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ORIGINAL RESEARCH

Analysis of microRNAs and their precursors in bovine early embryonic development

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ABSTRACT: In animals, the maternal-to-embryonic transition (MET) occurs in the first days of early development and involves the degradation of maternal transcripts that have been stored during oogenesis. Moreover, precise and specific control mechanisms govern the adequate synchronization of the MET events to promote the activation of the embryonic genome. These mechanisms are not well understood, but it is believed that microRNAs (miRNAs) could be one of the mechanisms involved. After a microarray screening study, we analysed the expression of specific miRNA during oocyte maturation and early embryo development until preimplantation stages. Two differentially expressed candidates were selected for further analysis. Mature and precursor forms of miR-21 and miR-130a were quantified by qRT–PCR in pools of 20 oocytes at GV (germinal vesicle), GV breakdown and metaphase II stages as well as in pools of embryos at the 2-cell, 4-cell, 8-cell and blastocyst stages. The results showed a linear increase during the 1–8 cell stage for the mature forms of miR-130a and miR-21 ($P < 0.05$ and $P < 0.003$, respectively) and for the precursor form of miR-130a ($P < 0.002$). To see if this increase was due to minor transcriptional activity, 2-cell embryos were exposed to α-amanitin for 30–34 h. Results showed a significant decrease in miR-21, pre-miR-21, miR-130a and SRFS3 in α-amanitin-treated embryos ($P < 0.05$). Considering the potential regulatory role of these miRNA, the bovine genome was screened to identify putative targets with a 3′UTR exact seed match. This study suggests that miRNAs could be important players in the MET, as expression profiles suggest a potential regulation role during early development steps.

Key words: bovine / early development / miRNA / transcription

Introduction

Early development gives rise to multiple changes in the genetic material of the embryo. In the first days after fertilization, the embryo is dependent on maternally inherited and stored mRNAs until the activation of its own mRNA transcription mechanisms (Schultz, 2002; Vasudevan *et al.*, 2006). This storage is very important, especially during the few days when transcription is absent or reduced since the embryo has to use the maternal heritage to generate new proteins. This period of time known as the maternal-to-embryonic transition (MET) underlies the passage between the usage of maternal genetic material stored in stabilized mRNAs and the activation of the new embryonic genetic material. It is a crucial step in the early development of all animals, and happens at the 4000- to 8000-cell embryo in frog, at the 2-cell embryo in mouse and at the 8-cell embryo in cow (Newport and Kirschner, 1982; Telford *et al.*, 1990; Schultz, 1993). This period of time brings about many changes, as was shown by protein modification on 2D gels by (Barnes and First, 1991), and requires effective and precise mechanisms to control the adequate synchronization of the early development.

MicroRNAs (miRNAs) are short non-coding RNAs of 19–24 nucleotides known to regulate genes by targeting their 3′-untranslated regions (3′UTRs) in an imperfect manner. The growing knowledge about miRNA mechanisms and involvement in many post-transcriptional regulation processes makes them interesting candidates for the control of maternal transcripts in the early embryo. MiRNAs are transcribed by the RNA polymerase II and possibly the RNA polymerase III enzymes (Borchert *et al.*, 2006; Pawlicki and Steitz, 2009). The first structure to be expressed is the primary miRNA (pri-miRNA), transcribed in the nucleus and cleaved by the microprocessor complex, an arrangement of the RNase-III-like enzymes Drosha and DGCR8. The precursor miRNA (pre-miRNA) produced by this cleavage is a double-stranded hairpin-shaped molecule, which is exported into the cytoplasm. The pre-miRNA is further cleaved by Dicer, another RNase-III-like enzyme, to produce the double-stranded miRNA duplex. The passenger strand (or the guide strand) is discarded and the remaining strand forms the mature miRNA, which will be integrated into the RNA-induced silencing complex (miRISC) to act as a recognition template to target mRNAs and regulate their

expression. Coupled with miRISC, miRNAs direct translational repression or promote the deadenylation process of mRNAs (Filipowicz *et al.*, 2008).

MiRNAs are interesting candidates for maternal mRNA degradation in the MET context because they regulate gene expression in a temporal- and spatial-specific manner. Recent studies showed the implication of miRNAs in the development of some species, especially in the degradation of maternal transcripts, by identifying their presence at distinct developmental stages prior to embryonic genome activation. For example, in *Caenorhabditis elegans*, miRNAs regulate and allow the transition between larval stages (Lee *et al.*, 1993). In zebrafish, miR-430 could be responsible for the degradation of a large number of maternal transcripts (Chen *et al.*, 2005; Giraldez *et al.*, 2006). MiR-427 is the *Xenopus* ortholog of this miRNA and leads to the deadenylation and depletion of hundreds of maternal mRNAs in the frog embryo (Watanabe *et al.*, 2005; Lund *et al.*, 2009). In the rainbow trout, some of the quantified miRNAs showed potential implication in the activation of the embryonic genome, with a particular consideration for miR-21. This miRNA was found in higher expression levels just before the genomic activation, a moment where an important regulatory mechanism must be applied (Ramachandra *et al.*, 2008). These up-regulated miRNA profiles at the embryonic genome activation (EGA) time, like miR-21, suggest the contribution of some miRNAs in embryonic development. Moreover (Ramachandra *et al.* (2008) suggest that these characteristic expression profiles support the hypothesis that miR-21 is processed during the EGA to act as a guide for maternal mRNA degradation.

