Chapter 4

Quantification of mRNAs encoding molecular motors and genes involved in chromosome segregation during final maturation of bovine oocytes in vivo

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Abstract

In the bovine as in many mammalian species, successful pregnancy following maturation, fertilization and culture in vitro of bovine oocytes remains relatively low compared to their in vivo counterparts. Moreover, it is well documented that not all oocytes obtained when using exogenous FSH hormone stimulation have a normal final maturation. The objective of this study was to investigate whether such differences in the oocytes and blastocysts developmental competence are associated with changes in oocyte mRNA levels of genes related to molecular motors, spindle formation and accurate chromosomal segregation throughout oocyte maturation (2 h pre LH, 6 and 22 h after LH) and at the blastocyst stage (day 7) in vivo. These genes include, 1) kinesin family member 3A 2) cytoplasmic dynein intermediate chain 3) Myosin regulatory light chain, 4) Formin-2, 5) partitioning defective protein 3, 6) Aurora-A. For this purpose, presumptive competent oocytes were selected after oFSH treatment on the basis of the steroid profile in the enclosing follicle. Moreover, to determine the role of these transcripts in developmental competence, mRNA expression was compared with that of non-competent oocytes from preovulatory follicles of the same group of cows, and to that of cultured oocytes from slaughterhouse ovaries at corresponding times of maturation and in vitro derived blastocysts.

Using quantitative real-time PCR, we detected for all above mentioned genes mRNA in the oocytes throughout the maturation process and the blastocyst stage. Formin-2 and Aurora-2 mRNA however, was only detected in the oocytes. Analysis of the mRNA levels within the oocytes scored as normal during the maturation did not reveal statistically differences. Relative abundance of all the mRNAs tested were significantly higher in the normal oocytes compared to that scored as deviant in the prematuration group. In the prematuration group, the mRNA for cytoplasmic dynein, Formin-2 and par-3 were significantly lower in the oocytes collected from slaughterhouse ovaries than those matured in vivo and scored as normal. At MII, Formin-2 and Aurora-A mRNA levels were significantly higher in the normal in vivo matured oocytes compared to the deviant or those matured in vitro. The level of par-3 mRNA was significantly higher ($P < 0.05$) in the in vivo derived blastocysts than in vitro produced blastocysts. The present results suggest an important role for these genes in spindle assembly, position and chromosome segregation. Furthermore it may be suggested that both the exogenous FSH induced intrafollicular environment and in vitro maturation and culture conditions may affect the developmental competence by affecting the expression of these genes.

***Key words*:** gene expression; bovine oocyte; blastocyst; KIF3A; par-3; Aurora-A; Formin-2

Introduction

Meiotic and developmental competence is acquired progressively during follicular and oocyte growth and is associated with a series of nuclear and cytoplasmic changes [1, 2]. The rate of successful pregnancy following maturation, fertilization and culture in vitro of bovine oocytes remains relatively low compared to their in vivo counterparts [3-5]. Moreover, it is well known that the hormonal environment of the follicles is altered in cows stimulated with exogenous gonadotrophin to a varying degree, depending partially on the type of protocol and the hormonal treatment used [6, 7]. Ovarian stimulation in mice has been shown to result in a significant increase in the frequency of oocyte spindle defects resulting in chromosomal errors [8-10]. Reduced fertility and pre- and post-implantation mortality have also been indicated as a consequence of using standard doses of gonadotrophins [11-15]. Clear evidence from several species indicated compromised developmental competence of an embryo to give rise to a viable healthy offspring, reflecting intrinsic oocyte defects that may include nuclear abnormality and suboptimal cytoplasmic maturation [16-20].

The intracellular trafficking of organelles complexes plays a major role in many cell and developmental processes. During maturation, oocyte ultrastructure studies have consistently demonstrated that translocation and transport organelle defects are likely to predominate in oocytes matured in vitro or obtained from animals stimulated with exogenous gonadotrophins [21-23]. Furthermore, the orientation and integrity of the meiotic spindle must be strictly controlled for proper chromosomal segregation. Misorientation of the spindle and errors in this process severely impairs chromosome segregation and may result in chromosomal aneuploidy. Therefore, the contribution of molecular motors and proteins involved in spindle formation and chromosome segregation to molecular events important for oocyte nuclear and cytoplasmic maturation and hence oocyte developmental competence is possible.

Organelle translocations in diverse organisms have some common molecular requirements, namely microtubule, actin, dynein, myosin, and kinesin. The directed transport of cellular components along the polarized microtubule and actin arrays use three major classes of motor proteins, kinesins [24], dyneins [25] and myosin which each move unidirectionally along the microtubule and actin tracks. These motors are required for multiple cellular tasks, such as the transport and positioning of organelles, the assembly of the meiotic spindle, chromosome movement [26] and mRNA translocation [27, 28].

Microtubules and actin filaments, the two key components of the cellular cytoskeleton, play important roles in all aspects of spindle assembly [29-33]. In somatic cells, several microtubule-dependent motors influence chromosome movement and positioning, in addition to its role in organelles translocation. These include a plus end directed motor, kinesin (KIF) [34, 35], and a minus end directed motor, cytoplasmic dynein. KIF3A is a member of the kinesin superfamily of motor proteins, producing movement necessary for transport of vesicles [36], positioning of nuclei, and proper segregation of genetic material. Targeted disruption of the KIF3A gene in mice uncovered an important role for KIF3 during development. The kif3A-/- mice do not survive beyond midgestation and exhibit apparent morphological abnormalities [37]. Similarly, the cytoplasmic dynein motor is a large complex composed of two heavy chain polypeptides, three intermediate chains and four light intermediate chains [38]. The cytoplasmic dynein motor has been implicated in vesicle transport, the perinuclear positioning of Golgi apparatus, chromosome movement on the mitotic spindle, separation of the mitotic spindle poles and nuclear positioning during cell division [39, 40]. In mammalian cells, dynein activity is proposed to control spindle length by transporting the MT-depolymerizing kinesin-13 KLP10A to the spindle poles [41]. Although the catalytic heavy chain is responsible for force production and dynamic interaction with microtubules, the intermediate chains have been implicated in linking the motor complex to membranous organelles [42, 43].

Actin-based motor myosin is central to the molecular events leading to proper cytoplasmic organelle translocation [44, 45]. Cytoplasmic myosin (Myosin II) has been demonstrated to participate in many cellular movements [46] in addition to its well-established role in cytokinesis [47, 48]. Myosin is a hetero-hexamer composed of two copies each of the heavy chain, essential light chain and regulatory light chain (MRLC) [49]. Biochemical and genetic studies have shown that MRLC regulates myosin activity by phosphorylating certain amino acids of myosin (for review see [50]) and play a direct role in force generating properties of myosin [46]. In Drosophila, mutations in the gene encoding MRLC disrupt cytokinesis in female germ cells [51] and showed defects in tissue movement during embryogenesis [52]. A recent study showed that in fertilized mouse oocytes, inhibition of myosin II or myosin light chain kinase (MLCK), which belongs to the family of Ca^{2+} /calmodulin dependent protein kinases and specifically phosphorylate myosin regulatory light chain inhibited second polar body formation and reduced cortical granules exocytosis [53].

Assembly of actin cables depend on two formin homologues, Bni1p and Bnr1p [54], members of a family of cytoskeletal regulatory proteins defined by conserved formin

homology (FH)1 and FH2 domains, for reviews, see [55]. Loss of Formin-2 (FMN2) function causes an oocyte maturation arrest at metaphase I, polyploidy and asymmetrical spindle positioning [56].

Partitioning defective protein (PAR-3) is known as an essential regulator of egg polarity, and to orient and position mitotic spindles relative to the anterior axis in the *C. elegans* embryo, [57]. Recent findings in mice oocytes showed, that Par-3 asymmetrically localized during meiosis and that its staining intensity increased on the cortical side of the MI spindle after germinal vesicle breakdown, which suggests an important role in defining the future site of the polar body extrusion [58].

Aurora-A is also an important regulator of spindle formation which plays an essential role for accurate chromosome segregation, and is known to be involved in the induction of centrosome duplication-distribution abnormalities and aneuploidy in mammalian cells [59, 60]. Mutation or disruption of the Aurora-A gene in various species leads to mitotic abnormalities, including centrosome separation, spindle aberrations, and chromosome segregation defects [61]. During maturation in mice oocytes, Aurora-A takes part in not only the initiation but also maintenance of meiotic spindle microtubule organization [62].

In vivo, a wide variety of growth factors, steroid and proteins are produced by granulosa cells in response to the LH surge [63]. Consequently, it seems logical that in vitro culture initiates a cascade of altered mRNA activity that leads to reduced oocyte developmental competence. Moreover, there is clear evidence indicating misrepresentation of mRNA expression being involved in molecular mechanisms of oocyte maturation [64] and early embryonic development due to the effect of in vitro culture conditions [65, 66]. Therefore, in this study, we used oocytes collected from cows stimulated with FSH classified as deviant and normal based on the steroid hormones profile, and oocytes collected from slaughterhouse ovaries and matured in vitro, to investigate whether the divergent culture conditions is accompanied by altered mRNA levels of the different selected genes playing important roles in organelles translocation and spindle integrity and formation.

