3 to 6 mm antral follicles. COC with more than three layers of compact CC surrounding the oocyte were selected and washed several times in TCM199/HEPES medium supplemented with 50 mg/l of gentamycin. Groups of 50 COC were subjected to IVM in 500 μ l of TCM199 serum-free medium supplemented with EGF (10 ng/ml), IGF-1 (19 ng/ml), fibroblast growth factor (2.2 ng/ml), hCG (5 UI/ml), pregnant mares serum gonadotrophin (10 UI/ml), insulin (5 μ g/ml),

transferrin (5 µg/ml), selenium (5 ng/ml), L-cystein (90 µg/ml), β-mercaptoethanol (0.1 mM), ascorbic acid (75 µg/ml), glycine (720 µg/ml), glutamine (0.1 mg/ml) and pyruvate (110 µg/ml) at 38.8 °C for 24 h in a humidified atmosphere containing 5% CO₂ as described earlier (Donnay *et al.* 2004). LiCl₂ (final concentration of 20 mM if not specified) or BIO at 0.5–50 µM was added in culture medium just before IVM. Oocytes were denuded by mechanical separation of CC either immediately after collection (immature oocytes at GV stage, 0 h of IVM) or after 3, 6, 10, 18 or 24 h of IVM. Oocytes were rinsed in PBS, frozen in liquid nitrogen and stored until RNA or protein extraction. In each experimental situation, 20–50 oocytes were analysed for their nuclear status. Meiotic status of oocytes at different times of maturation was established by lamin A/C immunofluorescence and chromatin labelling with Hoechst33342 or DAPI (Sigma, 1 µg/ml), followed by microscopic observation. Corresponding CC were centrifuged and pellets were kept frozen at –80 °C until use.

Granulosa cells from small follicles (<6 mm) were harvested by puncturing the follicles allowing expulsion of the cells. Cells were recovered by centrifugation, washed with fresh medium and counted in a haemocytometer. The culture medium used was McCoy 5A supplemented with 20 mM HEPES, penicillin (100 U/ml), streptomycin (100 mg/l), L-glutamine (3 mmol/l), 0.1% BSA, 0.1 µmol/l androstenedione, 5 mg/l transferrin, 20 µg/l selenium and 5% FBS. The cells were initially cultured for 48 h and then serum starved for next 24 h. Then cells were incubated in fresh culture medium with or without test reagents for the appropriate time. All granulosa cell cultures were performed under a water-saturated atmosphere of 95% air/5% CO₂ at 37 °C.

For RNA and protein extraction, we used samples of CC or entire COC, oocytes, primary culture of granulosa cells (5×10⁵ cells per sample) and 1 g biopsy from hypothalamic region of adult cow brain. All samples were frozen and kept at –80 °C before experiments.

Thymidine incorporation into granulosa cells

Granulosa cells (2×10⁵ viable cells/500 µl) were cultured in 24-well dishes in McCoy 5A medium and 10% FBS for 48 h and were then serum starved for 24 h. Next, 1 µCi/µl of [³H]-thymidine (Amersham Life Science) was added in the presence or absence of LiCl₂ (20 mM) and/or FSH (10^{–8} M) and IGF-1 (10^{–8} M). Cultures were maintained at 37 °C under 5% CO₂ in air. After 24 h of culture, excess of thymidine was removed by washing twice with PBS and the cells fixed with cold trichloroacetic acid 50% for 15 min and lysed by addition of 0.5 N NaOH. The radioactivity was determined in scintillation fluid (Packard Bioscience) by counting in a β-photomultiplier.

RNA and cDNA preparation and analysis

Total RNA preparation

Total RNA was extracted using TriZol reagent as advised by the manufacturer (Invitrogen). To avoid contaminations with genomic DNA, total RNA preparations were treated by RQ1 DNase (Promega) as described in the manufacturer's protocol.

