Title

Embryonic microRNAs are essential for bovine preimplantation embryo development

Journal

Proceedings of the National Academy of Sciences of the United States of America, 119(45)

ISSN

0027-8424

Authors

Paulson, Erika E
Fishman, Emily L
Ma, Jun
et al.

Publication Date

2022-11-08

DOI

10.1073/pnas.2212942119

Peer reviewed

PNAS RESEARCH ARTICLE | DEVELOPMENTAL BIOLOGY OPEN ACCESS

Embryonic microRNAs are essential for bovine preimplantation embryo development

Erika E. Paulson[a], Emily L. Fishman[a], Richard M. Schultz[b,c,1], and Pablo J. Ross[a,1]

Edited by Thomas Spencer, University of Missouri, Columbia, MO; received August 4, 2022; accepted September 26, 2022

MicroRNAs (miRNAs) are small, noncoding RNAs that regulate gene expression after transcription. miRNAs are present in transcriptionally quiescent full-grown oocytes and preimplantation embryos that display a low level of transcription prior to embryonic genome activation. The role of miRNAs, if any, in preimplantation development is not known. The temporal pattern of expression of miRNAs during bovine preimplantation development was determined by small RNA-sequencing using eggs and preimplantation embryos (1-cell, 2-cell, 4-cell, 8-cell, 16-cell, morula, and blastocyst). Embryos cultured in the presence of α-amanitin, which permitted the distinguishing of maternal miRNAs from embryonic miRNAs, indicated that embryonic miRNA expression was first detected at the two-cell stage but dramatically increased during the morula and blastocyst stages. Targeting *DGCR8* by a small-interfering RNA/morpholino approach revealed a role for miRNAs in the morula-to-blastocyst transition. Knockdown of DGCR8 not only inhibited expression of embryonically expressed miRNAs but also inhibited the morula-to-blastocyst transition. In addition, RNA-sequencing identified an increased relative abundance of messenger RNAs potentially targeted by embryonic miRNAs in DGCR8-knockdown embryos when compared with controls. Results from these experiments implicate an essential role for miRNAs in bovine preimplantation embryo development.

embryo | microRNA | DGCR8 | preimplantation development | bovine

Significance

This study assesses the role of embryonic microRNAs (miRNAs) in preimplantation embryo development using a bovine model, which is a more appropriate model to study the maternal-to-embryo transition during preimplantation development than the more commonly used rodent models. Using small RNA sequencing, a knockdown of embryonic miRNAs, and live embryo imaging, we demonstrate that embryonic miRNAs are essential for blastocyst development in bovine embryos. The results in this study are notably different from those observed in mouse and will likely be useful in future studies pursuing the role of specific miRNAs in preimplantation embryo development.

MicroRNAs (miRNAs) are small (~19 to 22 nt in length), noncoding RNAs that play important roles in nearly every cell type by regulating gene expression posttranscriptionally (1). Canonical miRNAs are initially transcribed by RNA polymerase II to form primary-miRNAs (pri-miRNAs), long stem-loop structures with single-stranded RNA extensions on both ends (2). Pri-miRNAs are recognized and cleaved in the nucleus by the microprocessor complex, comprised of the RNase III endonuclease Drosha and the double-stranded RNA-binding protein DGCR8, to form precursor-miRNAs (pre-miRNA) (~60 to 70 nt in length) (3). Pre-miRNAs are exported from the nucleus by Exportin-5, cleaved by Dicer, an RNase III endonuclease, resulting in a double-stranded miRNA duplex with 3′ overhangs (4, 5). The passenger strand is separated and released for degradation while the mature miRNA is bound by an Argonaute (AGO) protein to form the miRNA-induced silencing complex (miRISC) (6, 7). miRISC targets messenger RNA (mRNA) transcripts through Watson–Crick base-pairing between the miRNA and the transcript (8, 9). When base pairing is perfect and the miRNA is associated with AGO2, the transcript is cleaved by AGO2's endonuclease activity (7), essentially functioning as a small-interfering RNA (siRNA). In animals, however, miRNAs tend to bind imperfectly to the target mRNAs, and regardless of which AGO (AGO1-4) is associated with the miRNA, miRISC decreases expression of the mRNA target by translation inhibition, deadenylation, and 5′ to 3′ degradation of the mRNA (10, 11). Deadenylation and mRNA degradation are accomplished through AGO recruitment of a GW182/TNRC6 protein, which in turn recruits the CCR4-Not deadenylase complex, decapping proteins, and the 5′ -3′ exonuclease XRN1 (12–15).