Materials and Methods

Experimental design

At each time point of maturation, oocyte mRNA expression was compared with that of non-competent oocytes from preovulatory follicles of the same group of cows and with that of cultured oocytes from slaughterhouse ovaries at corresponding times of in vitro

maturation. Secondly, mRNA expression of the competent oocytes was compared with that of in-vivo derived blastocysts. Finally, mRNA expression of day 7 in-vitro produced blastocysts was compared to in-vivo derived counterparts.

Follicle development was stimulated in Holstein-Friesian cows using our standard protocol [65] with oFSH and a Crestar/GnRH-controlled LH surge. Cows were allocated at random to three experimental groups for ovariectomy (OVX): 1) at onset (2 h before LH), 2) after initiation (6 h after LH), and 3) at completion (22 h after LH) of final maturation to determine changes in mRNA expression throughout maturation in vivo. Oocytes were selected on the basis of follicle size, the steroid profile in the enclosing follicle as shown in table 2 and, were assigned to replicates for QPCR analysis in such a way that within a group replicates were equivalent with regards to steroid profile and represented maximum numbers of cows. In vivo blastocysts were collected from superovulated cows by flushing the uterus at day 7 after insemination. In-vitro oocytes were produced after maturation in vitro (IVM) using oocytes obtained from an abattoir, while in-vitro produced blastocysts were collected after fertilization and culture in-vitro of the in-vitro matured oocytes till the blastocysts stage. The experiment was carried out as approved by the Ethical Committee of the Veterinary Faculty of Utrecht University.

Animals and treatment

Normally cyclic Holstein-Friesian cows (n=36) were selected and treated for superovulation using the protocol as described before [65] with oFSH (Ovagen ICP, Auckland, New Zealand), prostaglandin (PG; Prosolvin; Intervet International B.V., The Netherlands) and a timed LH surge controlled by norgestomet/GnRH (Crestar ear implant/Receptal; Intervet International B.V.). Cyclicity was verified by measuring the concentration of progesterone in peripheral plasma during at least 6 weeks before FSH-stimulation [67] and the LH surge was monitored in plasma from 38 h after PG until OVX using a validated RIA with bovine LH (bLH-7981) for iodination and standards, and rabbit anti-bLH (8101) as antiserum [68]. Oocytes and follicular fluids were collected following OVX at 50, 58 and 74 h after PG corresponding with 2 h before, 6 and 22 h after the maximum of the LH surge, respectively.

Collection and selection of in-vivo preovulatory oocytes

For every treatment run with a group of 4 cows OVX was performed at 1 h intervals, the time needed to collect all oocytes and follicles from one cow by laparotomy through flank incision under local infiltration anesthesia [69]. Ovaries were collected in 0.9% (w/v)

NaCl at 37°C and immediately transported to the laboratory. The contents of each follicle > 9 mm were aspirated using an 18-ga winged infusion set needle attached to 15 ml polystyrene conical tube under low pressure by means of a suction pump, and were then immediately stored on ice at 4°C. The size of the follicles was calculated from the volume of follicular fluid after collection. After retrieval of the cumulus oocyte complexes (COC) under a stereo microscope, the follicular fluids were centrifuged 3,000 *g* for 10 min at 4°C and stored at -25°C until analysis for steroids. Collected COCs were rinsed twice in 700 µl PBS (Sigma-Aldrich, St. Louis, MO, U.S.A.) supplemented with 5% (w/v) polyvinyl alcohol (PVA; Sigma). Cumulus cells were removed by continuous pipetting after incubation for one minute with 100 µl 0.1% (w/v) hyaluronidase (Sigma-Aldrich, St. Louis, MO, U.S.A.) and denuded oocytes were checked for remaining cumulus cells and washed three times with PBS-PVA and stored individually in at -80°C until RNA extraction.

For each collection group relative to the LH surge, oocytes were sub-divided on the basis of the concentration of steroids in the fluid of the enclosing follicle. Briefly, oocytes from follicles with estradiol 17β > 0.9 µmol/L before LH, > 0.5 µmol/L 6 h after LH, and progesterone > 0.5 µmol/L 22 h after LH were considered to be competent (Table 2). Oocytes from follicles with unmistakably deviating steroid concentrations were assigned to the respective non-competent sub-groups, that is with estradiol < 0.37 µmol/L before and 6 h after LH, and with progesterone < 0.38 µmol/L 22 h after LH. The few oocytes that had heavy atretic features (expanded cumulus scattered in dark clumps in a jelly-like matrix) were excluded.

Blood sampling

Heparinized blood samples were collected from the jugular vein every day during the experimental cycle, every 3 h starting 12 h before removal of the second implant and every hour thereafter for 6 h. After immediate centrifugation at 4ºC, plasma was stored at -25ºC.

RIA of steroids in follicular fluid

Concentrations of the steroid hormones estradiol 17β and progesterone in follicular fluid were determined in aliquots of 1 to 25 µL fluid dependent of the hormone and the size of the follicle by solid-phase ^{125}I RIA methods (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA, U.S.A; estradiol 17β: TKE2; progesterone: TKPG) as validated for blood plasma of cows [67] with slight modifications such as extraction with diethyl ether (BDH Laboratory Supplies, Poole, England).

In vitro maturation (IVM) of immature oocytes

Bovine ovaries were collected from abattoirs, and transported in a thermos flask within 3 hours of collection. After washing with water and saline solution, cumulus oocyte complexes (COCs) were aspirated from follicles 3-6 mm in diameter, washed in HEPES-buffered TCM-199 (Gibco BRL, Paisley, UK), and selected on the basis of their morphology for in vitro maturation according to the density of their cumulus cell layers, and randomly allocated in groups of 50 COCs per well to a 4-well culture plate (Nunc A/S, Roskilde, Denmark). In vitro maturation of the COCs was performed in 500 μl TCM-199 per well, supplemented with 10% (v/v) fetal calf serum, 4 μg FSH/ml, and 6 μg LH/ml (Sioux Biochemical Inc., Iowa, USA), and 0.1 mM cysteamine (Sigma Chemical Co., St louis, USA) for 22 h at 38.5ºC under an atmosphere of 5% CO2 in air with maximum humidity. At the beginning (0 h), 6 h and 22 h of the maturation, 13 oocytes from three different batches were denuded from their cumulus cells by vortexing and stored at -80ºC.

In vitro embryo production (IVP)

Procedures for in vitro maturation were performed as described previously. After maturation, oocytes in COCs were fertilized in vitro according to the procedure described by Parrish et al.[70] with minor modifications [71] using frozen-thawed semen from a bull of proven fertility. The presumptive zygotes were freed from cumulus cells 20 h after IVF by vortexing, and a maximum of ten zygotes was placed in a 20 μl droplet of synthetic oviductal fluid (SOF) medium [72] supplemented with essential and non-essential amino acids (Sigma-Aldrich, St. Louis, MO, U.S.A.), 0.1% (w/v) BSA (Sigma-Aldrich), under oil (Reproline medical GmBH, Rheinbach, Germany) and cultured at 39°C, in humidified air containing 5% CO_2 and 7% O_2. On day 4 after IVF the number of cells per cleaved embryo was scored and all cleaved embryos were transferred to fresh SOF droplets. The developmental stage of the embryos was assessed at day 7 after IVF. Four groups of 5 expanded blastocysts were rinsed in PBS and stored at -80°°C until RNA extraction.

Collection of in-vivo derived blastocysts

Superovulation was induced using an eCG/monoclonal anti-eCG/PG treatment scheme [67]. Cows were inseminated with one straw into each uterine horn, 10 h after the LH peak. Seven days later the embryos were non-surgically recovered, embryonic developmental stage and general morphological appearance were assessed by stereo microscopy, once

qualified to those in morphological grades I and II [73], frozen for storage in liquid nitrogen until use.

RNA isolation, precipitation

Total RNA was prepared from at least three replicates at each maturation stage, except for 22 h normal, 6 and 22 h deviant oocytes, of which 20, 18 and 11 oocytes were collected, resulting in 2, 2 and 1 replicate, respectively. Each replicate containing from 9 to 13 pooled oocytes. Four replicates of day 7 blastocysts were used, each containing 5 blastocysts. The RNA was then isolated using microspin column and DNA was digested with Dnase1 to eliminate possible genomic DNA contamination according to manufacturer's instruction (Absolutely RNA Microprep Kit, Stratagene, San Diego, CA, USA), the RNA was recovered by two subsequent 50-µl elutions with warmed (60°°C) elution buffer provided in the kit.
RNA was then precipitated with 250 µl of 100% ethanol (EtOH) and 10 µl of 3 M sodium acetate pH 5.2, using 1 µl of 1 mg/ml linear acrylamide (Ambion, Austin, TX) as co-precipitant. The mixture was chilled at -80^{o}C for 30 min, centrifuged for 20 min at 4^{o}C at 16000 g. The pellet was then washed with 75% EtOH and resuspended in 15 µl of water for real-time PCR analysis.