RT-PCR

RT was performed on RNA amounts corresponding to 10 oocytes or on 1 µg of total RNA from CC and brain. Complementary DNA was extended from oligo (dT)15 primers during 1 h at 37 °C by mouse Moloney leukaemia virus reverse transcriptase (Invitrogen) as described in the user's manual. For RT-PCR analysis 1% of total RT reaction was used as a template. PCRs were performed using reagents from Interchim (Montluçon, France). In the negative control reaction, RNA amounts were directly subjected to PCR. Bovine GSK3α- and GSK3β-specific primers at a final concentration 0.5 µM (GSK3A: sense 5'-TGGCAGTCGACCGATAACACA and antisense 5'-GTTG-GACGGTGTGAATCGACAT; GSK3B: sense 5'-TCCTTCAAATA-CCACAGCAGCC and antisense 5'-TTCCAATCGTGACCAG-TGTTGC, Genbank accession numbers NM_001102192 and DT844404 respectively) were used to perform 35 cycles of PCR using the same cDNAs as templates. As a positive control of cDNA quality, 28 cycles of β-actin-specific PCR (primers 5'-GCGTGACATCAAGGAGAAGC and 5'-TGGAAGGTGGA-CAGGGAGGC) were performed on all cDNA samples. To confirm the specificity of amplified fragments, PCR products were cloned and sequenced.

Protein analysis

Antibodies

GSK-3β rabbit MAB, phospho-Ser^{21/9}-GSK-3α/β, phospho-p44/42 MAPK rabbit polyclonal antibody (MAPK3/MAPK1 phosphorylated at Thr²⁰²/Tyr²⁰⁴ respectively) and phospho-p38 (phospho-MAPK14) phosphorylated at Thr¹⁸⁰/Tyr¹⁸² were purchased from Cell Signalling (Danvers, MA, USA). Total MAPK42 (MAPK1 or ERK2, C14) and p38 α (MAPK14, C20) rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). α-Tubulin (TUBA) MAB (clone DM1) was from Sigma. All primary antibodies used were raised against highly homologous human or mice (MAPK14) antigens and therefore cross-reacted with corresponding bovine proteins. HRP-conjugated rabbit anti-mouse, goat anti-rabbit and goat anti-mouse IgG antibodies were purchased from Lab Vision (Fremont, CA, USA). Lamin A/C MAB was from Ozyme (Saint Quentin Yvelines, France); Alexa Fluor488 goat anti-rabbit IgG and Alexa Fluor594 goat anti-mouse IgG were from Molecular Probes (Carlsbad, CA, USA).

Western immunoblotting

Groups of a definite number of bovine oocytes, COC, cumulus or granulosa cells were lysed and then frozen–thawed in 20 µl of Tris-saline-EGTA buffer (pH 7.5) supplemented with 2 mM sodium orthovanadate and 1 µl/ml of protease inhibitor cocktail (Sigma) then thawed–frozen three times by rapid incubation in liquid nitrogen followed by immersion in a warm water bath at 30 °C. Before loading, concentrated reducing Laemmli buffer containing 80 mM dithiothreitol at final concentration was added to all protein extracts and samples were boiled for 8 min.

Proteins extracts were resolved on 10% SDS-PAGE gels and transferred onto nitrocellulose membranes. Blots were blocked with 5% dry milk in Tris-buffered saline/0.1% Tween 20 for 1 h

at room temperature and probed with various antibodies overnight at 4 °C. Dilutions were 1/1000 for all primary antibodies. After extensive washes in 1×TBS/Tween 0.1%, immunoreactivity was detected using the appropriate HRP-conjugated secondary antibodies (diluted 1/5000, incubated 1 h at room temperature) and revealed by ECL Plus kit according to the manufacturer's instructions (Amersham Biosciences). Densitometry was performed by scanning the original radiographs and then analysing the bands with Scion Image software Beta 4.0.2 (Fuji PhotoFilm, Valhalla, NY, USA). At least three blots were analysed for each experimental condition. Quantified data was subjected to one-way ANOVA and/or Student's *t*-test at a minimum level of significance of $P < 0.05$.