The degradation of non-translated mRNAs is also reported to be a critical step for allowing EGA in the mouse (Alizadeh *et al.*, 2005). The mechanisms involved in this degradation are still unclear and Schier was one of the first to hypothesize the potential role of miRNAs as a control mechanism in the degradation of maternal mRNAs (Schier, 2007). Also in the mouse, Dicer knocked-out oocytes and embryos are fated to die: oocytes cannot complete the first meiosis and embryos stop their development before the gastrulation stage (Bernstein *et al.*, 2003; Murchison *et al.*, 2007). These infertility phenotypes, which underscore the importance of miRNAs in early development, have been weakened by genomic analysis of miRNA binding sites in the 3′UTR of maternal mouse transcripts (Ma *et al.*, 2010). This study showed that miRNAs seem to have a very minimal impact on the abundance of mouse maternal miRNAs. The lethal phenotype caused by the loss of Dicer could be explained by the fact that Dicer also affects the biogenesis of siRNAs and thus the expression of important genes that are not necessarily regulated by miRNAs (Bernstein *et al.*, 2003; Ma *et al.*, 2010). Therefore, the roles of miRNAs in the early development are supported by studies in zebra fish, frog and rainbow trout, but less so by the studies in mouse. Very few studies have investigated the role of miRNAs during the bovine maternal-to-zygotic transition (Tesfaye *et al.*, 2009; Tripurani *et al.*, 2010).

To further assess the roles of miRNAs in the course of bovine early development, we identified differentially expressed miRNAs and then quantified selected candidates in oocytes and preimplantation stage embryos.

Materials and Methods

Sample collection

In vitro maturation

Follicles of 5 mm in diameter were aspirated from ovaries of slaughtered dairy cattle. The collected cumulus–oocyte complexes (COCs) were washed four times in TLH (HEPES-buffered Tyrode's lactate solution) and selected to be placed in 50-μl drops of maturation medium covered with mineral oil. The oocytes at the germinal vesicle stage (GVs) were collected after the TLH washes. The COCs was matured in TCM-199 medium supplemented with 10% fetal calf serum, pyruvate and gentamycin. The petri dishes containing the oocytes (Nunc, Denmark) were placed at 39°C under 5% CO_2 with a maximum humidity level. After 6 h of maturation, the oocytes at GV breakdown stage (GVBDs) were collected and the remaining oocytes were kept in incubation for 24 h for fertilization. All the GVs, GVBDs and MII (metaphase II) collected were mechanically denuded by agitation for about 5 min in PBS (phosphate-buffered saline) to remove their cumulus cells before the freezing step.

In vitro fertilization

Before IVF, MII-stage oocytes were collected. The remaining matured oocytes were washed two times in the TLH medium and then placed by groups of 5 in 48-μl drops of medium covered with mineral oil. The fertilization medium was composed of TL Stock (Tyrode's lactate solution) supplemented with 0.6% acid-free BSA (bovine serum albumin), 0.2 mM pyruvic acid, 10 μg/ml heparin and 50 mg/ml gentamycin. About 10 min before fertilization, 2 μl of 2 mM penicillamine, 1 mM hypotaurine and 250 mM epinephrine were added to each droplet. The semen used was a cryopreserved mixture of the ejaculates from three bulls (Centre d'insémination artificielle du Québec, St-Hyacinthe, Québec, Canada). The semen was thawed in 37°C water for 1 min and put on a progressive Percoll gradient (2 ml of 90% Percoll covered with 2 ml of 45% Percoll) and then centrifuged at 700*g* for 30 min at 26°C. The pellet of live spermatozoa was resuspended in the IVF medium after being counted on a haemocytometer to obtain a final concentration of 10^6 cells per ml. Two microlitres of the sperm suspension was added to each droplet and the dish was replaced for incubation under 5% CO_2 at 38.5°C with high humidity for 15–18 h.

In vitro culture

All the zygotes were mechanically denuded by gentle repeated pipetting, washed three times in TLH containing 0.4% BSA and placed by groups of 10 in 10-μl culture medium drops covered by mineral oil. To obtain blastocysts, the zygotes were cultured for 7 days in sequential medium of modified synthetic oviduct fluid (SOF) containing 0.4% BSA and amino acids, at 38.5°C in a 5% CO_2 reduced oxygen atmosphere (5%) with maximal humidity. After 72 h, the embryos were transferred from SOF 1 to SOF 2 medium and, after 48 h in SOF 2, were transferred in SOF 3 medium for the last 48 h of culture. Two-cell embryos were collected after 30–35 h, 4-cell embryos after 40–45 h, 8-cell embryos after 50–90 h and blastocysts after 7 days post-IVF. Collected blastocysts were a mix of young, expanded and hatched. All stages from GV to blastocysts were collected in pools of 20 and placed in 1.5-ml tubes after being washed in PBS and stored in a minimal PBS volume at −80°C until RNA extraction. The procedure was repeated to obtain three replicates for each stage.

α-Amanitin-treated embryos

To collect a pool of 2-cell embryos with blocked transcription, half of the oocytes at the MII stage were fertilized and cultured in respective medium supplemented with α-amanitin. For this, a concentration of 25-μg/ml α-amanitin (Sigma, St. Louis, MO, USA) was added to IVF and SOF1 medium (Vigneault *et al.*, 2009). It was observed that a 100 μg/ml concentration of α-amanitin induces the arrest of poly-(A) tail synthesis in mouse embryos, from the 2-cell stage to the blastocyst stage (Kidder *et al.*, 1985). Therefore, we used a 25 μg/ml concentration to minimize unwanted effects on treated samples. To prepare negative untreated control pools, the remaining half of the oocytes were fertilized and cultured as described above, in an independent dish. Control and treated pools of 10 embryos each were collected and stored at −80°C until RNA extraction. These steps were repeated to obtain three replicates of treated embryos and three replicates of control embryos.