Real-time Polymerase Chain Reaction

Reverse Transcription and primers design

Total RNA was reverse-transcribed in a total volume of 20 µl using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) containing oligo-dT and random hexamer primers. Reactions were incubated for 5 min at 25°°C, 30 min at 42°C, and 5 min at 85°C.
Primer sets were designed by using Beacon Designer 4 software (PREMIER Biosoft International, Palo Alto, CA, U.S.A.), from bovine sequences from NCBI, the primers used in the study are shown in table 2. The specificity of the primers was confirmed by sequencing and confirmation of the PCR product size on a standard 2% agarose gel with ethidiumbromide (EtBr). The PCR products were sequenced after purification with the QIaquick PCR Purification Kit (Qiagen, Valencia, CA, U.S.A.) and quantification with a spectrophotometer (Nanodrop ND 1000, Isogen, IJsselstein, the Netherlands). PCR products were then diluted from 100 to 0.01 fg as standards to construct the standard curve.

Real-time Polymerase Chain Reaction

Real-time PCR was performed on a Bio-Rad MyiQ system using the 2X iQ SYBR Green Supermix reagents, following the manufacturer's protocols. Reactions were performed using 25 µl duplicate reactions with the quantity of diluted cDNA corresponding to 0.1 to 0.3 of an oocyte, depending on the mRNA abundance as determined using oocytes from slaughterhouse ovaries matured in vitro, and 0.5 µM of each primer. Each transcript was amplified from at least three different replicates. Each transcript was amplified from at least three different groups of pooled oocytes, except 22 h normal, 6 and 22 h deviant oocytes, where 2, 2 and 1 group were used, respectively (see table 2). Samples and standard curves were amplified on the same run with the same PCR master mix. The thermal cycling program starts with an initial denaturation step at 95°°C for 3 min, and is followed by 45 PCR cycles (dissociation for 5 sec at 95^{o}C, annealing for 5 sec at temperature showed in table 2, and elongation for 20 sec at 72^{o}C), one melting cycle consisting of 5 sec at 95^{o}C, 30 sec at 72^{o}C, and a step cycle up to 95^{o}C (0.3^{o}C/sec transition rate), and finally a cooling down cycle at 40^{o}C. Amplification of the GAPDH mRNA [74, 75] was performed for each reverse transcribed sample as an endogenous quantification standard. These raw CT values were then analyzed with a modified delta-Ct method using a PCR data analysis program, qBase (version 1.3.2) (http://medgen.ugent.be/qbase/) to obtain relative quantification values. PCR product was analyzed on a 1.5% agarose gel with EtBr to confirm amplification. Product sizes and annealing temperatures for each gene are presented in table 1.

Statistical analysis

Data are presented as the mean ± SEM. Initially, Expression analysis data of the oocytes from follicles with a normal profile were subjected to one-way ANOVA and LSD test was used as a post hoc for comparison of individual means to assess the effect of maturation stages (-2, 6, 22). Differences within each oocyte time group (follicles with a normal profile, deviant profile and oocytes matured in vitro) were analyzed using one-way ANOVA. Difference between in vivo and in vitro derived blastocysts was analyzed using Student's t-test. Differences were considered statistically significant at the 95% confidence level ($P < 0.05$).

Table 1. Information on the primers used for real-time PCR

Genes	GeneBank accession number	Oligos sequences	Product size (bp)	Annealing temperature (°C)
KIF3A	NM_007054	F 5'-ggtgtttgggtagtctctg -3' R 5'-cggcacctaacaacaacct-3'	162	56
Dynein	BC015038	F 5'-gagtgaagaaggttctgtgtac-3' R 5'-aggcttgttattcttagttgtcc-3'	198	58
MRLC	BT020978	F 5'-aaagagcaaagaccaagacc-3' R 5'-cctggagcctcgttcatc-3'	226	56
FMNL2	NM_052905	F 5'-tgaccataacacgctgctg-3' R 5'-cctcttctgcttgcttatatgc-3'	200	54
Par3	AF467002	F 5'-ggcttcctcaagcagaacac-3' R 5'-cgagcactgaaaggcactac-3'	350	55
Aurora-A	NM_001038028	F 5'-agagacattaagccagagaa-3' F 5'-atccgaccttcaatcatttcag-3'	152	55
GAPDH	U43284	F 5'-ccacgagaagtataacaacacc-3' R 5'-gccagtagaagcagggatg-3'	229	56

KIF3A, *Homo sapiens* kinesin family member 3A; Dynein, *Homo sapiens* cytoplasmic dynein intermediate polypeptide 2; MRLC, *Bos taurus* myosin regulatory light chain 2; FMNL2, *Homo sapiens* formin-like 2; Par-3, *Homo sapiens* partitioning-defective 3 ; Aurora-A, *Bos taurus* Aurora-A; GAPDH, *Bos taurus* glyceraldehyde-3-phosphate dehydrogenase

Results

The number of oocytes classified as normal and deviant based on steroid and follicle size is presented in table 2.

The mRNAs for all the genes studied were amplified in all of the total RNA samples isolated from the oocytes. The mRNAs for Aurora-A and formin-2 were not detected at the blastocyst stage. DNA sequencing confirmed the PCR products are indeed from the same genes.

Messenger RNA levels in Fig. 1, A represent relative values of KIF3A; QPCR analysis showed that KIF3A mRNA level was highly expressed during maturation but decreases sharply at the blastocyst stage. No significant change was observed during maturation between the three maturation stages. However, in the prematuration group oocytes, a significant lower level of mRNA was found in the oocytes scored as deviant after FSH stimulation compared to those scored as normal or those matured in vitro.

Table 2. Steroid concentrations in follicles with presumptive competent and non-competent oocytes recovered during maturation in FSH-stimulated cows with a controlled LH surge

	notes	2 h before LH (Pre)	6 h after LH (Post)	22 h after LH (Post)
Normal follicles	[1]			
- number		47	50	20
- estradiol 17β	[2]	1.64 ± 0.12	0.84 ± 0.04	0.11 ± 0.01
- progesterone		0.15 ± 0.01	0.51 ± 0.01	0.75 ± 0.05
- follicle diameter	[3]	13 ± 0.4	13.5 ± 0.3	13 ± 0.5
- number of cows	[4]	13	9	8
Deviant follicles				
- number		52	18	11
- estradiol 17β		0.21 ± 0.01	0.23 ± 0.03	0.19 ± 0.02
- progesterone		0.09 ± 0.004	0.58 ± 0.06	0.16 ± 0.03
- follicle diameter		12 ± 0.3	12 ± 0.4	12 ± 0.8
- number of cows		7	7	3

[1] competent: oocytes from functional follicles on the basis of steroid profile
[2] steroid concentrations ± SEM in μmol/L follicular fluid
[3] mean diameter in mm as calculated from volume of fluid of follicles from which oocytes were used
[4] number of cows from which oocytes were retrieved

Messenger RNA levels for cytoplasmic dynein (Fig. 1, B) tended to decrease during oocyte maturation. Low levels of mRNA were detected at the blastocyst stage. However, due to the large variation in the values, the decrease was not significant during maturation. In the prematuration group and oocytes collected 6 h after maturation, the highest levels of mRNA among all oocytes analyzed was found in the FSH oocytes scored as normal.

Messenger RNA of the MRLC (Fig. 1, C) was detected in all stages of the oocyte maturation as well as at the blastocyst stage. No differences were observed between oocytes scored as normal during maturation. Among the prematuration group oocytes, the mRNA level was expressed highly in the oocytes scored as normal compared to those scored as deviant as well as those matured in vitro were no significant difference observed. No statistically significant differences ($P < 0.05$) were observed between the in vivo derived blastocysts and in vitro produced blastocysts.

The oocytes express Formin-2 mRNA throughout the maturation, whereas Formin-2 mRNA was not expressed by the day 7 blastocysts (Fig. 1, D). This expression did not appear to change significantly during maturation. Within the prematuration group, oocytes scored as normal expressed approximately 18 fold more mRNA than oocytes scored as

deviant. Similarly, at 22 h of maturation, normal oocytes expressed approximately 8 fold mRNA more than the deviant ones.

The mRNA of the par-3 was present through the maturation period and at the blastocyst stage. However, as shown in Figure 1, E, levels of par-3 mRNA were not statistically different among oocytes classified as normal at different maturation stages. In vivo derived blastocysts showed significantly ($P < 0.05$) higher mRNA levels than their in vitro counterparts.

The Aurora-A mRNA levels were detected throughout the maturation but, were not statistically different among oocytes classified as normal (Fig. 1, F). During the prematuration period, the mRNA levels for Aurora-A significantly ($P < 0.05$) decreased in the oocytes scored as deviant compared to the normal oocytes. After 22 h of maturation, the same pattern of mRNA expression could be seen, but a significant decrease was also observed in the mRNA level of the in vitro compared to deviant oocytes.