Immunohistochemistry

Ovarian biopsies (quarters of whole including cortex with follicles <6 mm) were fixed for 12 h in a solution containing 50% of saturated picric acid, 3.7% formaldehyde and 5% of acetic acid. After serial dehydration steps, the samples were embedded in paraffin and serially sectioned at a thickness of 7 µm. Sections were deparaffined, re-hydrated and micro-waved for 5 min in antigen-unmasking solution (Vector Laboratories, Inc., AbCys, Paris, France) then left to cool at room temperature. After washing in a PBS bath for 5 min, sections were immersed in a peroxidase-blocking reagent for 10 min at room temperature to quench endogenous peroxidase activity (Dako Cytomation; Dako, Ely, UK). After three 5-min washes in a PBS bath, sections were blocked with 5% goat serum in PBS for 20 min and then incubated overnight at 4 °C with PBS/0.1% BSA containing GSK3β or phospho-GSK3α/β antibody (dilution 1/100). After washing thrice for 10 min, sections were incubated for 30 min at room temperature with a biotinylated goat anti-rabbit antibody. After serial washes, a 10 min staining was performed in a streptavidin peroxidase solution at room temperature as described in the kit manual (both Lab Vision Corporation). Immunoreactivity was revealed by incubation at room temperature with DAB. The slides were counterstained with haematoxylin, then dehydrated and mounted in Depex (Sigma). Negative controls were performed by replacing primary antibodies by rabbit IgG diluted in PBS/0.1% BSA at a final concentration of 2 µg/ml. Slides were observed using the Axioplan Zeiss transmission microscope.

Immunofluorescence of bovine oocytes

Oocytes were fixed for 10 min in a solution containing 50% of saturated picric acid, 3.7% of methanol stabilised-formaldehyde (Interchim) and 5% of acetic acid or in fresh solution of 4% paraformaldehyde in PBS pH 7.4, and then washed four times 15 min in PBS supplemented with 0.2% BSA and once 30 min in PBS/0.2% BSA/0.1% Triton. Blocking was performed in PBS supplemented with 2% BSA and 10% of goat inactivated serum during 2 h. Overnight incubation with primary antibodies (diluted 1/100 for phospho-Ser-GSK3α/β and GSK3β, 1/200 for lamin A/C) was performed at 4 °C with constant shaking. At least four 30 min washes in PBS/0.2% BSA were done, and oocytes were incubated with corresponding secondary fluorochrome-conjugated antibody (diluted 1/200)

for 2 h at room temperature. Five 20-min washes were then performed. Oocytes and embryos were put on slides and mounted with Mowiol supplemented with DABCO anti-fading and 1 µg/µl of DAPI (Sigma). Immunofluorescence was observed using Axioplan Zeiss fluorescent microscope supplied with appropriate filters.

Progesterone and oestradiol RIA

The concentration of progesterone in the culture medium of granulosa cells was measured after 48 h of culture by a RIA protocol as previously described (Tosca *et al.* 2005). Results were expressed as the amount of progesterone (ng/ml) secreted by granulosa primary cultured cells for 48 h per 100 µg of protein. Progesterone secreted by groups of COC was measured by using 50 µl of medium sampled at 0, 1, 3, 6, 10 and 24 h after beginning of IVM using the same protocol. Results were expressed as the amount of progesterone (ng/ml) secreted by groups of 50 COC for 24 h. Statistical treatment was performed by ANOVA at a minimum level of significance of $P < 0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was a part of the OVOGENAE program, sponsored by ANR and APIS-GENE.