RNA extraction

Total RNA containing miRNAs was extracted from each pool of 20 ($n = 3$) collected using miRNeasy mini kit (Qiagen, Mississauga, Canada) following the manufacturer's protocol. DNAse digestion was performed using the RNase-Free DNase Set, directly on the extraction column (Qiagen). To confirm RNA integrity, all samples were analysed by Eukaryote Total RNA Pico with a 2100 Bioanalyzer, using RNA 6000 Pico reagents and chips (Agilent Technologies, Santa Clara, CA) according to the manufacturer's protocol.

MiRNA identification

To analyse the miRNAs present in oocytes, the total RNA from 300 GVs and 300 MII was extracted as described above. All the microarray steps were performed by the miRNA Expression Profiling Microarray Service (LC Sciences, Houston, TX) with the mammalian chip release 11.0 comprising a total of 3272 known mammalian miRNAs, including 94 bovine miRNAs. The assay started with 200 ng total RNA, which was amplified in-house and labelled with Cy3 and Cy5. The positive miRNA on the array were used to blasts the miRBase search engine (mirbase.org). We then selected two miRNAs, one that was over-expressed and the other under-expressed in MII compared with the GV stage to process with the QPCR.

Control genes and normalization

The limited amount of material compared with somatic tissues and the absence of stable miRNAs for normalization have prompted us to develop a robust normalization method. In a first step, to assess the quality and integrity of our samples, we used messenger RNAs known to be stable in an embryo culture system. The H2A.1 gene has been shown to be a good internal control (Vigneault *et al.*, 2007). Additionally, Drosha mRNA, which has a role in miRNA biogenesis, was measured as a control, but also to follow its expression profile during oocyte maturation and early embryogenesis to possibly explain some miRNA profiles. Moreover, normalization of miRNA data still poses considerable challenges. Based on the literature and the acceptable expression profile obtained, we decided to use the U6 snRNA gene as an endogenous control to normalize the data, as used by the Exiqon miRCURY LNA miRNA PCR system and other studies (Li *et al.*, 2009; Tesfaye *et al.*, 2009; Roa *et al.*, 2010). The primers used are listed in Supplementary data, Table S1.

miRNA quantifications

The candidate miRNAs were selected based on their pattern of expression as seen in the microarray results or for their implication in certain pathways. These candidates were quantified at each stage of oocyte maturation (GV, GVBD, MII) until the blastocyst stage (2-cell, 4-cell, 8-cell, blastocyst). The data were determined by quantitative real-time PCR using the miScript PCR System (Qiagen) according to the manufacturer's protocol. The total RNA obtained from each pool of 20 ($n = 3$) was converted to cDNA using miScript reverse transcriptase mix (Qiagen). To optimize the small concentration of samples, the RT mixes were made with the maximum RNA required in the kit, 15 μl of total RNA, which approximately represents concentrations between 200 and 500 pg/μl.

Each selected miRNA and their specific stem loop were quantified by real-time PCR using a miRNA-specific primer and the miScript universal primer and only the precursor miRNA-specific primers for the stem loop (Qiagen). The miScript Precursor Assay targets the sequence which is present in both primary (pri-miRNA) and precursor (pre-miRNA), so the primers used to target the pri-miRNA and the pre-miRNA are indistinctly in the same quantification. Thus, the term 'precursor' or 'pre-miR' in this analysis will include both primary and precursor forms. The primers used for miRNAs and pre-miRNAs were designed by Qiagen and the exact sequences are not available, thus their catalogue number are listed in Supplementary data, Table S1.

With the differential values obtained between the mature and the precursor forms, the results from this study illustrate the importance of quantifying the corresponding precursor stem-loop sequence. Indeed, this additional analysis gives a more accurate overall view of the miRNA expression and completes the analysis of the mature form, which could not be attributed to processing instead of new transcription.

The Roche Lightcycler 2.0 was used to perform the quantitative analysis using miScript QuantiTect SYBRGreen PCR master mix (Qiagen). In preliminary experiments, optimization of the samples was achieved with different dilutions to test the abundance of target sequence and establish the right dilution for each primer. Quantifications were done in 20-μl reaction volumes containing 2 μl of 10 × specific primer assay, 10 μl of universal primer and 2 μl of the proper dilution of cDNA. Prior to the quantification, standard curves for all miRNAs, pre-miRNAs and for the two controls were set up with 10-fold serial dilutions of cDNA from a pool of granulosa cells purified using the QIAquick Gel Extraction Kit (Qiagen). The expression of each miRNA was represented as a relative fold change, divided by the quantity of the reference gene U6.

α-Amanitin-treated embryos

For the pools of treated 2-cell embryos ($n = 3$), the mature and precursor forms of the miRNAs, miR-21 and miR-130a, were measured using the same protocol. The data were normalized to U6 snRNA, and H2A.1 was also measured to verify the quality of the samples. Moreover, the SRFS3 gene was used as a positive control for the α-amanitin treatment. This gene is sensitive to α-amanitin and its synthesis is partially blocked in bovine embryos treated for 24 h in α-amanitin (Kanka *et al.*, 2009). The same quantifications were done on the control pools and the results were compared with see the impact of RNA polymerase II inhibition on the expression of the miRNAs.

Genome-wide miRNA target identification

The UMD3.1 assembly of the bovine genome (Zimin *et al.*, 2009) was screened in order to identify perfect matches with the seed region of the miRNAs [mir-21: AGCUUAU; mir-130a: AGUGCAA; retrieved on 'miRNA SNiPer' http://www.integratomics-time.com/miRNA-SNiPer (Zorc *et al.*, 2012)]. BEDTools (Quinlan and Hall, 2010) was used to filter out matches which did not fall within the 3′UTR of genes present within the RefSeq annotation track of the UCSC Genome Browser (Dreszer *et al.*, 2012). In order to obtain a conservation score of the seed region in the mRNA targets, UMD3.1 coordinates were converted

to bosTau4 (Liu et al., 2009) coordinates and the five-way (cow, dog, human, mouse and platypus) phastCons (Siepel et al., 2005) scores from the UCSC Genome Browser were extracted for each seed region. Candidates that were not significantly conserved were removed from the analysis. A score of 1 corresponds to a perfect conservation of the seed region. Finally, we selected target candidates for which there was evidence of expression within bovine oocytes or embryos (Robert et al., 2011). The web-based target prediction softwares MicroCosm Targets (version 5, http://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets/v5/search.pl) and TargetScan release 6.0 (http://www.targetscan.org/) were used to confirm the most conserved mRNAs as predicted human and bovine miRNA targets.