Figure 1. Messenger RNA levels of the molecular motors proteins and genes involved in chromosome segregation (A to F) in bovine oocytes collected from cows undergoing superovulation with FSH, scored as normal or deviant based on steroid profile and follicle sizes and oocytes collected from slaughterhouse ovaries and matured in vitro.
a, b, c: significantly different within the time groups (in vivo normal, in vivo deviant and in vitro oocytes) ($P < 0.05$).
* Significantly different from the oocytes

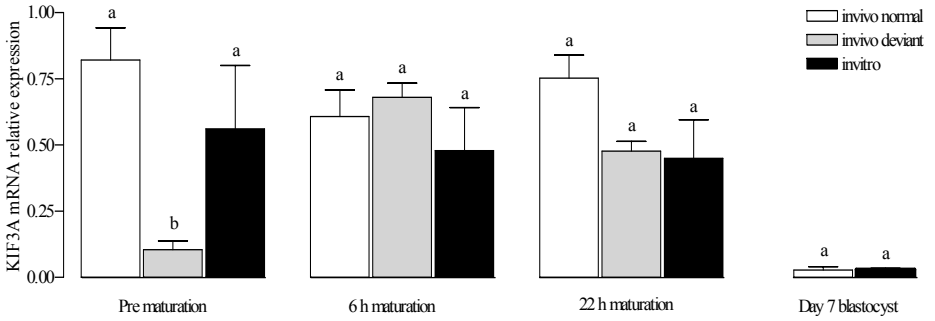

Fig. 1, A. KIF3A mRNA expression

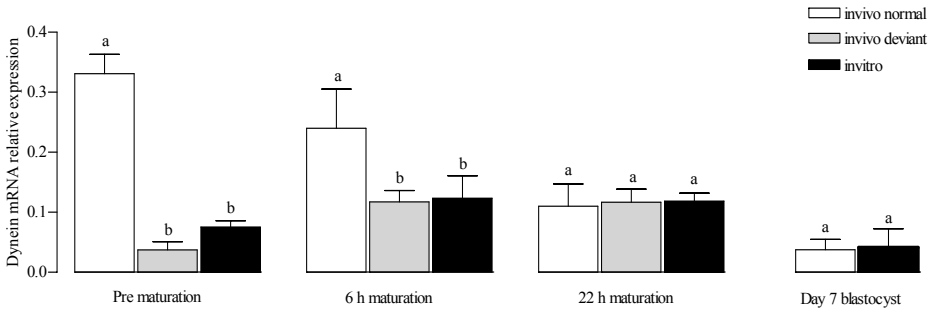

Fig. 1, B. Cytoplasmic dynein

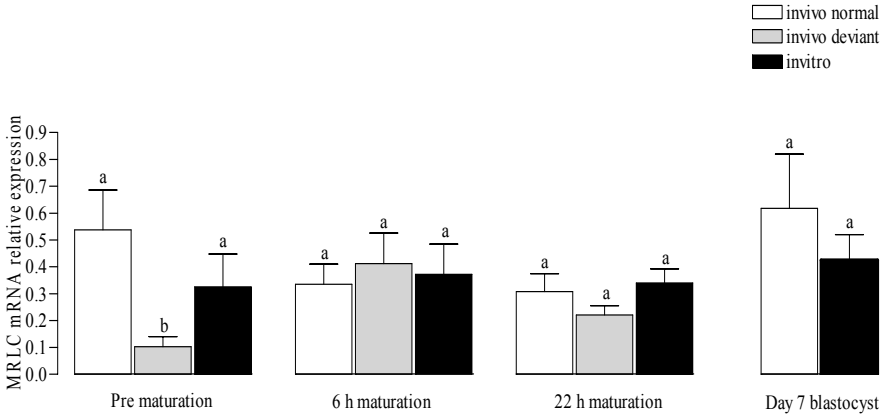

Fig. 1, C. Myosin regulatory light chain mRNA relative expression

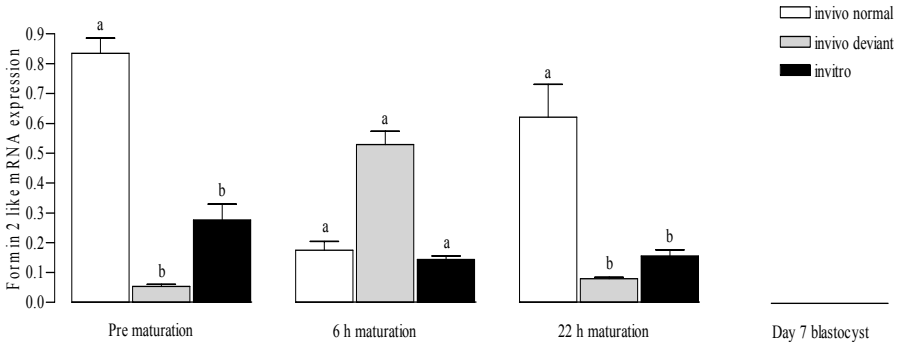

Fig. 1, D. Formin 2 like mRNA expression

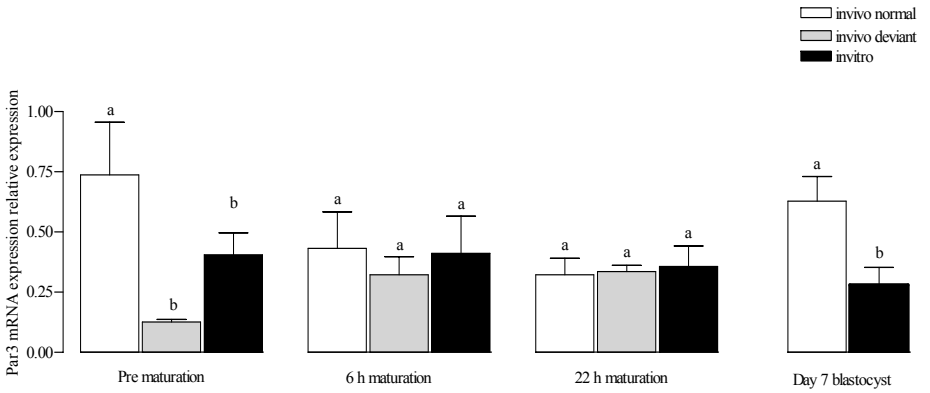

Fig. 1, E. Par3 mRNA expression

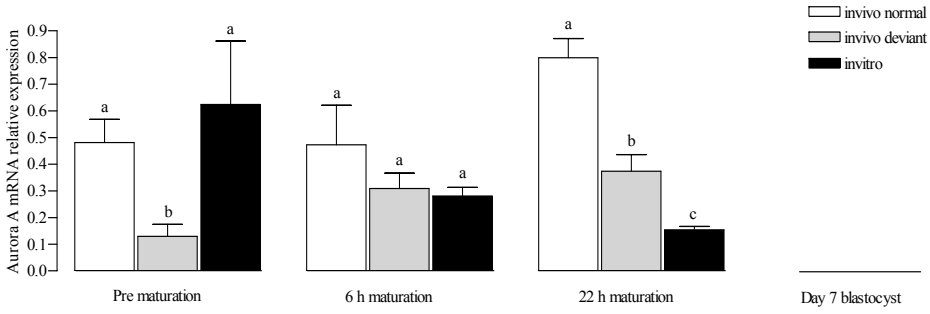

Fig. 1, F. Aurora-A mRNA expression

Discussion

At present, the nuclear and cytoplasmic pathways that contribute to the developmental competence of the bovine oocyte during maturation have not yet been resolved. In the current study, the identification of mRNAs of numerous molecular motors protein and genes involved in organelles translocation, spindle formation and chromosomal segregation in the oocyte have led to the suggestion that the expression of these mRNAs are needed to maintain proper nuclear and cytoplasmic maturation. In vitro and in vivo studies using somatic cells and knockout mouse models support that these genes have a conserved functional obligatory requirement to support nuclear and cytoplasmic maturation. The existence of these mRNAs in significant amount during maturation prompts the question which specific task(s) each individual mRNA performs in the oocyte because most of the studies came from experiments using somatic cells. However, meiotic tissues exhibit unique subcellular movements that may require specialized motors. Chromosome behavior during meiosis, particularly during the first division, differs markedly from that seen during mitosis, alignment and disjunction of chromosomes during meiosis I occurs by orientation of bivalents to the pole, not sister chromatids as in mitosis [76]. Therefore, due to cell type differences, considerable function diversity may exist. Further identification of mRNA for specific proteins in the different pathways should allow a more exact assessment of the role of these genes in oocyte maturation. Moreover, elucidating the functions and the mechanism by which these genes are regulated in the mammalian oocyte may contribute to our understanding of the molecular mechanisms of oocyte maturation.

It is well documented that not all oocytes in a stimulated cycle have a normal final maturation. Aberrations in cytoplasmic maturation as well as in timing of nuclear maturation were seen by Hyttel et al [77] in the bovine oocyte. It has been reported that only 72% of the oocytes collected at the end of maturation reach the MII stage of meiosis after exogenous gonadotrophins stimulation [78], while this percentage increases to more than 90% in the oocytes matured in vitro. The mechanism by which the superovulation techniques increase the incidence of meiotic arrest and aneuploidy is not known [14]. However, different possibilities can explain the higher incidence of aneuploidy in mammalian oocytes stimulated with exogenous gonadotrophins. Compromised follicular vascularity around the follicle may result in an under-developed capillary bed with reduced blood flow. An adequate blood supply in tissues is required to supply oxygen for aerobic metabolism, nutrients and hormones, and to eliminate CO_2 and other metabolic byproducts. Follicle diameter, estradiol concentration in follicular fluid and the vascular area have been shown to be closely associated with each other [79]

In the process of oocyte maturation there is a fundamental requirement for stable distribution of cellular components to different specific destinations at specific times. To achieve this task, cells employ molecular motors and two sets of tracks: microtubules and actin filaments. The motor domains of kinesins and dyneins have ATP-binding and microtubule-binding sites [80].

