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ORIGINAL RESEARCH

Reverse transcription priming methods affect normalisation choices for gene expression levels in oocytes and early embryos

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ABSTRACT: Mammalian oocytes and embryos rely exclusively on maternal mRNAs to accomplish early developmental processes. Since oocytes and early embryos are transcriptionally silent after meiotic resumption, most of the synthesised maternal mRNA does not undergo immediate translation but is instead stored in the oocyte. Quantitative RT-PCR is commonly used to quantify mRNA levels, and correct quantification relies on reverse transcription and the choice of reference genes. Different methods for reverse transcription may affect gene expression determination in oocytes. In this study, we examined the suitability of either random or oligo(dT) primers for reverse transcription to be used for quantitative RT-PCR. We further looked for changes in poly(A) length of the maternal mRNAs during oocyte maturation. Our data indicate that depending on the method of reverse transcription, the optimal combination of reference genes for normalisation differed. Surprisingly, we observed a shortening of the poly(A) tail lengths of maternal mRNA as oocytes progressed from germinal vesicle to metaphase II. Overall, our findings suggest dynamic maternal regulation of mRNA structure and gene expression during oocyte maturation and early embryo development.

Key words: maternal mRNA /bovine embryo /qRT-PCR /reverse transcription /gene expression /poly(A) tail

Introduction

Early embryonic development is primarily controlled by maternal mRNAs, which are produced by the oocyte during oogenesis (Vastenhouw *et al.*, 2019). Unlike in somatic cells, most mRNA synthesised in an oocyte is not immediately translated but is stored for future events (Hamatani *et al.*, 2004). After breakdown of the germinal vesicle (GV), the chromatin in an oocyte becomes condensed, resulting in transcriptional silencing of meiotic oocytes (Tan *et al.*, 2009; Dumdie *et al.*, 2018). This period of reduced transcription lasts until the maternal to zygotic transition (MZT), when the zygotic genome is activated and maternal mRNA is largely degraded. Before the MZT, oocytes and embryos therefore rely on maternal mRNA to accomplish dynamic events accompanying final oocyte maturation, fertilisation and early embryo development (Winata and Korzh, 2018). In short, precise regulation of maternal mRNA dynamics plays an essential role during maturation of the oocyte and early embryo development. Precise regulation of maternal mRNA expression ensures that dynamic events required for successful oocyte maturation and early embryo development can be accomplished; quantitative reverse transcription PCR (qRT-PCR) is a useful method for monitoring the regulation of (maternal) mRNA abundance or expression. It is routinely used in life science research because of its sensitivity and cost-effectiveness and has proven to be a useful method to quantify gene expression levels in sparse or limited RNA samples such as that from oocytes and embryos.

One of the key steps in qRT-PCR is the synthesis of complementary DNA (cDNA) by reverse transcription, which should result in a cDNA pool that quantitatively reflects the original mRNA copy number (Stangegaard *et al.*, 2006). There are two major priming strategies for reverse transcription used in qRT-PCR, namely random primers and oligo(dT) primers. Random primers with randomly ordered base

sequences can potentially anneal to any RNA species, at any position (from 5′ to 3′). Reverse transcription based on oligo(dT) primers can only anneal to the 3′ poly(A) tail of RNA. Maternal mRNA is, however, usually stored in a short poly(A) tail state (Mendez and Richter, 2001; Stangegaard *et al.*, 2006). It is therefore possible that, depending on the method of synthesis, cDNA may not quantitatively reflect the population of maternal mRNA available for translation.

Normalisation is an essential step when analysing gene expression to account for factors such as the total amount of mRNA recovered from different samples (Evans *et al.*, 2018). Even though selection of reference genes for normalisation in preimplantation embryos has been documented in various mammalian species (Goossens *et al.*, 2005; Kuijk *et al.*, 2007; Mamo *et al.*, 2007), the possible effect of different reverse transcription priming strategies has not been analysed in detail.

Translational control of maternal mRNA is achieved mainly by polyadenylation and deadenylation, and in particular by regulating the length of the poly(A) tail (Eichhorn *et al.*, 2016; Winata and Korzh, 2018). The regulation of maternal mRNA availability has been investigated primarily in *Drosophila*, *Xenopus* and the mouse (Sallés *et al.*, 1994; Mendez and Richter, 2001; Richter, 2007; Morgan *et al.*, 2017). It has been reported that, in immature oocytes, maternal mRNA is kept in a relatively short poly(A) tail state, stored in cytoplasmic granules (Anderson and Kedersha, 2009; Winata and Korzh, 2018). Since the oocyte is transcriptionally silent during and after its nuclear maturation, it has been hypothesised that stored maternal mRNA is released from RNA granules when translation is required (Anderson and Kedersha, 2009; Kotani *et al.*, 2013; Winata and Korzh, 2018).

Oocyte progression through meiosis is highly dependent on the activity of maturation-promoting factor (MPF), a complex of the regulatory subunit cyclin B, coded for by the *CCNB* gene and the catalytic subunit cyclin-dependent kinase 1 (CDK1). An increase in MPF activity from germinal vesicle breakdown (GVBD) to the metaphase I (MI) and metaphase II (MII) stages is achieved by temporally controlled synthesis of cyclin B from stored maternal mRNA (Ihara *et al.*, 1998; Mendez and Richter, 2001; Nakahata *et al.*, 2003; Nagahama and Yamashita, 2008). Recently, it has been reported that a high *CCNB* mRNA translation rate is associated with elongation of the *CCNB* poly(A) tail in mouse oocytes (Kotani *et al.*, 2013; Daldello *et al.*, 2019). Cyclin A (coded by *CCNA*) can also bind to CDK1 and regulate the activity of MPF during oocyte maturation (Li *et al.*, 2019). Moreover, a recent study demonstrated that cyclin A1 expression prevents segregation of chromosomes and anaphase entry (Radonova *et al.*, 2020).

We hypothesised that different priming strategies for reverse transcription may result in different fidelities of cDNA generation when using samples from oocytes or embryos. We chose cyclin genes to study, because they are highly regulated during oocyte maturation, which might lead to different gene expression patterns if different reverse transcription strategies were performed. We also focused on *CDK2* and *EIF4A3*, since they play important role during oocyte maturation and early embryo development. We therefore extracted mRNA from bovine oocytes and embryos, and synthesised cDNA using random primers and oligo(dT) primers. We used qRT-PCR to examine the gene expression patterns of cyclin genes and examined poly(A) tail length of various genes in oocytes from the GV to the MII stage (Supplementary Fig. S1). Our data uncovered differences in poly(A)tail length of mRNA during oocyte maturation and early embryo development. It is concluded that one should be critical in deciding which primer-type to use for reverse transcription when gene expression levels are examined in oocytes and pre-MZT embryos.