Statistical analysis

For the quantifications of all studied stages, one-way ANOVA with Tukey *post hoc* test were used with GraphPad Prism to analyse the differences in expression levels between stages for all miRNAs and control genes. Correlations were established by linear regressions (Pearson, r). Finally, t-tests were used to determine the significance of differences in miRNAs and control genes for the α-amanitin experiment within stages between the treated and the untreated 2-cell embryo pools. All differences with a P-value < 0.05 were assumed significant.

Results

miRNA identification

The microarray assay allowed the detection of (according to Sanger miRBase release 11.0) 491 miRNAs including 31 bovine miRNAs at the GV stage and 442 miRNAs including 26 bovine miRNAs at the MII stage (GEO access number: GSE35567). Moreover, 122 miRNAs presented differential expression between those 2 stages (Supplementary data, Table S2), including 11 bovine miRNAs. Among the 122 differentially expressed miRNA, 14 were identified has having a recognition sequence in bovine genes after we blasted the sequences found on our Bluechip V3 microarray slide (GEO#GPL11247). We focused our research on two of these miRNAs, miR-21 and miR-130a, which showed fold changes of 1.91 and -1.83, respectively. Moreover, the high expression level of miR-21 in the rainbow trout embryo suggests its implication in maternal transcript degradation (Ramachandra et al., 2008). Three other control micro RNA genes were used to assess stage variations: miR-125, miR-30 and miR-9.

Temporal correlation of the miRNAs expression levels

Quantification of mir-21 and mir-130a in the bovine early development from GV oocyte stage to the blastocyst embryo showed some interesting expression profiles. The results obtained for mature miR-21 demonstrated a positive correlation between the GV stage up to the 8-cell embryo ($P < 0.003$ and $R^2 = 0.4256$). The precursor of miR-21, as shown in Fig. 1, did not show such a clear correlation, but did show significant increase between MII stage and 2-cell embryo ($P < 0.05$). Moreover, the precursor assay clearly suggests new expression of this miRNA, as well as its primary and precursor forms. For miR-130a, a similar correlation from GV stage to 8-cell embryo was observed ($P < 0.05$ and $R^2 = 0.2084$; Fig. 1). The expression level of the pre-miR-130a followed a parallel temporal expression, based on the mature miR-130a, except for the higher GV stage expression level. The expression profile of pre-miR-130a, as seen in Fig. 2, showed a positive linear rise between GVBD stage and 8-cell embryo ($P < 0.002$ and $R^2 = 0.3329$).

The level of both mature miRNAs showed temporal variations over the course of the maternal-to-zygotic transition (Fig. 1). The expression levels of both miRNAs decreased at the blastocyst stage, right after the augmentation at the 8-cell stage. In a more quantitative analysis of these expression profiles, the ratios of the mature-to-precursor forms for the measured miRNAs were calculated. For miR-21, the ratio increased up to 10 times from the initial GV stage until the 8-cell and blastocyst stages in a regular pattern. The mature form of miR-21 was significantly increased in comparison with the precursor form, particularly at the 2-cell stage where the mature form increased up to 10 times after the MII stage and the precursor form increased up to 5 times. For miR-130a, the ratio increased from the GV stage until the 2-cell stage, to decrease until the blastocyst stage. Compared with the microarray results we obtained, the difference between the GV and MII stages in the QPCR results was not significant. As for miR-21, the concentration of the mature form of miR-130a was also higher in the 2-cell embryo than in the MII oocyte (Fig. 2).

Quantification of the two control genes, also reported in Fig. 1, showed expected results for the H2A.1 and Drosha genes (Vigneault et al., 2007). The expected expression profiles obtained for these two mRNAs support the quality and the integrity of the samples and of the extraction procedure. The three other miRNAs which were measured, miR-9, miR-30 and miR125 did not show any significant variations during embryo development (results not shown).

For the two miRNAs, miR-21 and miR-130a, the primary and precursor forms from 10 to 100 times show less expression than the mature form. This comparison is illustrated in Fig. 2 where ratios of mature and precursor forms are seen to fluctuate between each stage of the early development. For miR-21, the mature form is always present at a higher concentration. This is also true for miR-130a, except for the GV and blastocyst stages.

Quantification of miR-21 and miR-130a in 2-cell embryos treated with α-amanitin

Treatment of α-amanitin inhibits the RNA polymerase II enzyme which is believed to initiate the minor transcriptional activity that can occur in cow at the 2-cell stage (Memili et al., 1998). As expected, results showed a significant decrease in the level of the mature miR-21 caused by the treatment ($P < 0.02$) and, as seen in Fig. 3, a decrease for its precursor. This decrease, however, was not found to be statistically significant ($P < 0.07$); nevertheless a trend to decreased levels of mature and precursor forms of the miR-21 in the treated embryos is shown. The mature form of miR-130a also showed a significant decrease in the treated 2-cell embryos ($P < 0.02$). The expression level for its precursor is too low to be visible in Fig. 3 and, like pre-miR-130a, the decrease in abundance between untreated and treated embryos demonstrated a visible trend despite not being statistically significant. We used the SFRS3 gene as a positive control, based on a study by Kanka who observed a significant decrease in the expression level of this gene in 4-cell and 8-cell bovine embryos after 24 h of incubation with α-amanitin (Kanka et al.,