Preimplantation development prior to major embryonic genome activation (EGA), which occurs in a species-specific manner (e.g., two-cell in mouse, four-cell in porcine, eight-cell in bovine, four- to eight-cell in human), occurs initially in the absence and then in the presence of a low level of transcription (16–19). This early phase of development is supported by maternally derived proteins and mRNAs. Associated with EGA is degradation of maternal mRNAs, which appears critical for continued development (20). Because miRNAs are important posttranscriptional negative regulators of gene expression, their presence in oocytes and embryos suggests a role in development. In zebrafish, *Xenopus*, and *Drosophila*, miRNAs are required to clear maternal products which is essential for successful embryonic development (21–23).

Author affiliations: [a]Department of Animal Science, University of California, Davis, CA 95616; [b]Department of Anatomy, Physiology, and Cell Biology, School of Veterinary Medicine, University of California, Davis, CA 95616; and [c]Department of Biology, University of Pennsylvania, Philadelphia, PA 19104

Author contributions: E.E.P., R.M.S., and P.J.R. designed research; E.E.P., E.L.F., and P.J.R. performed research; E.E.P., E.L.F., and P.J.R. analyzed data; and E.E.P., E.L.F., R.M.S., and P.J.R. wrote the paper.

Competing interest statement: P.J.R. is an employee of STgenetics.

This article is a PNAS Direct Submission.

[1]To whom correspondence may be addressed. Email: rschultz@sas.upenn.edu or pross@ucdavis.edu.

This article contains supporting information online at http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2212942119/-/DCSupplemental.

Published November 2, 2022.

A role for miRNAs in early mammalian development, however, is less clear. In mouse, DGCR8-deficient oocytes are devoid of miRNAs, as expected, but DGCR8-deficient oocytes mature normally, and following fertilization, are capable of developing to the blastocyst stage at an incidence similar to controls (24). miRNAs do not appear to function in mammalian oocytes (i.e., lead to mRNA degradation) because the transcriptome of DGCR8-deficient oocytes is essentially the same as wild-type oocytes (24), a finding consistent with the inability of endogenous oocyte miRNAs to target microinjected *Luc* mRNAs harboring miR-30 binding sites in the 3′UTR for translation inhibition or degradation (25).

Mouse maternal miRNAs initially constitute a small portion of the overall small RNA population, composed of miRNAs, endogenous-siRNAs, and PIWI-interacting RNAs, among others. Their low concentration may underlie their inability to target mRNAs harboring miRNA-binding sequences (26). miRNA abundance increases following EGA with miRNAs becoming the dominant class of small RNAs during preimplantation development and gaining the ability to target mRNAs (27, 28). The ability of miRNAs to target mRNAs appears to be important for mouse development because DGCR8$^{-/-}$ embryos die by embryonic day 6.5 (29).

Although mouse has historically been the model system to study preimplantation development, there is growing evidence that large animal models are more appropriate in terms of understanding human preimplantation development, likely because of the timing of critical transitions, such as major EGA. Such differences in timing could reflect differences in the reliance on maternally derived mRNAs to support the early cleavage stages and molecular mechanisms that govern the onset of EGA and the associated dramatic reprogramming of gene expression that is critical for continued development (30).