Materials and methods

Bovine in vitro embryo culture and sample collection

Bovine ovaries were collected from a local slaughterhouse, rinsed with water and kept in 0.9% NaCl (B. Braun, Melsungen, Germany) supplemented with penicillin/streptomycin (100 µg/ml) (Gibco, Paisley, UK) at 30°C during processing. Cumulus–oocyte complexes (COCs) were aspirated from follicles with a diameter of 2–8 mm and identified using a stereomicroscope. The COCs were matured *in vitro* as described previously (Brinkhof *et al.*, 2015) and for subsequent analysis, GV oocytes were collected immediately after COC recovery while GVBD, MI and MII stage oocytes were collected at 6, 12 and 23 h of *in vitro* maturation, respectively; in all cases, cumulus cells were removed by vortexing. *In vitro* fertilisation was done as described (Brinkhof *et al.*, 2015). In short, after 23 h maturation, COCs were transferred to fertilisation medium. Motile sperm cells were introduced into the fertilisation medium at a final concentration of 1×10^6 per ml, and this was considered as day 0. After incubation with sperm for 20–22 h, presumptive zygotes were denuded of their cumulus cells by vortexing for 3 min, and then cultured further in synthetic oviductal fluid (SOF) (Brinkhof *et al.*, 2017) in a humidified atmosphere containing 5% CO_2 and 7% O_2 at 39°C. At day 5 after fertilisation, developing embryos were transferred to fresh SOF for further culture until day 8.

RNA extraction and cDNA generation

Oocytes or embryos in pools of 50 were rinsed in PBS and stored in 200 µl RLT lysis buffer (Qiagen, Valencia, CA, USA) at −80°C until RNA extraction. Total RNA isolation was performed using the RNeasy Micro Kit (Qiagen) according to the manufacturer's instruction. Reverse transcription was carried out directly after RNA isolation, using two different priming strategies. Reverse transcription (RT) mixtures were prepared from 10 µl of the RNA sample, 4 µl of 5 × RT buffer (Invitrogen, Breda, the Netherlands), 10 mM DTT (Invitrogen), 0.5 mM dNTP (Promega, Leiden, the Netherlands), 8 units RNAsin/RNAse inhibitor (Promega) and 150 units Superscript III reverse transcriptase (Invitrogen) in a total volume of 20 µl, supplemented with 1.8 units per ml random primers (Invitrogen) or 2.5 µM oligo(dT) primers (Invitrogen) respectively. Minus-RT controls were made up of the same reagents as above, using primers but without the reverse transcriptase. The mixtures were incubated at 70°C for 5 min, followed by 1 h at 50°C and 5 min at 80°C. Samples were subsequently stored at −20°C until further analysis.

Quantitative PCR

All amplification reactions were performed on three independent cDNA samples or -RT blanks in duplicate, following the manufacturer's protocol of the CFX detection system (Bio-Rad, Hercules, CA, USA). The reaction mixture contained 1 µl cDNA, 9 µl RNAse- and DNAse-free water (Invitrogen) and 10 µl iQ SYBR Green supermix

(Biorad) with a final primer concentration of 500 nM. Initial denaturation took place for 3 min at 95°C, followed by 40 cycles of 95°C for 20 s, the primer specific annealing temperature (Supplementary Table S1) for 20 s, and elongation at 72°C for 20 s. To determine the quantitative amplification efficiency, standard curves for each primer pair were made by four-fold dilution series of cDNA from 400 oocytes or 100 blastocysts.

Poly(A) tail assay

Poly(A) tail assays were performed as described (Rassa *et al.*, 2000) with a few modifications. Total RNA was extracted from 50 GV or MII oocytes as described above. After denaturation at 70°C, the mRNA was ligated with 50 pmol of primer GB-135 (5′-P-GGTCACCTTGATCTGAAGC-NH_2-3′) (Eurogentec, Maastricht, the Netherlands) at 37°C for 1 h in a total volume of 20 µl using T4 RNA ligase (New England Biolabs, Ipswich, MA, USA). GB-135 contained a 3′ amino modification to block ligation at this end. To inactivate RNA ligase, samples were boiled at 100°C for 5 min and cooled on ice. Reverse transcription was performed as described above using 50 pmol of primer GB-136 (5′-GCTTCAGATCAAGGTGACCT TTTT-3′) (Eurogentec), and the anchored cDNA was used for amplification. First round amplification was performed using a gene-specific primer (P1) (Supplementary Table S2) and GB-136. The product of the first round amplification was used as template for the second round amplification, using a gene-specific primer starting after the 3′ site of P1 (P2) (Supplementary Table S2) and GB-136. The PCR was performed as described above with 40 cycles for first round amplification and 20 cycles for second round amplification. Samples were electrophoresed on 1.0% agarose (Eurogentec) gels and visualised with ethidium bromide (Invitrogen).

Transformation and DNA sequencing

The PCR products resulting from the second round amplification as described above were inserted into the pGEM-T Easy vector (Promega) using T4 DNA ligase (New England Biolabs) and transformed into *Escherichia coli* JM109 competent cells (Promega) by heat-shock transformation according to the manufacturer's instructions. Transformants were selected on LB/Ampicillin/IPTG/X-Gal plates according to the manufacturer's instructions. At least 12 clones from each reaction were examined by digestion with Eco52I (Thermo Fisher Scientific, Eindhoven, the Netherlands) restriction followed by sequencing in case of the correct insert size.

DNA sequencing reactions were conducted using T7 primer (5′-TAATACGACTCACTATAGGG-3′) (Eurogentec) according to the manufacturer's instructions for the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Beverly, MA, USA), and determined using a 3500XL Genetic Analyser (Applied Biosystems). DNA sequencing results were analysed using Sequencing Analysis Software v6.0 (Thermo Fisher Scientific) and aligned by cluster W method using DNASTAR Lasergene 14 (DNASTAR, Madison, WI, USA).

Statistical analysis

Stability analysis for the reference genes was performed using geNorm (Vandesompele *et al.*, 2002). Data from the PCRs were tabulated in Microsoft Excel and statistical differences were examined using GraphPad Prism 7 (https://www. graphpad.com/scientific-software/prism/). Pools of embryos from three biological replicates were analysed for gene expression. Normal distributions of data sets were determined by the Shapiro–Wilk tests. Differences between two groups were analysed by the Mann–Whitney test or, in the case of multiple groups, by one-way ANOVA followed by a *post hoc* Tukey test. In bar graphs, differences between groups are indicated with different letters above the bars, with statistical significance set as $P < 0.05$. Asterisks indicate levels of significance (*$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$ and ****$P < 0.0001$). Error bars indicate standard deviation.

Results

Stability of potential reference genes during oocyte maturation

As different priming strategies for reverse transcription in oocytes may result in different pools of cDNA, we hypothesised that suitable reference genes for normalisation are different between cDNA synthesised using random primers and cDNA generated using oligo(dT) primers. To determine whether the optimal set of stably expressed reference genes differs in differently generated cDNA samples from GV, GVBD, MI and MII oocytes, expression of six commonly used reference genes (*ACTB*, *GAPDH*, *HPRT1*, *RPL15*, *SDHA* and *YWHAZ*) was analysed using qRT-PCR and the software packages of geNorm (Vandesompele *et al.*, 2002).