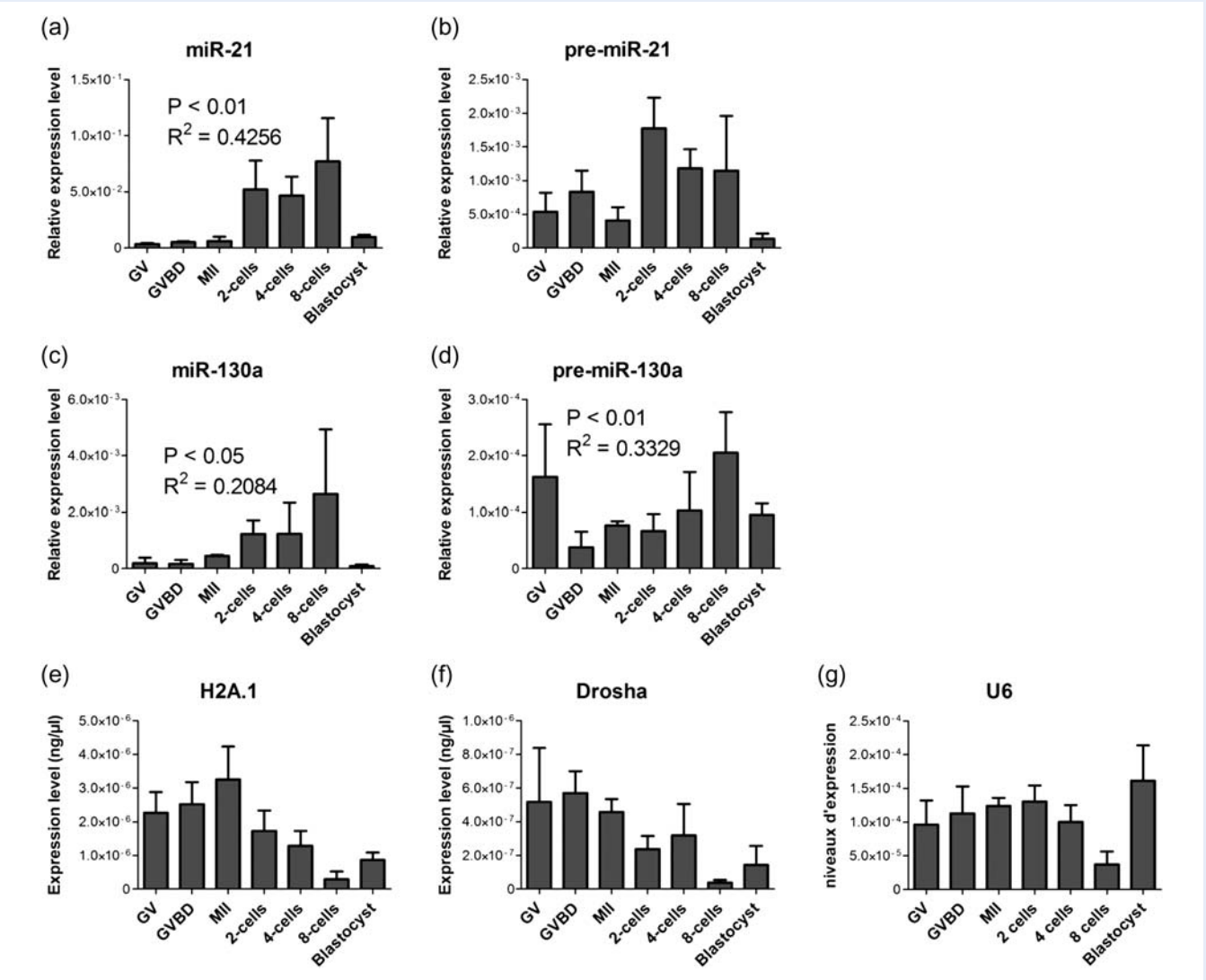

Figure 1 Expression profiles of (**a**) miR-21, (**b**) pre-miR-21, (**c**) miR-130a, (**d**) pre-miR-130a, (**e**) H2A.1, (**f**) Drosha and (**g**) U6 in the early bovine embryo development, as revealed by qRT–PCR quantifications. For miR-21, a correlation was observed in the mature form ($P < 0.003$; $R^2 = 0.4256$). The correlation was not clearly present in the precursor form. For miR-130a, a correlation was observed for the mature form ($P < 0.05$; $R^2 = 2084$) as well as in the precursor form except at the GV stage ($P < 0.002$; $R^2 = 0.3329$). Expression profiles of the two control genes can be supported by earlier study (for H2A.1) or a known trend (for Drosha and the U6 profile which confirms its value for normalization.

2009). Consistent with this study, our results demonstrated a significant decrease ($P \ll 0.01$) in the expression level of SFRS3 in the treated bovine 2-cell embryos (Fig. 3). Moreover, the expression level for the other control gene, H2A.1, did not show any significant change ($P \leq 0.05$). As this gene is not expected to be transcribed in the 2-cell embryo, it can be used as a negative control for the α-amanitin treatment as it should not be affected by the treatment.