Kinesins (KIFs) are responsible for intracellular trafficking of vesicles and organelles along microtubules and for the dynamics of chromosomes and microtubules in mitosis and meiosis [81, 82]. All KIFs have a globular motor domain that contains a microtubule binding sequence and an ATP-binding sequence [24]. Kinesin-2 family is a heterotrimeric complex composed of a KIF3A/3B heterodimer and kinesin associated protein 3 (KAP3) [83]. The importance of KIF3A mRNA expression was shown in *Drosophila melanogaster* females that were homozygous for KIF3A mutations. In these females, the sperm and embryo nucleus did not fuse [84] and consequently no further development could take place. In mammals, Marszalek and others [37] demonstrated that mouse null- embryos die at 10 days post coitum. KIF3A null mouse embryos apparently do have some competence, but fail to develop any further. Recently, immunostaining analysis of Hela cells revealed intense KIF3A staining localized at the centrosomes in interphase and, when chromosomes began to condense and the mitotic spindle was formed in prometaphase, KIF3A was localized mainly at the spindle microtubules and was also localized around the cellular cortex [34]. Our results showed that KIF3A was predominately expressed in the oocyte during maturation implying an important function in the molecular events which are important for the progression of meiosis. Moreover, within the prematuration group, deviant oocytes showed significantly low levels of KIF3A mRNA, as discussed previously, it is possible that these oocytes represent the group of the oocytes that lack the ability to resume meiosis. Interestingly, KIF3A has been reported to participate in neuronal cell polarity through the transport of the par-3-aPKC complex at the tip of the neurites [85]. The coexpression of KIF3A and par-3 in the oocytes of the present study may indicate that they are associated with each other, and thus KIF3A may play an important role in polar body extrusion through correct positioning of the par-3.

Cytoplasmic dynein, the main microtubule minus-end-directed motor proteins, is involved in spindle pole organization, nuclear migration during mitosis, the positioning and functioning of the endoplasmic reticulum, Golgi apparatus, mRNA transport [80, 86] and bidirectional motion of lipid droplets [87]. In contrast to kinesin and myosin heads, dynein has multiple ATP binding sites in each head (for review see [80], therefore its mechanics is strongly altered by the ATP concentrations [88]. Homozygous *C. elegans* are sterile and

many postembryonic cell lineages undergo stochastic failed division. In the same experiment, using RNAi specifically to inhibit function of the dynein light intermediate chain 1 (DLIC-1) gene product, the majority of embryos from wild-type injections underwent failed pronuclear migration [89]. In the present study, cytoplasmic dynein intermediate chain (DIC) mRNA was decreasing during final maturation of the oocytes scored as normal. These results are in agreement with previous finding using cDNA array hybridization [90], that demonstrated a two-fold decrease in the mRNA of cytoplasmic dynein (light polypeptide) during in vitro maturation of bovine oocytes. Moreover, near the germinal vesicle breakdown during *Xenopus* oocyte maturation (approx. 6 h post LH surge in bovine), phosphorylation has been shown to increase dramatically, indicating that previously synthesized mRNA had been present in large quantity [91].
A role for decreasing oocyte DIC mRNA in mitochondrial translocation is also possible. During maturation of mice oocytes, mitochondria translocate to the perinuclear region during the resumption of meiosis and formation of the first metaphase plate, cytoplasmic dynein has been shown to play a role in mitochondrial translocation, disruption of dynein function in Hela cells, which leads to the retreat of mitochondria from the cell periphery towards the nucleus, and the formation of long, interconnected mitochondria [92]. In vitro matured oocytes and vivo matured oocytes classified as deviant collected pre and 6 h after the LH surge have revealed significantly lower mRNA levels compared to the normal oocytes. In the present study, estradiol levels within the preovulatory follicles pre and 6 h after LH were used as the main criteria to select presumptive competent from deviant oocytes, in addition to follicle size. Interestingly, estrogen has been shown to induce the transcription and expression of DLC-1 in breast cancer cells in a dose dependent manner. Further, DLC-1 interacts directly with estrogen receptors (ER) and such interaction is required for the transactivation-promoting activity of DLC-1, suggesting an important role for dynein in the action of ER. Conversely, DLC-1 downregulation compromised the ER-transactivation activity and also its nuclear accumulation [93]. Together, these data may explain the lower abundance of cytoplasmic dynein in the in vitro and in vivo deviant oocytes. Moreover, because of the complex structure of dynein, comparing to other motors, it' is possible that its function requires extensive regulation.

Myosin is a motor protein, which has actin-binding sites and ATP-binding sites and is primarily regulated by phosphorylation of the MRLC in mammalian cells [94]. The protein kinase responsible for phosphorylation of MRLC is Ca2+/calmodulin-dependent myosin light chain kinase (MLCK) [95]. Inhibition of MLCK and myosin II activity in mice oocytes revealed important roles in the position of the meiotic spindles, second polar body formation and cortical granule exocytosis [53]. Moreover, at the blastocyst stage, inhibition

of MLCK suggests a regulatory role in compaction, formation of the blastocyst and maintenance of the differentiated state during preimplantation development of mouse [96]. In the present investigation, the higher expression of this mRNA during bovine oocyte maturation and the blastocyst stages suggests important functions during both stages. Moreover, oocytes scored as deviant from the stimulated cows at the prematuration stage, had relatively low amounts of MRLC compared to those scored as normal and oocytes collected from the slaughterhouse ovaries and matured in vitro. Therefore, it may suggest that ovarian factors did not appear to be necessary for maintaining MRLC mRNA levels or, effect of superovulation treatment may affect the stability of the mRNA. However, as mammalian and non mammalian MRLC motor activity is regulated by MLCK phosphorylation, it is reasonable to assume that MRLC function is not regulated at the transcription level.

Formins are a family of conserved proteins and play an important role in actin nucleation processes, the rate-limiting step for actin filaments assembly. All formins of animals, plants and fungi contain a unique, highly conserved formin homology domain, FH2, which interacts with actin, and a domain rich in Pro-FH1 located N terminally to the FH2 domain, which binds to the actin-associated protein profilin, (for review see, [55, 97]). Formins also participate in MT processes, although their role is less clear. During spindle alignment in budding yeast, actin filaments nucleated by the formin Bni1p (Bud neck involved) are thought to provide tracks for the transport of MTs bearing Bim1p and Kar9p at their plus ends to the cortex by a myosin motor, Myo2p [98]. The *S. cerevisiae* Bni1 mutant, exhibit defects in cytoplasmic MT alignment and the positioning of mitotic spindle [99]. Recent analysis of Bni1 in *C. albicans*, by Li et al.[100] confirmed the role of Bni1 in the regulation of cell polarity. Their report shows that Bni1 is required for correct alignment and positioning of the mitotic spindle and that Bni1 defects correlate with the mislocalization of the *C. albicans* Kar9 homolog, the protein that can link cortical actin to astral microtubules. In the present study, we quantified FMNL2 which consist of FDD, FH1 and FH2 domains [101]. Moreover, because formins, myosins and *S. cerevisiae* Kar9p, which has homology with adenomatous polyposis coli (APC) (detected in bovine oocytes, our unpublished results), are all identified in the oocyte in the present study, it is plausible that bovine oocytes have a conserved pathway for delivering MT ends to the cortex, a process needed for correct orientation and positioning of the spindle. It will be interesting to elucidate the mechanisms that ensure proper spindle position and chromosome segregation. In addition, the expression of formin during oocyte maturation and the lack of the expression at the blastocyst stage suggests a distinct regulatory role during meiosis.

One of the most striking findings of the present study was the lower expression of formin both in the oocytes scored as deviant from stimulated cows and those matured in vitro compared to the normal oocytes. Females *Fmn2*-/- mice oocytes have failed to progress through MI and to correctly position the MI spindle at the cortex of the oocyte. Fertilization of the ovulated oocytes from the formin-2 lacking mice resulted in 74% polyploidy embryos, with similar spontaneous abortions [56]. In contrast to the somatic cells, the oocytes have much larger nuclei and, chromosomes at MI have to travel longer distance to the spindle, therefore capture of the chromosomes by MT is likely to be inefficient [29]. In starfish oocytes, it has been shown that MT alone are not able to capture chromosomes more than 40 μm away from the centrosomes, and therefore, polymerized actin network is necessary for delivering chromosomes within the capture distance of microtubule asters, given that actin-depolymerizing or stabilizing drugs cause chromosome loss and aneuploid eggs [29]. Together, the lower level of formin in the present study in deviant oocytes from stimulated cows and in vitro matured oocytes may explain partly the higher incidence of aneuploidy using both systems. However, future analysis of mammalian oocyte actin and formin function should provide a better understanding whether a similar mechanism exists in mammalian oocytes.