Using geNorm, gene expression stability can be evaluated by the average expression stability (M value), with a low M value indicating high stability of expression. In cDNA samples synthesised using random primers, *RPL15* and *SDHA* were the most stably expressed, followed by *ACTB*, *HPRT1*, *GAPDH* and *YWHAZ* (Fig. 1A). The highest stability of gene expression in cDNA samples synthesised using oligo(dT) primers was recorded for *HRPT1* and *YWHAZ*, followed by *ACTB*, *SDHA*, *RPL15* and *GAPDH* (Fig. 1C).

To determine the optimal number of reference genes for accurate normalisation, the pairwise variation (V) was calculated between two sequential normalisation factors. In cDNA samples synthesised with either random primers and oligo(dT) primers, the inclusion of a third gene improved normalisation (high $V_{2/3}$ value), but the inclusion of a fourth gene had little effect (low $V_{3/4}$ value) (Fig. 1B and D). Overall, the optimal combination of reference genes for normalisation in cDNA samples differed between cDNA synthesised using random primers and oligo(dT) primers. With random primers, the combination of *RPL15*, *SDHA* and *ACTB* was favoured, whereas in cDNA samples synthesised using oligo(dT) primers, it was *HPRT1*, *YWHAZ* and *ACTB*.

Gene expression patterns during oocyte maturation

We next focused on the expression patterns of *CCNA1*, *CCNB1*, *CCNB2*, *CDK2* and *EIF4A3* in oocyte cDNA samples synthesised using random primers and oligo(dT) primers, since these genes play important roles during oocyte and early embryo development (Mendez and Richter, 2001; Jansova *et al.*, 2018). In cDNA samples synthesised with random primers, the expression of *CCNA1*, *CCNB1*, *CCNB2*, *CDK2* and *EIF4A3* was relatively stable throughout maturation from GV to MII

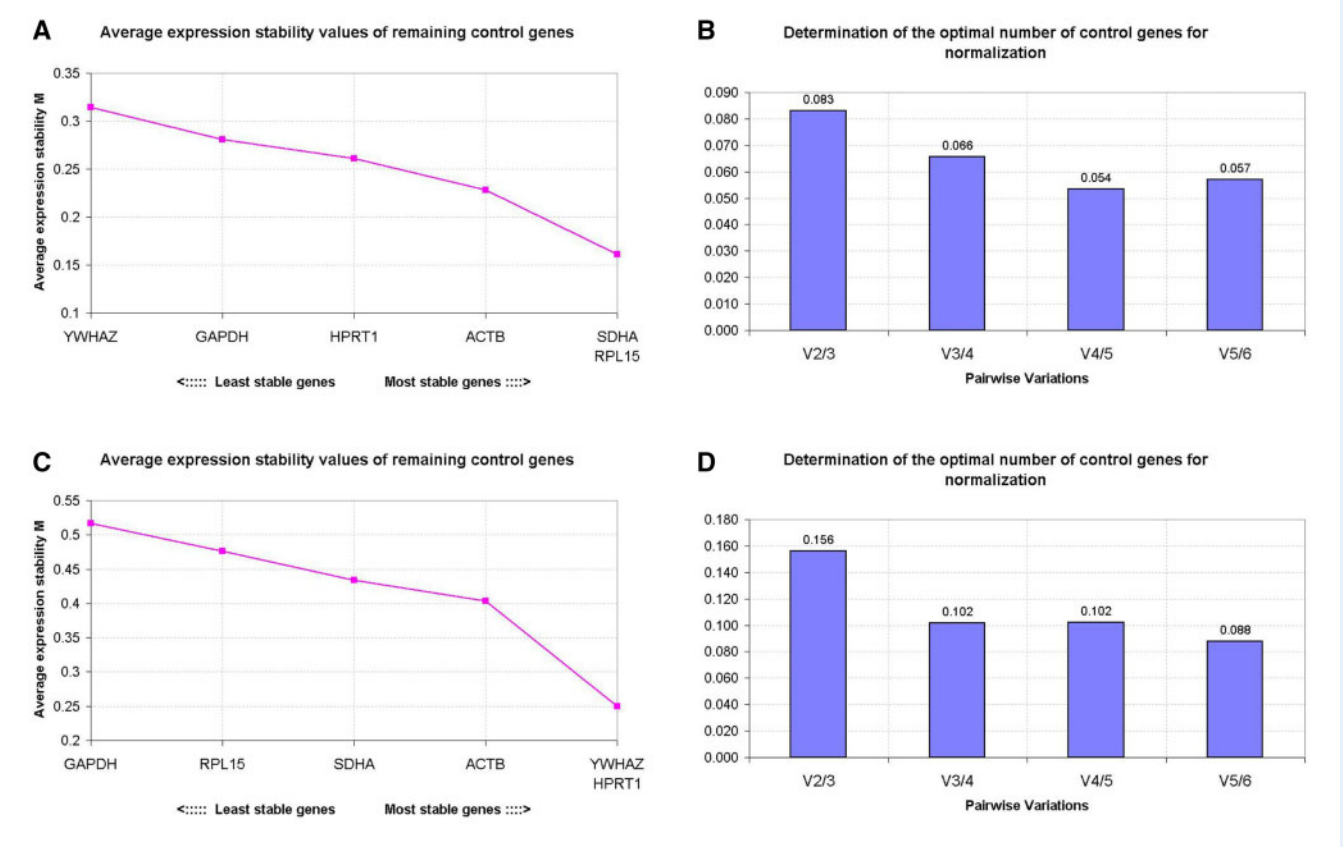

Figure 1. Stability of potential reference genes during oocyte maturation. The calculation of the average expression stability (M value) of candidate reference genes as determined by quantitative RT-PCR. The *y*-axis presents the M value and the *x*-axis presents the ranking of reference genes in order of increasing stability (from left to right); cDNA samples were synthesised with random primers (**A**) or oligo(dT) primers (**C**). The optimal number of reference genes for normalisation was determined by Pairwise variation (V) between the normalisation factors (Vn and Vn + 1). The optimal number of reference genes is shown for cDNA samples synthesised with random primers (**B**) or oligo(dT) primers (**D**). *Y*-axis represents the v-value indicating the pairwise variation between two sequential normalisation factors. Samples were derived from pools of 50 oocytes with three biological replicates.

(Fig. 2A–E). Even though expression of *CCNA1* increased significantly from the GV to the MI stage, the GV/MI ratio of *CCNA1* expression was only 1.32 (Fig. 2A). In cDNA samples synthesised using oligo(dT) primers, the expression level patterns for *CCNA1*, *CCNB1*, *CDK2* and *EIF4A3* were similar to those for cDNA synthesised using random primers (Fig. 2A–C and E). By contrast, the expression of *CCNB2* decreased significantly from the MI to the MII stage (Fig. 2C).

We then examined the relative expression of *GAPDH* in oocytes from GV to MII, since *GAPDH* expression was not used for normalisation. The patterns of apparent oocyte *GAPDH* expression were similar from GV to MII in cDNA samples synthesised with random primers (Fig. 2F). However, to our surprise, *GAPDH* expression levels decreased stepwise from the GV to MII stage in cDNA samples synthesised using oligo(dT) primers (Fig. 2F).