There is also growing evidence that miRNAs are critical for preimplantation development in larger species with delayed EGA, relative to mouse. In pig, for example, where EGA occurs during the four-cell stage (18, 31), suppression of miR-21 activity in parthenotes inhibits development beyond the four- to eight-cell stage (32). Reporter assays in bovine embryos indicate that miR-212 and miR-196a regulate FIGLA and NOBOX in eight-cell embryos, respectively (33, 34), and that miR-218 and miR-449b function in blastocysts to regulate pluripotency and cell differentiation markers, as determined by luciferase assays (35). Inhibiting miR-130b also results in a decreased incidence of development to the morula and blastocyst stages in bovine in vitro fertilization (IVF) embryos (36). Differential expression of miR-34a and miR-345 in bovine somatic cell nuclear transfer embryos compared with IVF embryos correlated with changes in predicted target transcripts at the morula stage (37, 38).

Although these studies indicate specific miRNA function during preimplantation embryo development, to date no study has addressed the global role of miRNAs in bovine preimplantation development. To this end, we first characterized expression of miRNAs during bovine preimplantation development from the egg to the blastocyst stages and then assessed miRNA function by knockdown (KD) of DGCR8 in preimplantation embryos, thereby inhibiting miRNA biogenesis. Results of these experiments indicate that miRNA biogenesis is associated with EGA and that miRNAs are essential for the morula to blastocyst transition.

Results

Small RNA-Sequencing Libraries. We first characterized the expression profile of small RNAs during bovine in vitro preimplantation development at metaphase II (MII) oocyte, zygote (1C), 2-cell (2C), 4-cell (4C), 8-cell (8C), 16-cell (16C), morula (M), and blastocyst (BL) stages using a recent method that requires a small number of embryos and is free from biases associated with ligation (39). To identify newly transcribed small RNAs, zygotes were cultured in the presence of α-amanitin and embryos collected at the 2-cell (2α), 4-cell (4α), and 8-cell (8α) stages. Libraries were sufficiently sequenced such that the average number of reads for each of the four replicates for each developmental stage ranged from 8.5 to 10.7 million reads (*SI Appendix*, Fig. S1*A*). Reads were mapped to the *Bos taurus* genome (ARS-UCD1.2.97), normalized to reads per kiobase per million (RPKM), and categorized by RNA type (Dataset S1). For each sample, >80% of the reads mapped to the genome (*SI Appendix*, Fig. S1*B*), with 60 to 90% of the annotated reads mapping to protein-coding regions (*SI Appendix*, Fig. S2*A*), and the rest distributed to noncoding RNA regions (*SI Appendix*, Fig. S2 *A–C*). The number of reads mapping to protein-coding regions exhibited a modest trend to decrease following EGA (*SI Appendix*, Fig. S1*A*). The documented decline in siRNAs during preimplantation development (27, 28) that map to coding regions could be a contributing factor to this trend. In the breakdown of reads mapping to noncoding RNAs, there was an apparent increase in the proportion of small RNAs, including miRNAs, small nuclear RNAs (snRNAs), and small nucleolar RNAs (snoRNAs) following EGA (*SI Appendix*, Fig. S1 *A* and *B*). Principle component analysis (PCA) based on genome mapping showed most samples had similar expression profiles, except for morula and blastocyst samples that exhibited a distinct profile (*SI Appendix*, Fig. S2*D*). Taken together, these results show an increase in many regulatory noncoding RNAs after EGA, including miRNAs.