Genome-wide miRNA target identification

It is commonly recognized that miRNA targeting involves seven to eight nucleotides at the 5′ end of the miRNA, called the 'seed region', and the 3′UTR of the mRNA (Lewis *et al.*, 2003; Doench and Sharp, 2004; Brennecke, *et al.*, 2005). From our genome-wide screen, 391 and 619 genes were detected with an exact 3′UTR match for miR to the seed regions of miR-21 and miR-130a, respectively (Supplementary data, Tables S3 and S4). Moreover, 24 and 77 mRNAs appeared to have a perfect conservation of the seed region for miR-21 and miR-130a, respectively. Among them, WWP1, DNAJA2, PITX2, MAPK10, NFIB, STAG2, PELI, PCBP1 and SPRY2 are also described as miR-21 targets in human according to the MicroCosm Targets database (Griffiths-Jones *et al.*, 2008) and only C19H17orf39 and TFDP1 were not classified as miR-21 targets in bovine using TargetScan (Lewis *et al.*, 2003). Seventeen mRNAs out of the 77 putative miR-130a targets were predicted as human miR-130a targets in MicroCosm and only 11 mRNAs were not described as miR-130a targets in bovine by TargetScan (SNAP25, TBCEL, UBE4B, CHD9, CFL2, ROBO1, GADD45A, RAD51B, RPS6KA5, NDST1 and TLE). Two mRNAs were not retrieved (C2H1orf144 and LGALSL).

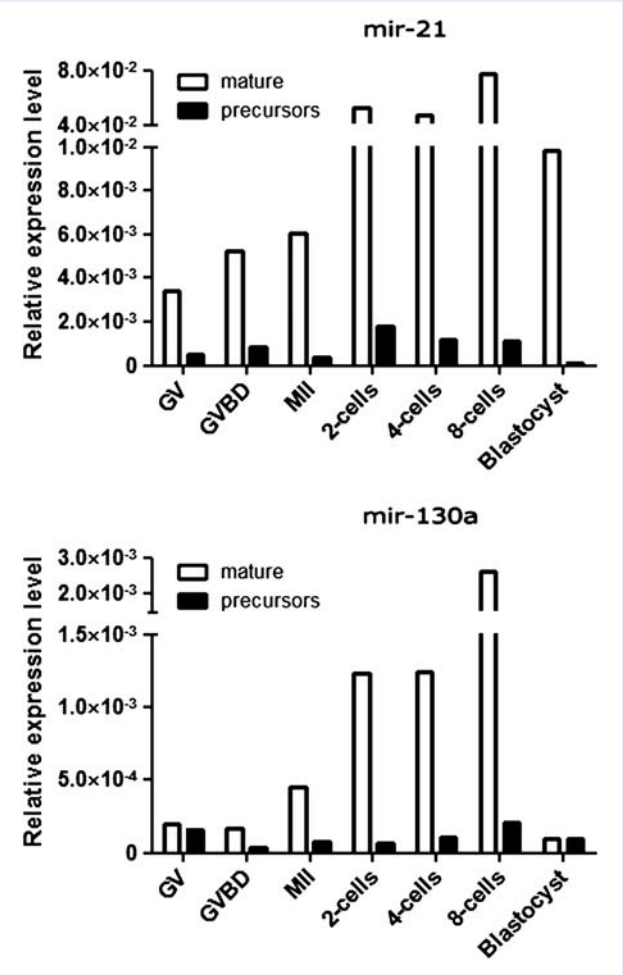

Figure 2 Expression profiles of miR-21 and miR-130a to compare mature and precursor forms relative expression levels in the bovine early development, as revealed by the q-RT–PCR. For miR-21, the mature form is always in a higher expression level than the precursor form. For miR-130a, mature and precursor forms are equally found at the GV and blastocyst stages and in the higher expression level at other stages.

Discussion

The first interesting results are the significant rise in expression profiles obtained for mature miR-21 and miR-130a from 1 to 8 cells. These unexpected patterns could indicate an activity of miRNAs on mRNAs carrying a seed sequence in their 3′UTR during the first segmentations of the embryo. In another study, the quantification of miR-10 and miR-424 in early bovine development showed a steady expression level from the GV oocyte until the 16-cell embryo (Tripurani *et al.*, 2010). Other profiles were presented for miR-125, miR-127 and miR-145, which showed an increased expression level by the 4- and 8-cell embryo (Tesfaye *et al.*, 2009). Moreover, miR-196a increases steadily from the 2- to the 8-cell embryo (Tripurani *et al.*, 2011). All these results could propose an implication for these specific miRNAs during the MET. Additionally, this previous study showed the potential evidence of a direct negative regulation of a maternal transcript, the NOBOX element, by miR-196a (Tripurani *et al.*, 2011). Our results are the first demonstration that some miRNA could originate from *de novo* transcription during the pre-MET phase in bovine.

In all these cases, a reduced expression level of these miRNAs was observed at the blastocyst stage and emphasized the expression period of the mature miRNAs during or before the MET. Those profiles were interesting because the expression level increased from the 1- to 8-cell embryo stage instead of decreasing like total mRNA profiles. As mentioned earlier for the trout and the zebrafish study, these specific augmentations of certain miRNAs during the MET suggest a putative role in the destruction of the maternal target mRNAs at the MET (Giraldez *et al.*, 2006, Ramachandra *et al.*, 2008).

In accordance with these results, the calculated ratios of mature-to-precursor forms can additionally illustrate this particular expression pattern at the MET, especially with miR-130a where the mature form decreased to an equal concentration to the precursor form at the blastocyst stage. Consequently, this marked decrease in the mature form at the blastocyst stage suggests a role for the miRNA during the MET.

With these differential values obtained between the mature and the precursor forms, our results illustrate the importance of quantifying the corresponding precursor stem-loop sequence. Indeed, this additional analysis gives a more accurate overall view of the miRNA expression and completes the analysis of the mature form, which could not be attributed to processing instead of new transcription.