We therefore examined whether the expression levels of other potential reference genes also varied from GV to MII in cDNA samples synthesised using oligo(dT) primers. Because RNA was extracted from groups of 50 oocytes, the relative expression levels were first compared without normalisation. Unexpectedly, the expression levels of both the reference and other (target) genes examined decreased as oocytes matured from the GV to the MII stage (Fig. 3A–K). Due to a high standard deviation among the three biological replicates, these differences were not statistically significant, expect in the case of *RLP15* ($P < 0.05$), but the pattern was consistent for all genes examined. For comparison, the expression levels of reference and target genes, from GV to MII in cDNA samples synthesised using oligo(dT) primers, are shown in Supplementary Fig. S5.

Poly(A) tail length regulation during oocyte maturation

Because oocytes rely for a relatively long time on maternal mRNA during and after oocyte maturation, it is not unlikely that the poly(A) tail length is regulated during oocyte maturation. We therefore conducted poly(A) tail length assays (Rassa *et al.*, 2000) to investigate differences in the length of the poly(A) tails of *GAPDH*, *CCNB1*, *CCNB2* and *HPRT1* mRNA at different stages of oocyte maturation. For the poly(A) tail length assays, a nested PCR was performed using gene-specific forward primers with the addition of GB136 to increase the specificity of the PCR products (Fig. 4A). Second round PCR products

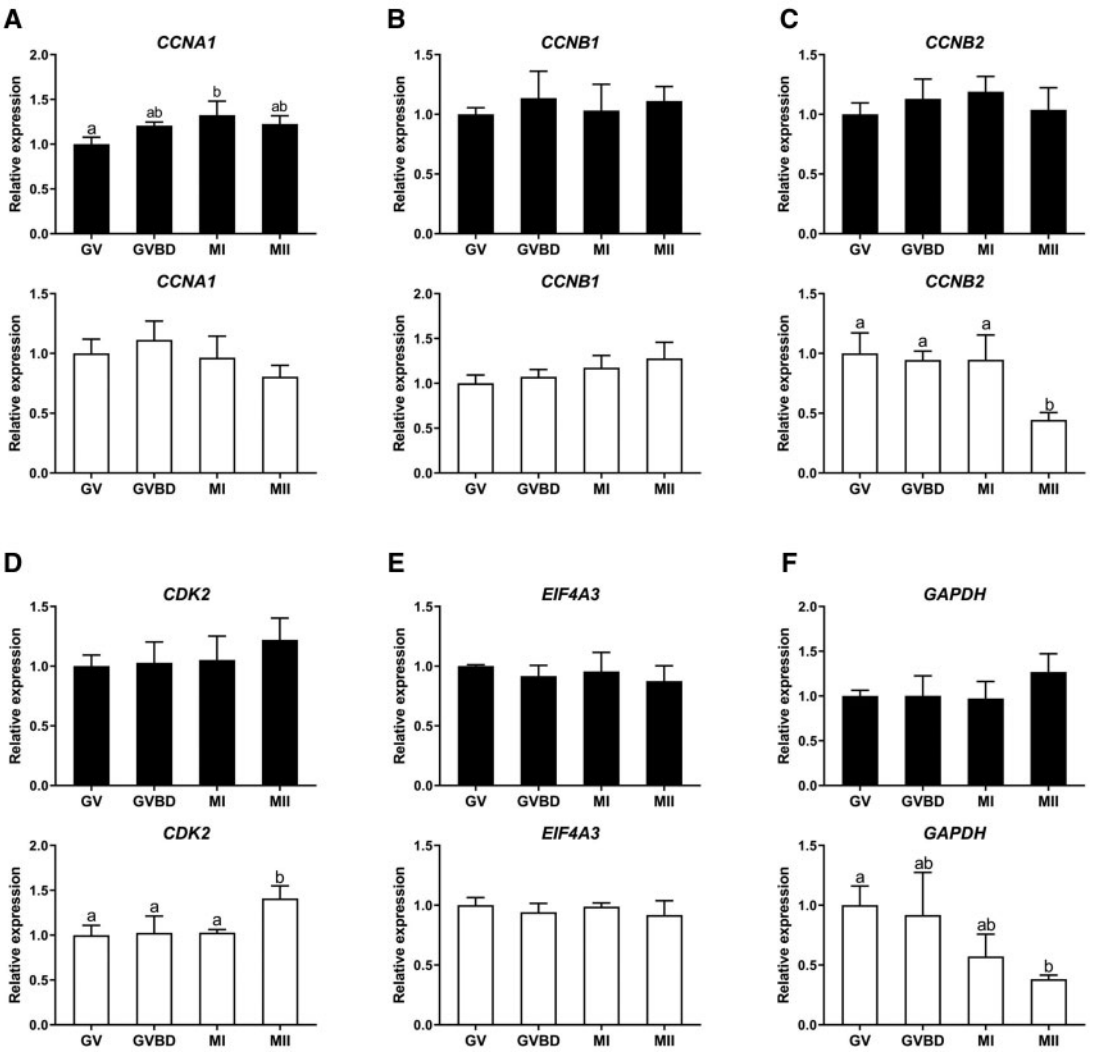

Figure 2. Gene expression patterns during oocyte maturation. The relative expression of genes in cDNA samples synthesised using random (black bars) or oligo(dT) primers (white bars) from bovine GV to MII oocytes. (**A**) *CCNA1*, (**B**) *CCNB1*, (**C**) *CCNB2*, (**D**) *CDK2*, (**E**) *EIF4A3*, (**F**) *GAPDH*. GV, GVBD, MI and MII, respectively refer to germinal vesicle, germinal vesicle breakdown, metaphase I and metaphase II stages. Relative expression in GV oocytes was set as 1. Significant differences between groups are indicated by different letters above the bars ($P < 0.05$) and error bars represent standard deviation. *ACTB*, *RPL15* and *SDHA* were used for normalisation in cDNA samples synthesised using random primers; *ACTB*, *HPRT1*, and *YWHAZ* were used for normalisation in cDNA samples synthesised using oligo(dT) primers. Samples were collected from pools of 50 oocytes with three biological replicates.

were then size-separated by agarose gel electrophoresis. The PCR products from GV oocytes showed slightly slower mobility than products from the MII oocytes in all of the genes we examined (Fig. 4B, Supplementary Fig. S2), indicating that mRNA in GV oocytes contained longer poly(A) tails than in MII oocytes.

To further confirm the decrease in poly(A) tail length during oocyte maturation, we performed DNA sequencing of amplicons (Fig. 4A). We determined that the length of the poly(A) tails ranged from 0 to 29 nucleotides (Fig. 4C and D). We further compared the poly(A) tail length of *GAPDH*, *CCNB1*, *CCNB2* and *HPRT1* mRNA in oocytes at the GV and MII stages. Consistent with the agarose gel electrophoresis results, significantly longer poly(A) tails were detected for *GAPDH*, *CCNB1* and *CCNB2* mRNA in GV oocytes, compared with MII oocytes (Fig. 4E). The poly(A) tail of *HPRT1* was also shortened in MII compared to GV oocytes but this difference was not statistically significant (Fig. 4E).