Temporal Pattern of miRNA Expression during Bovine Preimplantation Development. Given the well-established role of miRNAs in posttranscriptionally regulating transcript abundance and the apparent increase in miRNAs following EGA, we focused our attention on this class of small RNAs. Many bovine miRNAs are not annotated in the most current version of the bovine genome, although their sequences are known and held in databases, such as miRBase. Accordingly, trimmed reads were also mapped to the *B. taurus* miRBase, which is the most up-to-date miRNA database, being more comprehensive for miRNA analysis than just mapping to the genome. A range of 28,000 to 85,000 reads were annotated in this analysis (*SI Appendix*, Fig. S3*A*). Although the number of annotated reads was similar across all stages, there was a large increase in the percentage of miRNA reads at the blastocyst stage (*SI Appendix*, Fig. S3*B*), presumably reflecting an increase in miRNA expression and a shift in the small RNA population toward miRNAs being the dominant class of small RNAs at this stage, similar to mouse (27). miRNA reads were normalized to reads per million (RPM), and miRNAs were only considered present at a specific stage if they were represented in at least three of the four replicates. The total number of miRNAs identified at each developmental stage ranged from 125 to 210 (*SI Appendix*, Fig. S3*C*), representing a total of 291 known *B. taurus* miRNAs (Dataset S2). Because small RNA-sequencing (RNA-seq) has potential biases introduced in the RNA extraction, library preparation, and amplification steps, we validated by qPCR expression of three embryonically expressed and one oocyte-expressed miRNA. For all miRNAs tested, qPCR fold-change closely matched the fold-change determined by RNA-seq (*SI Appendix*, Fig. S3*D*), which is consistent with the higher accuracy of the small RNA-seq methods used (39).

We next compared miRNA expression between each developmental stage and across development as a whole. Prior to EGA, PCA of miRNAs expressed in each of the 44 samples (four replicates per oocyte/embryo stage) showed a similar miRNA expression profile with a different expression pattern emerging after EGA, particularly in morula and in blastocysts (Fig. 1*A*), a finding consistent with embryonic expression of miRNAs following EGA. Likewise, visualization by heat map also suggested embryonic expression of miRNA commencing during EGA with a distinct population of miRNAs being expressed in morulae and blastocysts (Fig. 1*B*). A differential expression analysis between each consecutive stage identified specific miRNAs that were up-regulated or down-regulated ($P < 0.05$) during each transition with a greater number of down-regulated miRNAs than up-regulated miRNAs from the MII oocyte through eight-cell stage, before major EGA (Fig. 1*C*). About half of the two-cell and eight-cell and all of the four-cell up-regulated miRNAs were α-amanitin–sensitive, indicating their embryonic origin. Very few (<10%) of the down-regulated miRNAs in these stages were α-amanitin–sensitive (Fig. 1*C*). A total of 15 embryonic miRNAs were expressed prior to major EGA. After major EGA, there was a shift to increased miRNA expression, with the greatest increase observed during the morula-to-blastocyst transition. Overall, an increase in 84 miRNAs was observed from eight-cell to blastocyst. Taken together, these results indicate that a subset of miRNAs are expressed before major EGA, but become the dominant class of small RNA after major EGA, with significant up-regulation during the morula-to-blastocyst transition.

DGCR8-KD Embryos Have Decreased Expression of miRNAs. To assess the role of newly expressed miRNAs in bovine preimplantation development, in particular the morula-to-blastocyst transition, we employed an siRNA/morpholino (MO) approach to target DGCR8, a component of the microprocessor complex that is essential for biogenesis of canonical miRNAs. Prior to knocking down DGCR8, we profiled *DGCR8* transcript abundance during bovine preimplantation development (Fig. 2*A*). The expression profile was consistent with continuous degradation of maternally derived *DGCR8* transcript initiating during oocyte maturation, with embryonic expression clearly occurring during the 16-cell to morula stages.

To decrease miRNA abundance, DGCR8 was knocked down by microinjecting a combination of siRNA/MO targeting *DGCR8* mRNA 6 h after initiating IVF. We used a combined approach to maximize the likelihood of decreasing DGCR8 protein abundance; the MO would immediately inhibit translation of *DGCR8* mRNA, whereas the siRNA would decrease *DGCR8* transcript abundance over time.