References

- Acevedo N, Ding J & Smith GD 2007a Insulin signaling in mouse oocytes. *Biology of Reproduction* **77** 872–879.
- Acevedo N, Wang X, Dunn RL & Smith GD 2007b Glycogen synthase kinase-3 regulation of chromatin segregation and cytokinesis in mouse preimplantation embryos. *Molecular Reproduction and Development* **74** 178–188.
- Ali A, Hoefflich KP & Woodgett JR 2001 Glycogen synthase kinase-3: properties, functions, and regulation. *Chemical Reviews* **101** 2527–2540.
- Baatout S, Jaussi R, Michaux A, Buset J, Schoonjans W & Jacquet P 2007 Intracellular signal transduction in mouse oocytes and irradiated early embryos. *In Vivo* **21** 587–592.
- Baluch DP & Capco DG 2008 GSK3β mediates acentromeric spindle stabilization by activated PKC zeta. *Developmental Biology* **317** 46–58.
- Bartolome J, Hernandez J, Landaeta A, Kelleman A, Sheerin P, Risco CA & Archbal LF 2002 The effect of interval from day of administration of bovine somatotropin (bST) to synchronization of ovulation and timed-insemination on conception rate of dairy cows with and without ovarian cysts. *Theriogenology* **57** 1293–1301.
- Coenen K, Massicotte L & Sirard MA 2004 Study of newly synthesized proteins during bovine oocyte maturation *in vitro* using image analysis of two-dimensional gel electrophoresis. *Molecular Reproduction and Development* **67** 313–322.
- Cohen P & Frame S 2001 The renaissance of GSK3. *Nature Reviews. Molecular Cell Biology* **2** 769–776.
- Cohen P, Alessi DR & Cross DA 1997 PDK1, one of the missing links in insulin signal transduction? *FEBS Letters* **410** 3–10.
- Donnay I, Faerge I, Grondahl C, Verhaeghe B, Sayoud H, Ponderato N, Galli C & Lazzari G 2004 Effect of prematuration, meiosis activating sterol and enriched maturation medium on the nuclear maturation and competence to development of calf oocytes. *Theriogenology* **62** 1093–1107.