Reference gene selection and gene expression patterns in morulae and blastocysts

Our next aim was to identify the most suitable reference genes for normalisation, and to examine expression patterns for specific target genes in morulae and blastocysts. Maternal mRNA is largely degraded

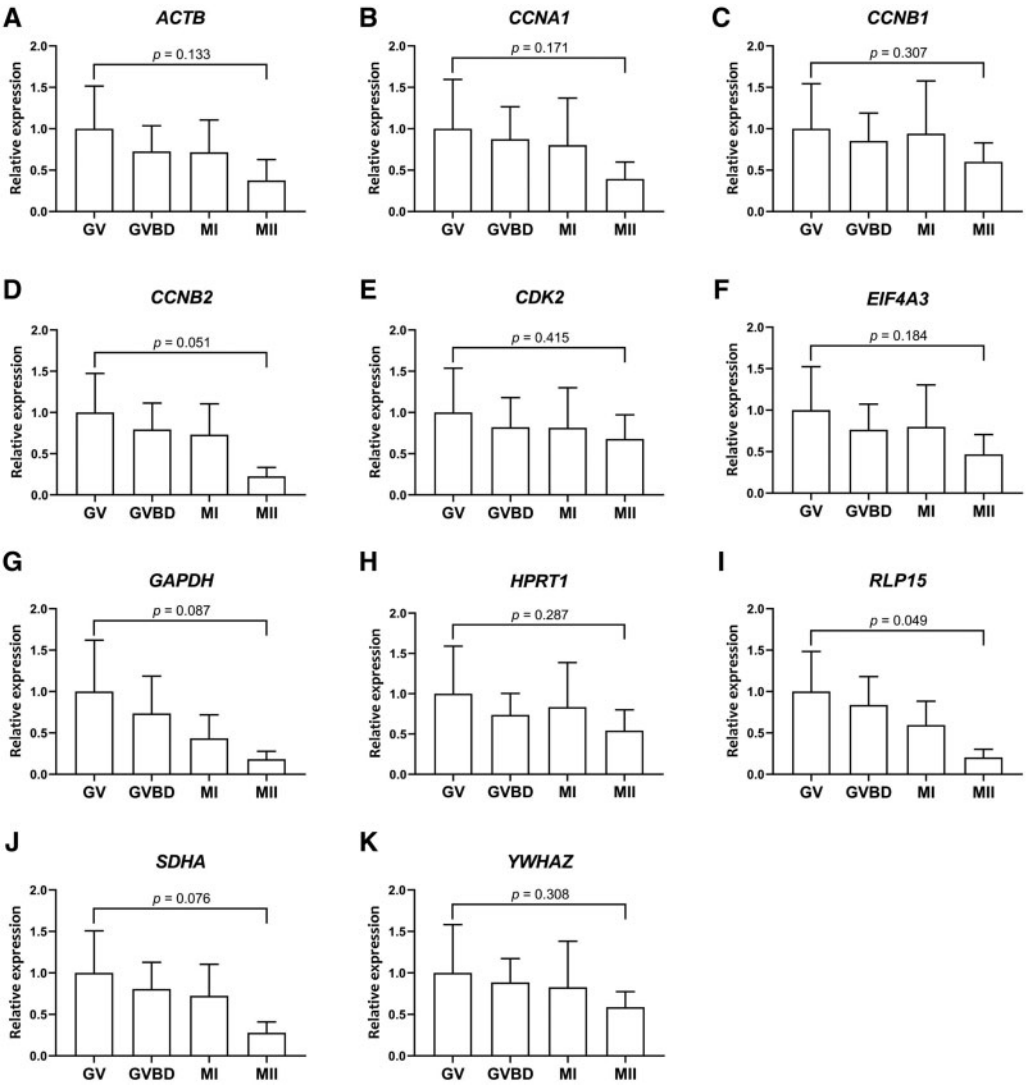

Figure 3. Gene expression patterns during oocyte maturation using oligo(dT) primers. Relative expression in cDNA samples synthesised using oligo(dT) primers in maturing bovine oocytes, as determined by quantitative RT-PCR without normalisation. (**A**) ACTB, (**B**) CCNA1, (**C**) CCNB1, (**D**) CCNB2, (**E**) CDK2, (**F**) EIF4A3, (**G**) GAPDH, (**H**) HRRT1, (**I**) RLP15, (**J**) SDHA, (**K**) YWHAZ. GV, GVBD, MI, MII, respectively refer to germinal vesicle, germinal vesicle breakdown, metaphase I and metaphase II stages. Absolute expression in GV oocytes is set at 1. Error bars represent standard deviation. Samples were collected from pools of 50 oocytes with three biological replicates.

soon after major zygotic genome activation, which occurs at around the 8-cell stage in cattle embryos, similar to that in human embryos (Niakan *et al.*, 2012). *SDHA* and *YWHAZ* were the most stably expressed genes when cDNA was synthesised using random primers, while *RPL15* and *YWHAZ* were most stably expressed in cDNA synthesised using oligo(dT) primers (Fig. 5A and C). However, the three most stably expressed candidate reference genes were the same for cDNA synthesised using random primers or oligo(dT) primers, namely *RPL15*, *SDHA* and *YWHAZ* (Fig. 5A–D).

We therefore used a combination of *RPL15*, *SDHA* and *YWHAZ* to normalise gene expression in embryos. The expression patterns of *CCNA1*, *CCNB1*, *CCNB2*, *CDK2* and *EIF4A3* in morulae and blastocysts were very similar between cDNA samples synthesised using random primers and oligo(dT) primers (Fig. 6A–E). Surprisingly, the expression of *GAPDH* increased markedly from morula to blastocyst in cDNA samples synthesised using random primers, but did not differ in cDNA samples synthesised using oligo(dT) primers (Fig. 6F).

We also included 8-cell embryos to identify the best reference genes for normalisation and expression patterns from 8-cell embryos to blastocysts. The three most stably expressed reference genes were still *RPL15*, *SDHA* and *YWHAZ* in cDNA samples synthesised with random primers while *GAPDH*, *SDHA* and *YWHAZ* were the most suitable reference genes for normalisation in cDNA samples synthesised with oligo(dT) primers (Supplementary Fig. S3). Expression patterns of