DGCR8 mRNA was significantly decreased by day 2 (four-cell stage) of development compared with control, noninjected embryos, and embryos injected with control siRNA/MO (control-injected) with the knockdown maintained, although to a lesser degree, through day 5 (morula stage) of development (Fig. 2*B*). As anticipated and serving as another control, the abundance of *DGCR8* mRNA was not affected when embryos were injected with only the *DGCR8*-targeting MO (Fig. 2*B*). The combined microinjection approach revealed that DGCR8 protein was significantly reduced on day 5 of development compared with control embryos and control-injected embryos, as measured by immunofluorescence (Fig. 2*C*). The extent of the decrease in the amount of DGCR8 protein was sufficient to perturb miRNA biogenesis because expression of embryonically expressed miR-371 and miR-7 was markedly decreased (>90%) by day 5 of development (Fig. 2*D*). These miRNAs were selected from small RNA-seq results as highly expressed

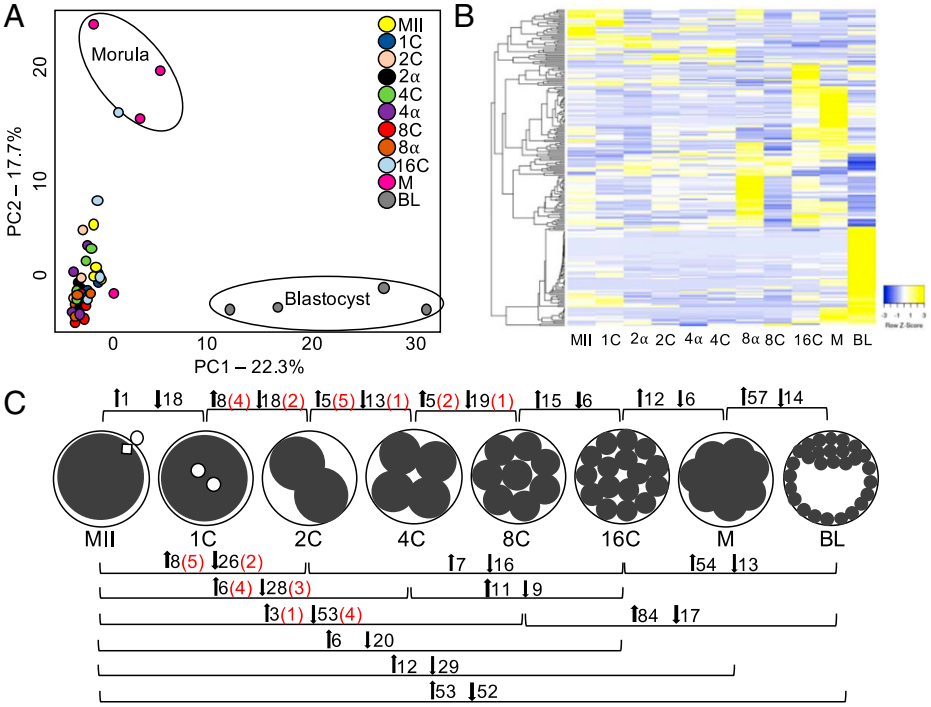


Fig. 1. Differential expression of miRNAs during bovine preimplantation development. (*A*) PCA of all 44 small RNA libraries (four replicates per oocyte/embryo stage) based on miRNA expression alone (color key top right). (*B*) Pearson correlation heat map of miRNA expression across developmental stages. (*C*) Number of up-regulated and down-regulated miRNAs in each cleavage transition, represented by black numbers next to up or down arrows, respectively. Red numbers in parenthesis next to up-regulated or down-regulated miRNA number represents the number of those miRNAs that are α-amanitin sensitive. Metaphase-II egg (MII), zygote (1C), 2-cell embryo treated with α-amanitin (2α), 2-cell embryo (2C), 4-cell embryo treated with α-amanitin (4α), 4-cell embryo (4C), 8-cell embryo treated with α-amanitin (8α), 8-cell embryo (8C), 16-cell embryo (16C), morula (M), and blastocyst (BL).