As seen earlier, the miRNAs are derived from a longer primary miRNA which contains a hairpin cleaved by Drosha and becomes the precursor form. Then, this precursor form is exported to the cytoplasm by Exportin-5 and cleaved by Dicer to give a double-stranded RNA which contains the mature miRNA. Proteins involved in the maturation steps such as Drosha, Dicer or Exportin-5 can represent possible limiting factor in the miRNA biosynthesis pathway (Yi *et al.*, 2003). In such a case, the precursor form is stuck in the nucleus and cannot be matured in the cytoplasm. This is why a quantification of only the mature form does not give all of the information about the biogenesis processing of the miRNA. Thus, in addition to the quantification of the mature form, a quantification of the precursor and primary forms could indicate whether the processing is normal or if some step of the biogenesis is problematic or abnormal.

Recent studies showed that some miRNAs present specific deregulated expression in cancerous tissues. The majority show dysregulation of the expression of the mature forms, but one study also quantified the precursor forms. The results showed that pre-miR-143 and pre-miR-145 are expressed in both colorectal tumors and normal colorectal tissues while the mature forms of these two miRNAs are only expressed in healthy tissues (Michael *et al.*, 2003). Therefore, the absence of miR-143 and miR-145 in cancerous tissues suggests they are possibly involved in colorectal cancer genesis, acting like tumor suppressors. Moreover, in oesophageal cancer, the mature-to-precursor ratios of miR-21, miR-143, miR-145 and miR-205 are higher in cancerous cells than in adjacent healthy tissues where the ratios show equal concentrations (Akagi *et al.*, 2011). These results indicate that the dys-regulation of some miRNA may be correlated with abnormal gene expression which leads to diseases.

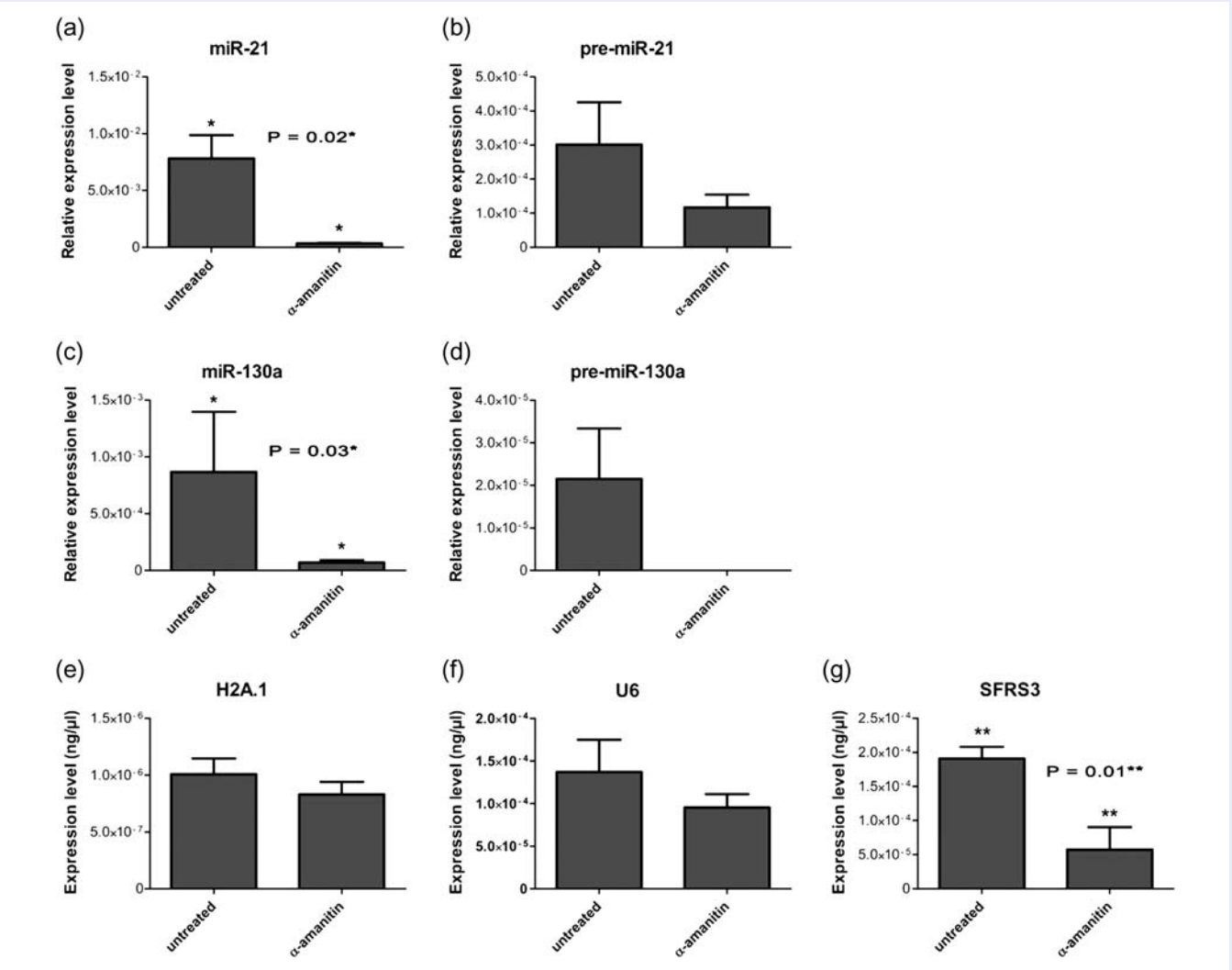

Figure 3 Expression profiles of (**a**) miR-21, (**b**) pre-miR-21, (**c**) miR-130a, (**d**) pre-miR-130a and of the control genes, (**e**) H2A.1, (**f**) U6 and (**g**) SFRS3 in bovine 2-cell embryos treated with α-amanitin, compared with untreated 2-cell embryos, as revealed by qRT–PCR quantifications. Results showed significant diminution of miR-21 and miR-130a in embryos treated with α-amanitin ($P < 0.02$ and 0.03, respectively). As expected, control genes H2A.1 and U6 did not change with the treatment and SFRS3 decreases very significantly ($P < 0.01$).