Par-3 gene is a major component of an important complex regulating cell polarity. Orientation and positioning of the mitotic spindle relative to the anterior-posterior axis is a well known function of par3-par6-atypical protein kinase C (aPKC) complex in *C. elegans*. These changes are believed to originate from asymmetric forces applied by par proteins through the microtubules [102, 103]. Mutations in the par3 gene affect the asymmetric distribution of the protein involved in cell fate determination and the orientation of mitotic spindles in successive cell cycles in *C. elegans* [104]. In mammalian oocytes, par-3 protein has been shown to localize to the anterior half of the mouse oocyte and, at MI and MII, par-3 is associated with the spindle and enriched in the central subdomain of the actin cap overlying the meiotic spindles [58]. The findings in the present study of decreased par-3 mRNA levels especially in oocytes classified as deviant from stimulated cows may indicate a disturbed function of polar body emission, because most of these oocytes defined by our selection criteria may not be able to resume meiosis [78].
Results from mammalian epithelial cells have shown that par-3, par-6 and aPKC complex are asymmetrically localized to tight junctions (TJ) [105], and this complex in addition to Cdc42 may provide an essential cue for the formation of the TJ complex [106]. During embryonic development, par-3, par-6 and aPKC have been shown to be localized only at the blastocyst stage in TJ. The same study found that par-6 and aPKC are targeted at TJ as soon as they become established while par-3 is targeted at them only when the blastocoels

appears, suggesting that par-3 is involved in the maintenance of tight junction (TJ) [107]. The significantly lower mRNA levels of par-3 in the in vitro produced blastocysts were the major finding that differed from the in vivo derived blastocysts in the current investigation. Cell adhesion is critically important for the cellular development as well as for the integrity of tissue. It is known that establishment of cell contacts during compaction in mouse embryo is mediated by E-cadherin, one component of TJ [108], that has been shown to perturbed in fragmented mouse embryos [109]. Accordingly, lower Par-3 mRNA levels in the in vitro produced blastocysts may contribute to the loss of TJ integrity, higher embryo fragmentation, defects in cell-cell adhesion and lose of the normal cellular polarity and probably also in the initial attachment of the embryo. Additionally, given the conserved function of the genes involved in polarity in eukaryotic and known conservation between polarization of the mammalian embryos and other cells, it seems likely that this system will be used in mammalian oocytes and early embryonic development [110].

The Aurora-A protein kinase, encoded by the STK15 oncogene is of major interest since it is located at the centrosome from S-phase to mitosis [111], involved in centrosome separation [112] and maturation as well as in bipolar spindle assembly and stability [113]. Increased levels of Aurora-A mRNA have been shown to correlate with chromosome instability in tumors [114]. In the mice oocytes, a higher and stable quantity of Aurora-A protein has been shown throughout maturation stages, which was localized to the spindle poles of meiotic spindle from MI to MII stage [62]. This indicates an important role for Aurora-A in the regulation of spindle formation and accurate chromosome segregation and may explain the lower levels of Aurora-A mRNA levels in MII oocytes matured in vitro. Moreover, in breast tumor cells, it has been shown that poly(A) tails of cyclin B1 and cyclin dependent kinase 1 (Cdk1) mRNA were synergistically elongated by Aurora-A and cytoplasmic polyadenylation element binding protein (CPEB). Therefore, the higher level of Aurora-A mRNA during maturation might strongly indicate an important role in meiotic resumption pathway, similar to the egg maturation process in frogs [115].

In conclusion, we studied a set of important genes expected to be involved in developmental competence of bovine oocytes and early embryonic development. Its apparent expression in the oocyte indicates that these genes have an important developmental role. One such role may be to enhance nuclear maturation by supporting spindle formation and accurate chromosomal segregation, the other, by supporting proper cytoplasmic maturation through correct organelles translocation. The detailed physiological significance of mRNA expression of most of these genes is largely unknown in the oocyte.

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References

1. Fair T, Hyttel P, Greve T. Bovine oocyte diameter in relation to maturational competence and transcriptional activity. Mol Reprod Dev 1995; 42: 437-442.
2. Hyttel PF, T. Callesen, H. Greve, T. Oocyte growth, capacitation and final maturation in cattle. Theriogenology 1997; 47: 23-32.
3. Merton JS, de Roos AP, Mullaart E, de Ruigh L, Kaal L, Vos PL, Dieleman SJ. Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. Theriogenology 2003; 59: 651-674.
4. van de Leemput EE, Vos PL, Zeinstra EC, Bevers MM, van der Weijden GC, Dieleman SJ. Improved in vitro embryo development using in vivo matured oocytes from heifers superovulated with a controlled preovulatory LH surge. Theriogenology 1999; 52: 335-349.
5. Rizos D, Ward F, Duffy P, Boland MP, Lonergan P. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. Mol Reprod Dev 2002; 61: 234-248.
6. Dieleman SJ, Bevers MM. Folliculogenesis and oocyte maturation in superovulated cattle. Mol Reprod Dev 1993; 36: 271-273.
7. Fortune JE, Hinshelwood MM, Roycroft J, Vincent SE. Superovulation in cattle: effects of purity of FSH preparation on follicular characteristics in vivo. Bull Assoc Anat (Nancy) 1991; 75: 55-58.
8. Van Blerkom J, Davis P. Differential effects of repeated ovarian stimulation on cytoplasmic and spindle organization in metaphase II mouse oocytes matured in vivo and in vitro. Hum Reprod 2001; 16: 757-764.
9. Van Blerkom J. The origin and detection of chromosomal abnormalities in meiotically mature human oocytes obtained from stimulated follicles and after failed fertilization in vitro. Prog Clin Biol Res 1989; 296: 299-310.
10. Van Blerkom J. Occurrence and developmental consequences of aberrant cellular organization in meiotically mature human oocytes after exogenous ovarian hyperstimulation. J Electron Microsc Tech 1990; 16: 324-346.
11. McKiernan SH, Bavister BD. Gonadotrophin stimulation of donor females decreases post-implantation viability of cultured one-cell hamster embryos. Hum Reprod 1998; 13: 724-729.
12. Elmazar MM, Vogel R, Spielmann H. Maternal factors influencing development of embryos from mice superovulated with gonadotropins. Reprod Toxicol 1989; 3: 135-138.

13. Spielmann H, Vogel R. Genotoxic and embryotoxic effects of gonadotropin hyperstimulated ovulation on murine oocytes, preimplantation embryos and term fetuses. Ann Ist Super Sanita 1993; 29: 35-39.
14. Vogel R, Spielmann H. Genotoxic and embryotoxic effects of gonadotropin-hyperstimulated ovulation of murine oocytes, preimplantation embryos, and term fetuses. Reprod Toxicol 1992; 6: 329-333.
15. Ertzeid G, Storeng R. The impact of ovarian stimulation on implantation and fetal development in mice. Hum Reprod 2001; 16: 221-225.
16. Sanfins A, Plancha CE, Overstrom EW, Albertini DF. Meiotic spindle morphogenesis in in vivo and in vitro matured mouse oocytes: insights into the relationship between nuclear and cytoplasmic quality. Hum Reprod 2004; 19: 2889-2899.
17. Sanfins A, Lee GY, Plancha CE, Overstrom EW, Albertini DF. Distinctions in meiotic spindle structure and assembly during in vitro and in vivo maturation of mouse oocytes. Biol Reprod 2003; 69: 2059-2067.
18. Albertini DF, Sanfins A, Combelles CM. Origins and manifestations of oocyte maturation competencies. Reprod Biomed Online 2003; 6: 410-415.
19. Combelles CM, Racowsky C. Assessment and optimization of oocyte quality during assisted reproductive technology treatment. Semin Reprod Med 2005; 23: 277-284.
20. Almeida PA, Bolton VN. The relationship between chromosomal abnormality in the human preimplantation embryo and development in vitro. Reprod Fertil Dev 1996; 8: 235-241.
21. Abbott AL, Fissore RA, Ducibella T. Incompetence of preovulatory mouse oocytes to undergo cortical granule exocytosis following induced calcium oscillations. Dev Biol 1999; 207: 38-48.
22. Greve T, Callesen H, Hyttel P. Endocrine profiles and egg quality in the superovulated cow. Nord Vet Med 1983; 35: 408-421.
23. Takagi M, Kim IH, Izadyar F, Hyttel P, Bevers MM, Dieleman SJ, Hendriksen PJ, Vos PL. Impaired final follicular maturation in heifers after superovulation with recombinant human FSH. Reproduction 2001; 121: 941-951.
24. Hirokawa N, Takemura R. Kinesin superfamily proteins and their various functions and dynamics. Exp Cell Res 2004; 301: 50-59.
25. Hirokawa N, Noda Y, Okada Y. Kinesin and dynein superfamily proteins in organelle transport and cell division. Curr Opin Cell Biol 1998; 10: 60-73.
26. Karki S, Holzbaur EL. Cytoplasmic dynein and dynactin in cell division and intracellular transport. Curr Opin Cell Biol 1999; 11: 45-53.
27. Shav-Tal Y, Singer RH. RNA localization. J Cell Sci 2005; 118: 4077-4081.
28. Tekotte H, Davis I. Intracellular mRNA localization: motors move messages. Trends Genet 2002; 18: 636-642.
29. Lenart P, Bacher CP, Daigle N, Hand AR, Eils R, Terasaki M, Ellenberg J. A contractile nuclear actin network drives chromosome congression in oocytes. Nature 2005; 436: 812-818.
30. Van Blerkom J. Microtubule mediation of cytoplasmic and nuclear maturation during the early stages of resumed meiosis in cultured mouse oocytes. Proc Natl Acad Sci U S A 1991; 88: 5031-5035.
31. Plancha CE, Albertini DF. Hormonal regulation of meiotic maturation in the hamster oocyte involves a cytoskeleton-mediated process. Biol Reprod 1994; 51: 852-864.
32. Albertini DF. Regulation of meiotic maturation in the mammalian oocyte: interplay between exogenous cues and the microtubule cytoskeleton. Bioessays 1992; 14: 97-103.
33. Albertini DF. Cytoplasmic microtubular dynamics and chromatin organization during mammalian oogenesis and oocyte maturation. Mutat Res 1992; 296: 57-68.
34. Haraguchi K, Hayashi T, Jimbo T, Yamamoto T, Akiyama T. Role of the kinesin-2 family protein, KIF3, during mitosis. J Biol Chem 2006; 281: 4094-4099.