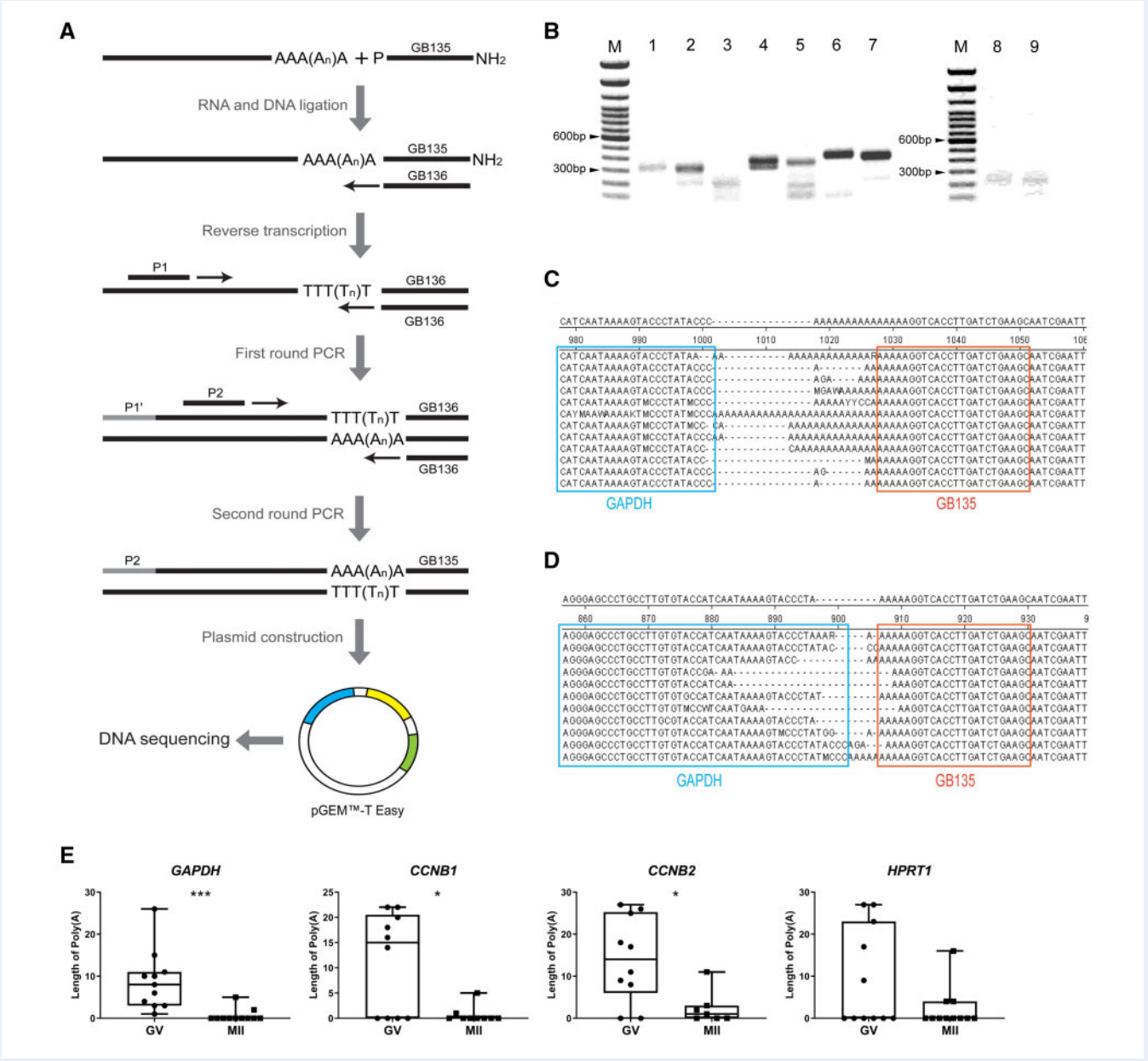

Figure 4. Poly(A) tail length regulation during oocyte maturation. (**A**) Schematic representation of the polyadenylation assay and poly(A) sequencing. mRNAs were ligated to a synthetic primer containing a 3′ amino blocking group (GB135). The ligation products are then used as templates for a reverse transcription reaction using GB136 primers complementary to GB135. To amplify cDNA and increase the specificity of the amplicons, first round amplification was performed using a gene-specific primer (P1) and GB136, and second round amplification was performed using a gene-specific primer which started after the 3′ site of P1 (P2) and GB136. The second round amplified product was then ligated with pGEMTM-T Easy plasmid, followed by transformation with JM109 cells before DNA sequencing. (**B**) Agarose gel electrophoresis of second round amplified products. Lane M: 100 bp ladder; Lane 1: positive control; Lanes 2–3: *CCNB1* at germinal vesicle (GV) and metaphase II (MII) stage; Lanes 4–5: *CCNB2* at GV and MII stage; Lanes 6–7: *GAPDH* at GV and MII stage; Lanes 8–9: *HPRT1* at GV and MII stage. Uncropped blots are provided is Supplementary figure S2. DNA sequencing result for *GAPDH* at GV stage (**C**) and MII stage (**D**) aligned by cluster W method. The end sequence of *GAPDH* is outlined with the cyan box and sequence of GB135 is included in the orange box. (**E**) Length of poly(A) tail of *GAPDH*, *CCNB1*, *CCNB2* and *HPRT1* at the GV and MII stages. Significant differences are indicated by asterisks (*$P < 0.05$, ***$P < 0.0005$) and error bars represent standard deviation. Samples were collected from pools of 50 oocytes with three biological replicates.

CCNB1, *CCNB2* and *CDK2* were similar from 8-cell embryos to blastocysts between cDNA samples synthesised using random primers and oligo(dT) primers (Supplementary Fig. S4B–D). On the hand, the expression of *CCNA1* and *EIF4A3* was down-regulated from the 8-cell embryo to the blastocyst stage in cDNA samples synthesised using random primers, whereas expression of *CCNA1* and *EIF4A3* was