- Fisher DL, Morin N & Doree M 1999 A novel role for glycogen synthase kinase-3 in *Xenopus* development: maintenance of oocyte cell cycle arrest by a beta-catenin-independent mechanism. *Development* **126** 567–576.
- Frame S & Cohen P 2001 GSK3 takes centre stage more than 20 years after its discovery. *Biochemical Journal* **359** 1–16.
- Fumoto K, Lee PC, Saya H & Kikuchi A 2008 AIP regulates stability of Aurora-A at early mitotic phase coordinately with GSK-3 β . *Oncogene* **27** 4478–4487.
- Goodarzi MO, Antoine HJ, Pall M, Cui J, Guo X & Azziz R 2007 Preliminary evidence of glycogen synthase kinase 3 beta as a genetic determinant of polycystic ovary syndrome. *Fertility and Sterility* **87** 1473–1476.
- Goodarzi MO, Jones MR, Chen YD & Azziz R 2008 AKT2: first evidence of genetic association with polycystic ovary syndrome. *Diabetes Care* **31** 2284–2287.
- Guo TB, Chan KC, Hakovirta H, Xiao Y, Toppari J, Mitchell AP & Salameh WA 2003 Evidence for a role of glycogen synthase kinase-3 beta in rodent spermatogenesis. *Journal of Andrology* **24** 332–342.
- Harrell NB, Teachey MK, Gifford NJ & Henriksen EJ 2007 Essential role of p38 MAPK for activation of skeletal muscle glucose transport by lithium. *Archives of Physiology and Biochemistry* **113** 221–227.
- Jope RS 2003 Lithium and GSK-3: one inhibitor, two inhibitory actions, multiple outcomes. *Trends in Pharmacological Sciences* **24** 441–443.
- Kaladchibachi SA, Doble B, Anthopoulos N, Woodgett JR & Manoukian AS 2007 Glycogen synthase kinase 3, circadian rhythms, and bipolar disorder: a molecular link in the therapeutic action of lithium. *Journal of Circadian Rhythms* **5** 3.
- Lau KF, Miller CC, Anderton BH & Shaw PC 1999 Expression analysis of glycogen synthase kinase-3 in human tissues. *Journal of Peptide Research* **54** 85–91.
- Lee SE, Kim JH & Kim NH 2007 Inactivation of MAPK affects centrosome assembly, but not actin filament assembly, in mouse oocytes maturing *in vitro*. *Molecular Reproduction and Development* **74** 904–911.
- Li S, Maruo T, Ladines-Llave CA, Kondo H & Mochizuki M 1994 Stage-limited expression of myc oncoprotein in the human ovary during follicular growth, regression and atresia. *Endocrine Journal* **41** 83–92.
- Liang CG, Su YQ, Fan HY, Schatten H & Sun QY 2007 Mechanisms regulating oocyte meiotic resumption: roles of mitogen-activated protein kinase. *Molecular Endocrinology* **21** 2037–2055.
- Liu L, Rajareddy S, Reddy P, Jagarlamudi K, Du C, Shen Y, Guo Y, Boman K, Lundin E, Ottander U *et al.* 2007 Phosphorylation and inactivation of glycogen synthase kinase-3 by soluble kit ligand in mouse oocytes during early follicular development. *Journal of Molecular Endocrinology* **38** 137–146.
- MacAulay K, Hajdich E, Blair AS, Coghlan MP, Smith SA & Hundal HS 2003 Use of lithium and SB-415286 to explore the role of glycogen synthase kinase-3 in the regulation of glucose transport and glycogen synthase. *European Journal of Biochemistry* **18** 3829–3838.
- Meijer L, Flajolet M & Greengard P 2004 Pharmacological inhibitors of glycogen synthase kinase 3. *Trends in Pharmacological Sciences* **25** 471–480.
- Morrison DL, Yee A, Paddon HB, Vilimek D, Aebersold R & Pelech SL 2000 Regulation of the meiosis-inhibited protein kinase, a p38MAPK isoform, during meiosis and following fertilization of Seastar oocytes. *Journal of Biological Chemistry* **275** 34236–34244.
- Parrott JA, Kim G & Skinner MK 2000 Expression and action of kit ligand/stem cell factor in normal human and bovine ovarian surface epithelium and ovarian cancer. *Biology of Reproduction* **62** 1600–1609.
- Pascreau G, Delcros JG, Morin N, Prigent C & Arlot-Bonnemains Y 2008 Aurora-A kinase Ser349 phosphorylation is required during *Xenopus laevis* oocyte maturation. *Developmental Biology* **317** 523–530.
- Pelech S, Jelinkova L, Susor A, Zhang H, Shi X, Pavlok A, Kubelka M & Kovarova H 2008 Antibody microarray analyses of signal transduction protein expression and phosphorylation during porcine oocyte maturation. *Journal of Proteome Research* **7** 2860–2871.
- Perdiguer E, Pillaire MJ, Bodart JF, Hennersdorf F, Frodin M, Duesbery NS, Alonso G & Nebreda AR 2003 Xp38gamma/SAPK3 promotes meiotic G(2)/M transition in *Xenopus* oocytes and activates Cdc25C. *EMBO Journal* **22** 5746–5756.