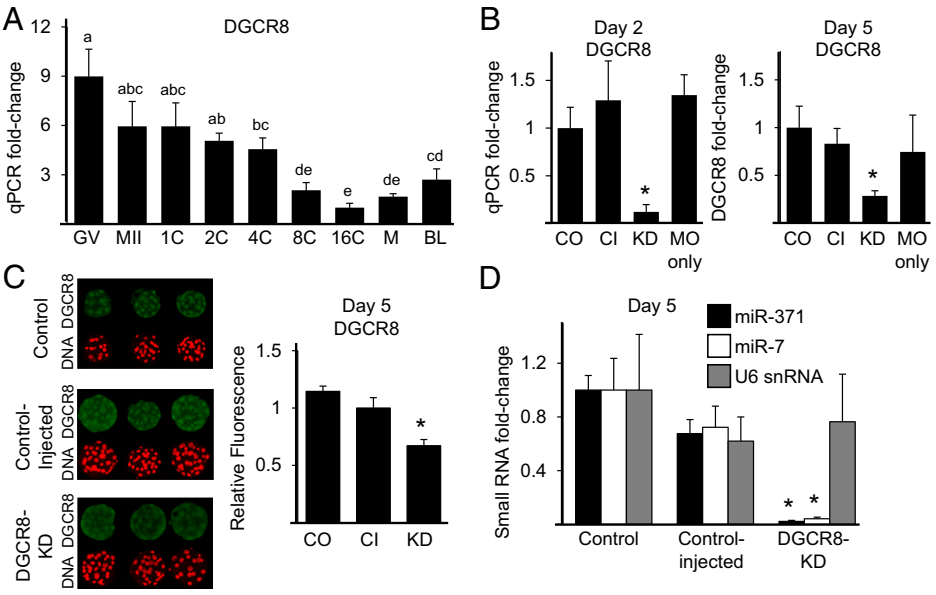


Fig. 2. Knockdown of DGCR8 in bovine embryos by siRNA/MO microinjection. (*A*) Relative *DGCR8* mRNA levels measured by qPCR across bovine preimplantation development. Four biological replicates were used with 10 embryos each. Different superscript letters (^{abcde}) indicate statistically different groups ($P < 0.05$). (*B*) Relative *DGCR8* mRNA levels measured by qPCR on day 2 (*Left*) and day 5 (*Right*) of embryo development in control embryos (CO), control-injected embryos (CI), embryos injected with *DGCR8*-targeting siRNA and MO, and embryos injected with *DGCR8*-targeting MO only (MO only). Four biological replicates were used with 20 embryos each. (*C*) Relative DGCR8 protein by immunocytochemistry in CO, CI, and KD embryos. Representative images (40X magnification) are shown (*Left*) and relative fluorescent intensity (*Right*). Three biological replicates were used with 20 embryos each. The KD group differs significantly from the CO and CI groups ($P < 0.05$). (*D*) Quantification of two embryonically expressed canonical miRNAs (bta-miR-371 and bta-miR-7) by qPCR on day 5 of embryo development in CO, CI, and KD embryos. Small, noncoding RNA U6 (U6 snRNA) was used as a negative control. Four biological replicates were used with 20 embryos each. Error bars represent SE. *$P < 0.05$.

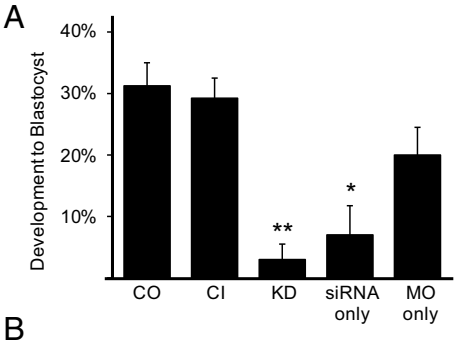
EGA-specific miRNAs. This decrease in miRNA abundance was due to decreased abundance of DGCR8 because no decrease in abundance of the small noncoding RNA U6 was observed; biogenesis of U6 is DGCR8-independent. Together, these results show that knockdown of DGCR8 results in a decrease in embryonic miRNA abundance by day 5 of development.