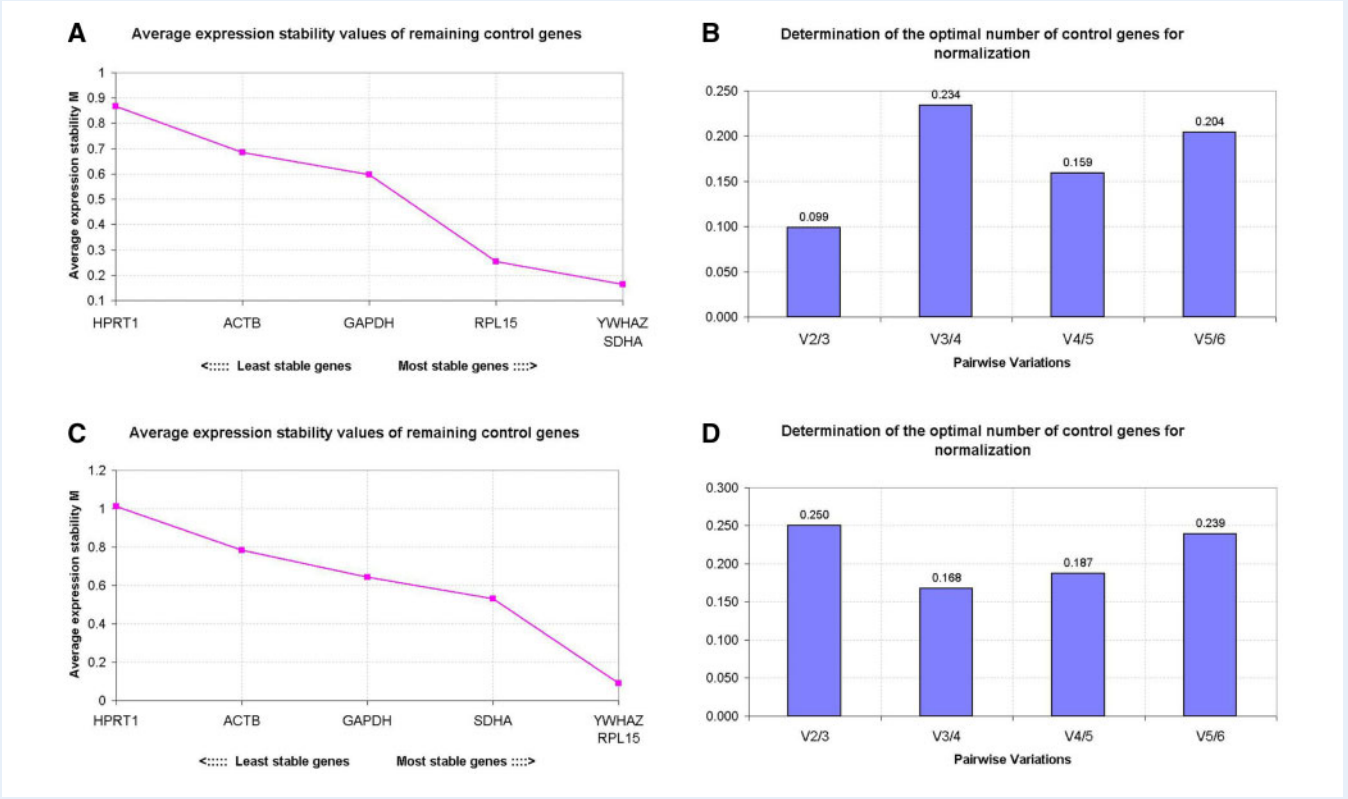

Figure 5. Reference gene selection in morulae and blastocysts. The geNorm analysis of the average expression stability (M value) of all candidate reference genes determined by quantitative RT-PCR; cDNA samples were synthesised using random primers (**A**) or oligo(dT) primers (**C**). More stable reference genes are positioned on the right side of the diagram, with less stable genes on the left side. The optimal number of reference genes for normalisation was determined by Pairwise variation (V) between the normalisation factors (Vn and Vn + 1). The optimal number of reference genes is shown for cDNA samples synthesised with random primers (**B**) or oligo(dT) primers (**D**). *Y*-axis represents the v-value indicating the pairwise variation between two sequential normalisation factors. Samples were collected from pools of 50 embryos with three biological replicates.

significantly elevated at the morula stage in cDNA samples synthesised using oligo(dT) primers (Supplementary Fig. S4A and E).

Discussion

Proper quantification of mRNA expression levels with qRT-PCR relies on the use of stably expressed reference genes (Goossens *et al.*, 2005; Kuijk *et al.*, 2007; Mamo *et al.*, 2007). A critical step in the process, in particular reverse transcription, has however been less well studied. The RNA concentration, quality and the type of reverse transcriptase can influence reverse transcription, but when samples received the same experimental handling and relative levels are determined, quantification can be reliable (Cholet *et al.*, 2020). Here, we demonstrate that the combination of genes optimal for normalisation is dependent on the priming strategy, i.e. random hexamers or oligo(dT), used for reverse transcription in oocytes. Bovine oocytes were used, since these can be collected in fairly large quantities from leftover slaughterhouse ovaries. In addition, the oocytes can be efficiently fertilised *in vitro* and embryos cultured to the blastocyst stage. No laboratory animals were therefore required, in accordance with the 3Rs of animal experimentation. Above all, the timing of bovine oocyte maturation and preimplantation development is very similar to that of human oocytes and embryos.

Selection of reference genes for normalisation is an essential step for accurate gene expression analysis. The software program geNorm was used to determine stability of gene expression. Comparisons with other programs such as BestKeeper and NormFinder, revealed that they gave very similar results (Spinsanti *et al.*, 2006; De Spiegelaere *et al.*, 2015). More importantly, geNorm is the most commonly used normalisation algorithm, because geNorm does not need large data sets and the raw data for this program do not need to be normally distributed (Mehdi Khanlou and Van Bockstaele, 2012). Even though the selection of reference genes for normalisation in bovine oocytes has been documented (Goossens *et al.*, 2005; Khan *et al.*, 2016; Caetano *et al.*, 2019), the effect of different reverse transcription priming strategies to the choice of reference genes has not been addressed in detail. It has been reported that the combination of suitable reference genes includes *HPRT1* and *B2M* in bovine oocyte cDNA samples synthesised using random primers (Caetano *et al.*, 2019), while the ideal reference genes were *ACTB* and *GAPDH* in bovine oocyte cDNA samples synthesised using oligo(dT) primers (Khan *et al.*, 2016).