- Salameh W, Helliwell JP, Han G, McPhaul L & Khorram O 2006 Expression of endometrial glycogen synthase kinase-3beta protein throughout the menstrual cycle and its regulation by progesterone. *Molecular Human Reproduction* **12** 543–549.
- Sarkisian M, Mendez R & Richter JD 2004 Progesterone and insulin stimulation of CPEB-dependent polyadenylation is regulated by Aurora A and glycogen synthase kinase-3. *Genes and Development* **18** 48–61.
- Sirard MA, Parrish JJ, Ware CB, Leibfried-Rutledge ML & First NL 1988 The culture of bovine oocytes to obtain developmentally competent embryos. *Biology of Reproduction* **39** 546–552.
- Swain JE, Ding J, Wu J & Smith GD 2008 Regulation of spindle and chromatin dynamics during early and late stages of oocyte maturation by aurora kinases. *Molecular Human Reproduction* **14** 291–299.
- Takenaka K, Moriguchi T & Nishida E 1998 Activation of the protein kinase p38 in the spindle assembly checkpoint and mitotic arrest. *Science* **280** 599–602.
- Thornton TM, Pedraza-Alva G, Deng B, Wood CD, Aronshtam A, Clements JL, Sabio G, Davis RJ, Matthews DE, Doble B *et al.* 2008 Phosphorylation by p38 MAPK as an alternative pathway for GSK3 β inactivation. *Science* **320** 667–670.
- Tomek W, Torner H & Kanitz W 2002 Comparative analysis of protein synthesis, transcription and cytoplasmic polyadenylation of mRNA during maturation of bovine oocytes *in vitro*. *Reproduction in Domestic Animals* **37** 86–91.
- Tosca L, Froment P, Solnais P, Ferre P, Foulle F & Dupont J 2005 Adenosine 5'-monophosphate-activated protein kinase regulates progesterone secretion in rat granulosa cells. *Endocrinology* **146** 4500–4513.
- Tosca L, Uzbekova S, Chabrolle C & Dupont J 2007 Possible role of 5'AMP-activated protein kinase in the metformin-mediated arrest of bovine oocytes at the germinal vesicle stage during *in vitro* maturation. *Biology of Reproduction* **77** 452–465.
- Uzbekova S, Arlot-Bonnemains Y, Dupont J, Dalbies-Tran R, Papillier P, Pennetier S, Thelie A, Perreau C, Mermillod P, Prigent C *et al.* 2008 Spatio-temporal expression patterns of aurora kinases A, B, and C and cytoplasmic polyadenylation-element-binding protein in bovine oocytes during meiotic maturation. *Biology of Reproduction* **78** 218–233.
- Vanholder T, Opsomer G & de Kruif A 2006 Aetiology and pathogenesis of cystic ovarian follicles in dairy cattle: a review. *Reproduction, Nutrition, Development* **46** 105–119.
- Vigneault C, McGraw S, Massicotte L & Sirard MA 2004 Transcription factor expression patterns in bovine *in vitro*-derived embryos prior to maternal–zygotic transition. *Biology of Reproduction* **70** 1701–1709.
- Vignerot C, Perreau C, Dalbies-Tran R, Joly C, Humblot P, Uzbekova S & Mermillod P 2004a Protein synthesis and mRNA storage in cattle oocytes maintained under meiotic block by roscovitine inhibition of MPF activity. *Molecular Reproduction and Development* **69** 457–465.
- Vignerot C, Perreau C, Dupont J, Uzbekova S, Prigent C & Mermillod P 2004b Several signaling pathways are involved in the control of cattle oocyte maturation. *Molecular Reproduction and Development* **69** 466–474.
- Villa-Diaz LG & Miyano T 2004 Activation of p38 MAPK during porcine oocyte maturation. *Biology of Reproduction* **71** 691–696.
- Wang X, Liu XT, Dunn R, Ohl DA & Smith GD 2003 Glycogen synthase kinase-3 regulates mouse oocyte homologue segregation. *Molecular Reproduction and Development* **64** 96–105.
- Welsh GI, Miller CM, Loughlin AJ, Price NT & Proud CG 1998 Regulation of eukaryotic initiation factor eIF2B: glycogen synthase kinase-3 phosphorylates a conserved serine which undergoes dephosphorylation in response to insulin. *FEBS Letters* **421** 125–130.
- Woodgett JR 1990 Molecular cloning and expression of glycogen synthase kinase-3/factor A. *EMBO Journal* **9** 2431–2438.
- Yao HB, Shaw PC, Wong CC & Wan DC 2002 Expression of glycogen synthase kinase-3 isoforms in mouse tissues and their transcription in the brain. *Journal of Chemical Neuroanatomy* **23** 291–297.
- Zarubin T & Han J 2005 Activation and signaling of the p38 MAP kinase pathway. *Cell Research* **15** 11–18.

Received 13 April 2009

First decision 21 May 2009

Accepted 21 May 2009