35. Miki H, Okada Y, Hirokawa N. Analysis of the kinesin superfamily: insights into structure and function. Trends Cell Biol 2005; 15: 467-476.
36. Takeda S, Yamazaki H, Seog DH, Kanai Y, Terada S, Hirokawa N. Kinesin superfamily protein 3 (KIF3) motor transports fodrin-associating vesicles important for neurite building. J Cell Biol 2000; 148: 1255-1265.
37. Marszalek JR, Ruiz-Lozano P, Roberts E, Chien KR, Goldstein LS. Situs inversus and embryonic ciliary morphogenesis defects in mouse mutants lacking the KIF3A subunit of kinesin-II. Proc Natl Acad Sci U S A 1999; 96: 5043-5048.
38. Pfister KK, Fisher EM, Gibbons IR, Hays TS, Holzbaur EL, McIntosh JR, Porter ME, Schroer TA, Vaughan KT, Witman GB, King SM, Vallee RB. Cytoplasmic dynein nomenclature. J Cell Biol 2005; 171: 411-413.
39. Reinsch S, Gonczy P. Mechanisms of nuclear positioning. J Cell Sci 1998; 111 (Pt 16): 2283-2295.
40. Vallee RB, Sheetz MP. Targeting of motor proteins. Science 1996; 271: 1539-1544.
41. Gaetz J, Kapoor TM. Dynein/dynactin regulate metaphase spindle length by targeting depolymerizing activities to spindle poles. J Cell Biol 2004; 166: 465-471.
42. King SM, Wilkerson CG, Witman GB. The Mr 78,000 intermediate chain of Chlamydomonas outer arm dynein interacts with alpha-tubulin in situ. J Biol Chem 1991; 266: 8401-8407.
43. Paschal BM, Mikami A, Pfister KK, Vallee RB. Homology of the 74-kD cytoplasmic dynein subunit with a flagellar dynein polypeptide suggests an intracellular targeting function. J Cell Biol 1992; 118: 1133-1143.
44. Wheatley S, Kulkarni S, Karess R. Drosophila nonmuscle myosin II is required for rapid cytoplasmic transport during oogenesis and for axial nuclear migration in early embryos. Development 1995; 121: 1937-1946.
45. Becker KA, Hart NH. Reorganization of filamentous actin and myosin-II in zebrafish eggs correlates temporally and spatially with cortical granule exocytosis. J Cell Sci 1999; 112 (Pt 1): 97-110.
46. Chaudoir BM, Kowalczyk PA, Chisholm RL. Regulatory light chain mutations affect myosin motor function and kinetics. J Cell Sci 1999; 112 (Pt 10): 1611-1620.
47. Ostrow BD, Chen P, Chisholm RL. Expression of a myosin regulatory light chain phosphorylation site mutant complements the cytokinesis and developmental defects of Dictyostelium RMLC null cells. J Cell Biol 1994; 127: 1945-1955.
48. Satterwhite LL, Lohka MJ, Wilson KL, Scherson TY, Cisek LJ, Corden JL, Pollard TD. Phosphorylation of myosin-II regulatory light chain by cyclin-p34cdc2: a mechanism for the timing of cytokinesis. J Cell Biol 1992; 118: 595-605.
49. Korn ED, Hammer JA, 3rd. Myosins of nonmuscle cells. Annu Rev Biophys Biophys Chem 1988; 17: 23-45.
50. Bresnick AR. Molecular mechanisms of nonmuscle myosin-II regulation. Curr Opin Cell Biol 1999; 11: 26-33.
51. Jordan P, Karess R. Myosin light chain-activating phosphorylation sites are required for oogenesis in Drosophila. J Cell Biol 1997; 139: 1805-1819.
52. Edwards KA, Kiehart DP. Drosophila nonmuscle myosin II has multiple essential roles in imaginal disc and egg chamber morphogenesis. Development 1996; 122: 1499-1511.
53. Matson S, Markoulaki S, Ducibella T. Antagonists of myosin light chain kinase and of myosin II inhibit specific events of egg activation in fertilized mouse eggs. Biol Reprod 2006; 74: 169-176.
54. Evangelista M, Pruyne D, Amberg DC, Boone C, Bretscher A. Formins direct Arp2/3-independent actin filament assembly to polarize cell growth in yeast. Nat Cell Biol 2002; 4: 260-269.

55. Evangelista M, Zigmond S, Boone C. Formins: signaling effectors for assembly and polarization of actin filaments. J Cell Sci 2003; 116: 2603-2611.
56. Leader B, Lim H, Carabatsos MJ, Harrington A, Ecsedy J, Pellman D, Maas R, Leder P. Formin-2, polyploidy, hypofertility and positioning of the meiotic spindle in mouse oocytes. Nat Cell Biol 2002; 4: 921-928.
57. Etemad-Moghadam B, Guo S, Kemphues KJ. Asymmetrically distributed PAR-3 protein contributes to cell polarity and spindle alignment in early C. elegans embryos. Cell 1995; 83: 743-752.
58. Duncan FA, Moss SB, Schultz RM, Williams CJ. PAR-3 defines a central subdomain of the cortical actin cap in mouse eggs. Dev Biol 2005; 280: 38-47.
59. Carmena M, Earnshaw WC. The cellular geography of aurora kinases. Nat Rev Mol Cell Biol 2003; 4: 842–854.
60. Kufer TA, Nigg EA, Sillje HH. Regulation of Aurora-A kinase on the mitotic spindle. Chromosoma 2003; 112: 159–163.
61. Adams RR, Carmena M, Earnshaw WC. Chromosomal passengers and the (aurora) ABCs of mitosis. Trends Cell Biol 2001; 11: 49-54.
62. Yao LJ, Zhong ZS, Zhang LS, Chen DY, Schatten H, Sun QY. Aurora-A is a critical regulator of microtubule assembly and nuclear activity in mouse oocytes, fertilized eggs, and early embryos. Biol Reprod 2004; 70: 1392-1399.
63. Bevers MM, Dieleman SJ, van den Hurk R, Izadyar F. Regulation and modulation of oocyte maturation in the bovine. Theriogenology 1997; 47: 13-22.
64. Watson AJ, De Sousa P, Caveney A, Barcroft LC, Natale D, Urquhart J, Westhusin ME. Impact of bovine oocyte maturation media on oocyte transcript levels, blastocyst development, cell number, and apoptosis. Biol Reprod 2000; 62: 355-364.
65. Knijn HM, Wrenzycki C, Hendriksen PJ, Vos PL, Herrmann D, van der Weijden GC, Niemann H, Dieleman SJ. Effects of oocyte maturation regimen on the relative abundance of gene transcripts in bovine blastocysts derived in vitro or in vivo. Reproduction 2002; 124: 365-375.
66. Corcoran D, Fair T, Park S, Rizos D, Patel OV, Smith GW, Coussens PM, Ireland JJ, Boland MP, Evans AC, Lonergan P. Suppressed expression of genes involved in transcription and translation in in vitro compared with in vivo cultured bovine embryos. Reproduction 2006; 131: 651-660.
67. Dieleman SJ, Bevers MM. Effects of monoclonal antibody against PMSG administered shortly after the preovulatory LH surge on time and number of ovulations in PMSG/PG-treated cows. J Reprod Fertil 1987; 81: 533-542.
68. Dieleman SJ, Bevers MM, Poortman J, van Tol HT. Steroid and pituitary hormone concentrations in the fluid of preovulatory bovine follicles relative to the peak of LH in the peripheral blood. J Reprod Fertil 1983; 69: 641-649.
69. Dieleman SJ, Kruip TA, Fontijne P, de Jong WH, van der Weyden GC. Changes in oestradiol, progesterone and testosterone concentrations in follicular fluid and in the micromorphology of preovulatory bovine follicles relative to the peak of luteinizing hormone. J Endocrinol 1983; 97: 31-42.
70. Parrish JJ, Susko-Parrish J, Winer MA, First NL. Capacitation of bovine sperm by heparin. Biol Reprod 1988; 38: 1171-1180.
71. Izadyar F, Zeinstra E, Colenbrander B, Vanderstichele HM, Bevers MM. In vitro maturation of bovine oocytes in the presence of bovine activin A does not affect the number of embryos. Anim Reprod Sci 1996; 45: 37-45.
72. van Wagtendonk-de Leeuw AM, Mullaart E, de Roos AP, Merton JS, den Daas JH, Kemp B, de Ruigh L. Effects of different reproduction techniques: AI MOET or IVP, on health and welfare of bovine offspring. Theriogenology 2000; 53: 575-597.