In our study, the quantification of the precursor as well as the mature forms of the two miRNAs selected serves to validate the profiles obtained. Our results show a concordant correlation for the pre-miR-130a which can validate the mature profile of miR-130a. For miR-21, the results highlight a significant augmentation of the precursor form between the MII stage and the 2-cell embryo, which could be associated with the hypothetical minor transcription observed at that stage in bovine embryos (Memili *et al.*, 1998). This augmentation is interesting because there is no other known transcription at the bovine 2-cell embryo stage (Kanka *et al.*, 2009). Therefore, the results obtained indicate temporal processing of the miRNA in the primary, precursor and mature forms.

As seen in Fig. 2, ratios of mature-to-precursor forms indicate that for miR-21, the mature form is always present at a higher concentration than the precursor form, while in the case of miR-130a, the mature form is higher than the precursor form at all stages except at the GV and blastocyst stages where they are in equal concentrations. In cancerous tissues, dysregulation and changing ratios of miRNAs could be caused by up- or down-regulation of some factors involved in the miRNA biogenesis machinery such as Drosha and Exportin-5 (Volk and Shomron, 2011). Based on the results obtained for the expression of Drosha, this factor is likely to be present in bovine embryos and support the observed rapid transition from the pre to mature forms.

Meanwhile, the unique conditions associated with transcriptional arrest might involve higher needs for gene regulation. Thus, in addition to the increase in mature and precursor forms at the 2-cell embryo, the increasing ratios suggest a pronounced processing of miR-130a and miR-21 during the MET to meet the higher needs of maternal mRNAs regulation.

We used α-amanitin on 2-cell embryos to assess the impact of RNA polymerase II inhibition on miRNA expression. This arrest of

transcription will possibly allow linking the significant augmentation of miR-21 and the hypothetical minor transcription. Experiments showed that miR-21 and miR-130a are sensitive to the treatment and suggest *de novo* transcription of these transcripts before the major EGA. This minor transcription was previously detected as a weak signal in the bovine zygote or in the 2-cell embryo (Hyttel *et al.*, 1996; Viuff *et al.*, 1996; Memili *et al.*, 1998). In cows, there is only one gene known to be transcribed at this potential minor activation: SFRS3, which is involved in nucleocytoplasmic export and in polyadenylation. This gene product is sensitive to α-amanitin treatment at the 4- and 8-cell stages (Kanka *et al.*, 2009). Our results show that SFRS3 gene was also sensitive to α-amanitin in the 2-cell embryo. These important results are quite surprising and support the presence of minor transcriptional activity in the early bovine embryo.

Previous studies in mouse suggest the possibility that miRNAs could not have such a relevant role in oocyte mRNA regulation (Tang *et al.*, 2007). Indeed, expression levels of miRNAs seem to be more important in the preimplantation embryos, around the MET rather than at the maturing oocyte stages. Meanwhile, different expression profiles have been obtained for other miRNAs during early development. Some of them showed higher levels in the oocyte while others showed a very weak expression level throughout early development and an augmentation only at the blastocyst stage (Tripurani *et al.*, 2010). These various results could suggest specific spatial and temporal actions of miRNAs, as well as different origins, i.e. maternal or embryonic.

In this study, we used a genome-wide screen in order to select miRNA gene targets. We were able to identify 391 targets for miR-21 and 619 for miR-130a that (i) perfect matches to the seed region of the miRNA in their 3′UTR, (ii) have conservation of the targeted sites among five mammal species and (iii) are expressed in the cells of interest (oocytes or embryos). Using TargetScan 22 out of the 24 candidates and 66 out of the 77 candidates with perfect conservation of the seed region target among the five selected mammals identified as bovine mir-21 and mir-130a targets, respectively. Moreover, some of them are described as miRNA targets in human by the MicroCosm software. Thus, the mRNA targets appeared to be identified with high confidence. However, in this analysis, we did not seek to identify targets that may be imperfect matches to the seed region or whose target lies in the coding sequence or 5′UTR of the transcript. Neither did we consider targets which may pair with the 11 central bases of the miRNA or be indirect targets (Lewis *et al.*, 2005; Shin *et al.*, 2010). The list of candidates presented here is not exhaustive and relevant genes for the early embryo development targeted by one of these miRNAs may be missing. Nevertheless our strategy revealed numerous new putative miRNA-regulated genes.

In conclusion, our results indicate that the increasing expression of the quantified miRNAs is correlated with the maternal-to-zygotic transition. From these trends of expression, we suggest that miR-21 and miR-130a are involved in gene regulation during this critical period of early development. Moreover, the miRNA machinery could be more active during the MET to make up for pronounced needs for regulation, particularly of the maternal mRNAs which have to be degraded. This period brings special needs and conditions of gene regulation that can explain the up-regulation of some miRNAs and their higher precursor-to-mature ratios.

In addition, the increasing expression level of the immature forms of miR-21 (primary and precursor) at the 2-cell embryo, together with its sensitivity to α-amanitin treatment, indicates the possibility that they represent a portion of the minor transcriptional activation. This study also associates the two miRNAs which present such trends with their targets in the 3′UTR of maternal mRNAs, disclosing potential regulation pathways. Finally, as the number of studies which show the importance of miR-21 in several cellular pathways increases, attention must be paid to the implication of this miRNA in the development process.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/.

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Authors' roles

E.M. performed the embryo experiment and the PCR analysis; I.D. did the micro-array experiment; M.G. worked on the identification of targets for the miRNA; E.F. designed the bioinformatic analysis used to find the targets of mir-21 and mir-130; M.-A.S. designed the experiment and co-wrote the paper with E.M.

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Conflict of interest

None declared.

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