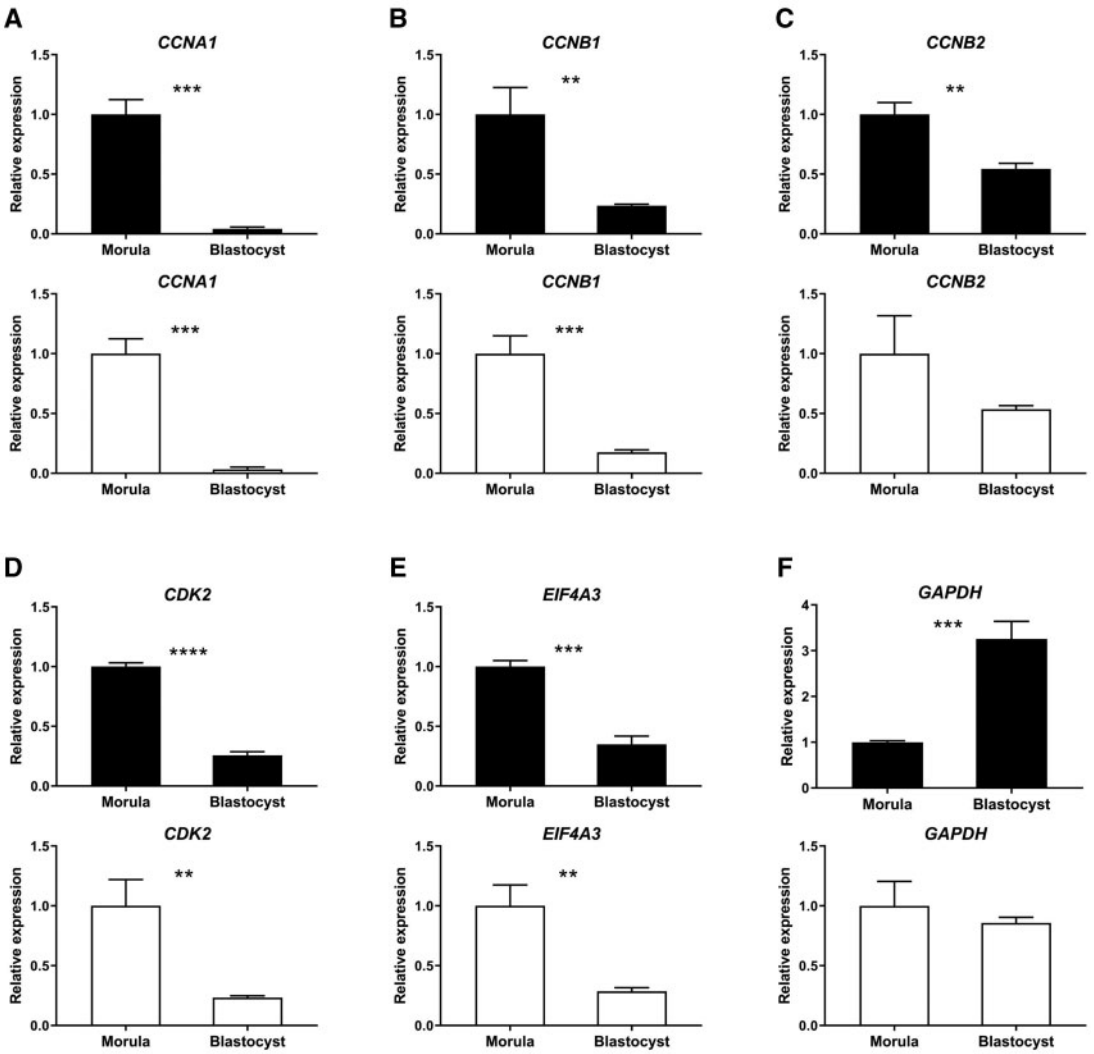

Figure 6. Gene expression patterns in morulae and blastocysts. The relative expressions of genes in cDNA samples synthesised using random primers (black bars) or oligo(dT) primers (white bars) in bovine morulae and blastocysts. (**A**) *CCNA1*, (**B**) *CCNB1*, (**C**) *CCNB2*, (**D**) *CDK2*, (**E**) *EIF4A3*, (**F**) *GAPDH*. Relative expression in morulae set at 1; $^{**}P < 0.005$, $^{***}P < 0.0005$ and $^{****}P < 0.0001$ indicate significant differences between morulae and blastocysts and error bars represent standard deviation. *RPL15*, *SDHA* and *YWHAZ* were used for normalisation for cDNA samples synthesised with both random primers and oligo(dT) primers. Samples were collected from pools of 50 embryos with three biological replicates.

Consistently, in our study, even though the same original mRNA and system were applied, suitable reference genes for normalisation were different if reverse transcription priming strategies were different.

In morulae and blastocysts, the combination of suitable reference genes seems to be less dependent on the priming strategy. This may be due to the large numbers of maternal mRNA transcripts present in oocytes and very early embryos, and the degradation of these maternal transcripts following the onset of zygotic gene expression at around the 8-cell stage in bovine embryos. To demonstrate that this difference is dependent on the method used for reverse transcription but not the efficiency of RNA extraction, we split RNA samples into two equal parts for the different reverse transcription strategies after extraction. Interestingly, it has been reported that *GAPDH*, *PPIA*, *ACTB*, *RPL15*, *GUSB* and *H2A.2* are not suitable reference genes for normalisation, because of their inconstant levels throughout preimplantation development (Ross *et al.*, 2010). In our study, the suitable reference genes for normalisation in oocytes were different from those in morulae and blastocysts even when reverse transcription is performed using the same priming strategy. We suggest using different combinations of reference genes for normalisation before and after zygotic genome activation, due to the switch from maternal to zygotic mRNA content.

In this study, we show that gene expression levels as determined by qRT-PCR change during oocyte maturation and can differ depending on the priming strategy used for reverse transcription. This is in agreement with previous studies in which relative gene expression was directly compared when reverse transcription was performed using

random primers and oligo(dT) primers (Thélie *et al.*, 2007; Gohin *et al.*, 2014). In cDNA samples synthesised using random primers, the expression was stable from the GV to MII stage in oocytes for every gene we examined, indicating that maternal mRNAs were not degraded after resumption of meiosis. Indeed, it has been documented that, at least in bovine oocytes, there is no decrease in total RNA content during meiosis (Lequarre *et al.*, 2004). We also detected a surge in absolute gene expression in oocytes between the GV and GVBD stages when no normalisation was applied, suggesting that large amounts of maternal RNA are synthesised within the 6 h prior to GVBD. Therefore, when gene expression levels are examined using qRT-PCR, reverse transcription using random primers is preferable.

In the mouse, high levels of cyclin B protein in MI and MII oocytes are achieved by control of maternal mRNA translation, which coincides with an elongation of the *CCNB1* mRNA (Kotani *et al.*, 2013). High levels of cyclin B have been observed in MI and MII oocytes (Wu *et al.*, 1997; Heikinheimo and Gibbons, 1998; Quetglas *et al.*, 2010). We detected consistent levels of *CCNB1* expression throughout oocyte maturation, while the detected *CCNB2* levels were significantly reduced from GV to MII oocytes when cDNA was synthesised with oligo(dT) primers. To address how cyclin B gene expression was regulated in oocytes, we analysed our qRT-PCR data without normalisation, since numbers of oocytes were equal in all groups. Both *CCNB1* and *CCNB2* expression, as well as reference gene expression levels could be seen to be down-regulated from the GV to the MII stage.

Associated with our qRT-PCR without normalisation data, the poly(A) tail lengths for *CCNB1*, *CCNB2* and *HPRT1* also decreased from the GV to the MII stage. In contrast, it has been reported that the poly(A) tail of *CCNB1* is elongated during oocyte maturation in the mouse, *Xenopus* and zebrafish (Mendez and Richter, 2001; Kotani *et al.*, 2013). A decrease in poly(A) tail length and a down-regulation of gene expression when normalisation was not applied was also observed for other genes, indicating a general deadenylation of maternal mRNA during bovine oocyte maturation. In agreement with our findings, it has been reported that the amount of poly(A) RNA is reduced by half, but that the total RNA content does not change during bovine oocyte maturation (Lequarre *et al.*, 2004). It is therefore possible that maternal mRNAs have long poly(A) tails to increase their stability during the long time of storage before meiosis resumption. Interestingly, other studies have shown that mRNAs have much longer poly(A) tails, up to 250 nucleotides (Vaur *et al.*, 2002; Lim *et al.*, 2016), than the poly(A) lengths we detected.

In conclusion, we observed that the genes used for normalisation of expression levels may differ in terms of apparent stability of expression in oocytes depending on the priming strategy used to synthesise the cDNA, and the priming strategy should therefore be tailored to the question addressed. In embryos at a stage beyond zygotic genome activation, such as morulae and blastocysts, the method of cDNA generation appears to be less critical to obtaining reliable qRT-PCR results. In general, the poly(A) tail of mRNA species synthesised before GVBD in oocytes seems to shorten during meiotic maturation to the MII stage in bovine oocytes.

Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online.

Data availability

The supporting data for this article are available in the article and the online supplementary material.

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Authors' roles

B.Y., H.T.A.v.T., and B.A.J.R. conceived and designed the experiments. B.Y. and H.T.A.v.T. performed the experiments. B.Y. collected and analysed the data. B.A.J.R. contributed the reagents, materials and analysis tools. B.Y., T.A.E.S. and B.A.J.R. wrote the manuscript. All authors read and approved the manuscript.

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Conflict of interest

None declared.

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