73. Robertson I, Nelson R. Certification of the embryos. Manual of the international embryo transfer society. Savoy, IL; 1998.
74. Bettegowda A, Patel OV, Ireland JJ, Smith GW. Quantitative analysis of messenger RNA abundance for ribosomal protein L-15, cyclophilin-A, phosphoglycerokinase, beta-glucuronidase, glyceraldehyde 3-phosphate dehydrogenase, beta-actin, and histone H2A during bovine oocyte maturation and early embryogenesis in vitro. Mol Reprod Dev 2005.
75. Goossens K, Van Poucke M, Van Soom A, Vandesompele J, Van Zeveren A, Peelman LJ. Selection of reference genes for quantitative real-time PCR in bovine preimplantation embryos. BMC Dev Biol 2005; 5: 27.
76. Petronczki M, Siomos MF, Nasmyth K. Un menage a quatre: the molecular biology of chromosome segregation in meiosis. Cell 2003; 112: 423-440.
77. Hyttel P, Callesen H, Greve T. Ultrastructural features of preovulatory oocyte maturation in superovulated cattle. J Reprod Fertil 1986; 76: 645-656.
78. de Loos FA, Bevers MM, Dieleman SJ, Kruip TA. Follicular and oocyte maturation in cows treated for superovulation. Theriogenology 1991; 35: 537-546.
79. Mattioli M, Barboni B, Turriani M, Galeati G, Zannoni A, Castellani G, Berardinelli P, Scapolo PA. Follicle activation involves vascular endothelial growth factor production and increased blood vessel extension. Biol Reprod 2001; 65: 1014-1019.
80. Mallik R, Gross SP. Molecular motors: Strategies to get Along. Curr Biol 2004; 14: 971-982.
81. Sheetz MP. Motor and cargo interactions. Eur J Biochem 1999; 262: 19-25.
82. Endow SA. Microtubule motors in spindle and chromosome motility. Eur J Biochem 1999; 262: 12-18.
83. Yamazaki H, Nakata T, Okada Y, Hirokawa N. Cloning and characterization of KAP3: a novel kinesin superfamily-associated protein of KIF3A/3B. Proc Natl Acad Sci U S A 1996; 93: 8443-8448.
84. Williams BC, Dernburg AF, Puro J, Nokkala S, Goldberg ML. The *Drosophila* kinesin-like protein KLP3A is required for proper behavior of male and female pronuclei at fertilization. Development 1997; 124: 2365-2376.
85. Nishimura T, Kato K, Yamaguchi T, Fukata1 Y, Ohno S, Kaibuchi K. Role of the PAR-3–KIF3 complex in the establishment of neuronal polarity. 2004; 6: 328-334.
86. Hirokawa N. Kinesin and dynein superfamily proteins and the mechanism of organelle transport. Science 1998; 279: 519-526.
87. Valetti C, Wetzel DM, Schrader M, Hasbani MJ, Gill SR, Kreis TE, Schroer TA. Role of dynactin in endocytic traffic:effects of dynamitin overexpression and colocalization with CLIP-170. Mol Biol Cell 1999; 10: 4107-4120.
88. Mallik R, Carter BC, Lex SA, King SJ, Gross SP. Cytoplasmic dynein functions as a gear in response to load. Nature 2004; 427: 649-652.
89. Yoder JH, Han M. Cytoplasmic Dynein Light Intermediate Chain Is Required for Discrete Aspects of Mitosis in Caenorhabditis elegans. Mol Biol Cell 2001; 12: 2921–2933.
90. Dalbies-Tran R, Mermillod P. Use of heterologous complementary DNA array screening to analyze bovine oocyte transcriptome and its evolution during in vitro maturation. Biol Reprod 2003; 68: 252-261.
91. Huang CF, Chang CB, Huang C, Ferrell JE. M phase phosphorylation of cytoplasmic dynein intermediate chain and $p150^{Glued}$. J Biol Chem 1999; 274: 14262-14269.
92. Varadi A, Johnson-Cadwell LI, Cirulli V, Yoon Y, Allan VJ. Cytoplasmic dynein regulates the subcellular distribution of mitochondria by controlling the recruitment of the fission factor dynamin-related protein-1. J Cell Sci 2004; 117: 4389-4400.

93. Rayala SK, den Hollander P, Balasenthil S, Yang Z, Broaddus RR, Kumar R. Functional regulation of oestrogen receptor pathway by the dynein light chain 1. Embo J 2005; 6: 538-545.
94. Komatsu S, Yano T, Shibata M, Tuft RA, Ikebe M. Effects of the regulatory light chain phosphorylation of myosin II on mitosis and cytokinesis of mammalian cells. J Biol Chem 2000; 275: 34512-34520.
95. Kamm KE, Stull JT. Dedicated myosin light chain kinases with diverse cellular functions. J Biol Chem 2001; 276: 4527-4530.
96. Kabir N, Yamamura H, Takagishi Y, Inouye M, Oda SI, Hidaka H. Regulation of preimplantation development of mouse embryos: Effects of inhibition of myosin light-chain kinase, a Ca^{2+}/calmodulin-dependent enzyme. J Exp Zool 1996; 274: 101-110.
97. Wasserman S. FH proteins as cytoskeletal organizers. Trends Cell Biol 1998; 8: 111–115.
98. Beach DL, Salmon ED, Bloom K. Localization and anchoring of mRNA in budding yeast. Curr Biol 1999; 9: 569–578.
99. Lee L, Klee SK, Evangelista M, Boone C, Pellman D. Control of mitotic spindle position by the Saccharomyces cerevisiae formin Bni1p. J Cell Biol 1999; 144: 947-961.
100. Li CR, Wang YM, De Zheng X, Liang HY, Tang JC, Wang Y. The formin family protein CaBni1p has a role in cell polarity control during both yeast and hyphal growth in *Candida albicans*. J Cell Sci 2005; 118: 2637–2648.
101. Katoh M, Katoh M. Identification and characterization of human FMNL1, FMNL2 and FMNL3 genes in silico. Int J Oncol 2003; 22: 1161-1168.
102. Severson AF, Bowerman B. Myosin and the PAR proteins polarize microfilament-dependent forces that shape and position mitotic spindles in *Caenorhabditis elegans*. J Cell Biol 2003; 161: 21-26.
103. Grill SW, Gonczy P, Stelzer EH, Hyman AA. Polarity control forces governing asymmetric spindle positioning in the *Caenorhabditis elegans* embryo. Nature 2001; 409: 630-633.
104. Guo S, Kemphues KJ. Molecular genetics of asymmetric cleavage in the early Caenorhabditis elegans embryo. Curr Opin Genet Dev 1996; 6: 408-415.
105. Dodane V, Kachar B. identification of isoforms of G proteins and PKC that colocalize with tight junctions. J Membr Biol 1996; 149: 199-209.
106. Joberty G, Petersen C, Gao L, Macara IG. The cell-polarity protein Par6 links Par3 and atypical protein kinase C to Cdc42. Nat Cell Biol 2000; 2: 531-539.
107. Virot S, Le T, Ohno S, Pawson T, Maro B, Louvet-Vallee S. Asymmetric distribution of PAR proteins in the mouse embryo begins at the 8-cell stage during compaction. Dev Biol 2005; 282: 307-319.
108. Rowlands TM, Symonds JM, Farookhi R, Blaschuk OW. Cadherins: Crucial regulators of structure and function in reproductive tissues. Rev Reprod 2000; 5: 53-61.
109. Alikani M. Epithelial cadherin distribution in abnormal human pre-implantation embryos. Hum Reprod 2005; 25.
110. Albertini DF, Barrett SL. The developmental origins of mammalian oocyte polarity. Semin Cell Dev Biol 2004; 15: 599-606.
111. Dutertre S, Descamps S, Prigent C. On the role of aurora-A in centrosome function. Oncogene 2002; 21: 6175-6183.
112. Giet R, McLean D, Descamps S, Lee MJ, Raff JW, Prigent C, Glover DM. Drosophila Aurora A kinase is required to localize D-TACC to centrosomes and to regulate astral microtubules. J Cell Biol 2002; 156: 437-451.
113. Giet R, Prigent C. The Xenopus laevis aurora/Ip11p-related kinase pEg2 participates in the stability of the bipolar mitotic spindle. Exp Cell Res 2000; 258: 145-151.

114. Miyoshi Y, Iwao K, Egawa C, Noguchi S. Association of centrosomal kinase STK15/BTAK mRNA expression with chromosomal instability in human breast cancers. Int J Cancer 2001; 92: 370–373.
115. Andresson T, Ruderman JV. The kinase Eg2 is a component of the Xenopus oocyte progesterone-activated signaling pathway. Embo J 1998; 17: 5627–5637.