Loss of Embryonically Expressed Canonical miRNAs Causes Developmental Arrest at the Morula Stage. The dramatic increase in miRNA expression during the morula-to-blastocyst transition suggests a potential role for miRNAs in this transition. To determine whether such is the case, DGCR8-KD embryos were cultured for 8 d to the blastocyst stage and the percentage of embryos that developed to blastocyst determined. Compared with control embryos and control-injected embryos, DGCR8-KD embryos had a significantly lower percentage of embryos develop to the blastocyst stage (Fig. 3*A*). For all experiments, groups of embryos were injected with either an siRNA or MO only targeting DGCR8, or together in combination. Both the MO alone or siRNA alone decreased the incidence of blastocyst formation, but the greatest decrease in development occurred when the siRNA and MO were used in combination ($P < 0.00001$).

The decreased incidence of development to the blastocyst stage could not be attributed to a developmental delay because, in some experiments, embryos were cultured longer but no increase incidence of blastocyst formation was observed. In addition, rescue experiments indicate the observed compromised development was unlikely due to off-targeting effects. Zygotes were microinjected with control MO and siRNA (Control), *DGCR8*-targetting MO and siRNA (DGCR8 KD), or *DGCR8*-targeting MO and siRNA and *DGCR8* copy RNA (cRNA; Rescue). Development was then monitored using live-imaging. Of the injected Control (total: 41), *DGCR8* KD (total: 65), and Rescue (total: 68) that cleaved to the two-cell stage, 37, 38, and 38, respectively, developed to the eight-cell stage, of which 23, 1, and 11, respectively, developed to the blastocyst stage. The observed rescue of 11 of 38 (29%) vs. 1 of 38 (3%) is significant ($P < 0.01$, χ^2) and the extent of rescue compared favorably with controls, 23 of 37 (62%). In toto, these results suggest that embryonic miRNAs are required for bovine embryo development to the blastocyst stage.

To determine whether the decreased incidence in development to the blastocyst stage was stage-specific, time-lapse imaging of control embryos, control-injected embryos, and DGCR8-KD embryos was conducted for 8 d with images taken every 5 min. DGCR8-KD embryos cleaved, on average, at the same times and same incidence to the 2-cell, 4-cell, 8-cell, 16-cell, and morula stages as control embryos and control-injected embryos (Fig. 3*B*). Strikingly, although the morulae looked similar morphologically in all three groups, only 10% of the DGCR8-KD morula formed blastocysts, whereas 83% of the control morula formed blastocysts (Fig. 3*C*). The number of embryos that reached the morula stage by day 5 compared with the total embryos cultured was not different between the three groups (Fig. 3*B*). Additionally, DGCR8-KD morulae underwent compaction like control embryos, but then underwent what appeared to be cell death, expelling dead cells (Movies S1–S3). Taken together, these results indicate that embryonic miRNAs are necessary for the morula-to-blastocyst transition.

Differentially Expressed Genes in DGCR8-KD Embryos Reflect Developmental Arrest. To identify gene-expression changes linked to the morula-stage developmental arrest in DGCR8-KD embryos, RNA-seq was conducted on pools of control embryos, control-injected embryos, and DGCR8-KD embryos on day 3 and day 5 of development, representing 8-cell/16-cell and morula stage embryos, respectively. Day 3 was chosen to capture expression at the beginning of major EGA, and day 5 chosen to capture expression prior to the observed developmental arrest in *DGCR8*-KD embryos. An average of 14.5 to 19.8 million reads



B

	Starting # of Embryos	2-cell		4-cell		8-cell		16-cell		Morula		Blastocyst	
		%	Time (h)	%	Time (h)	%	Time (h)	%	Time (h)	%	Time (h)	%	Time (h)
Control embryos	27	52	30 ±2.0	44	36 ±1.2	41	46 ±1.3	37	86 ±6.3	37	116 ±2.6	26	151 ±3.3
Control-injected embryos	33	52	30 ±1.1	52	41 ±1.7	39	51 ±4.1	33	91 ±7.1	30	108 ±3.1	24	155 ±3.7
DGCR8-KD embryos	42	52	31 ±0.9	52	38 ±1.2	48	53 ±4.4	45	91 ±5.2	38	119 ±2.6	5	185 ±3.0

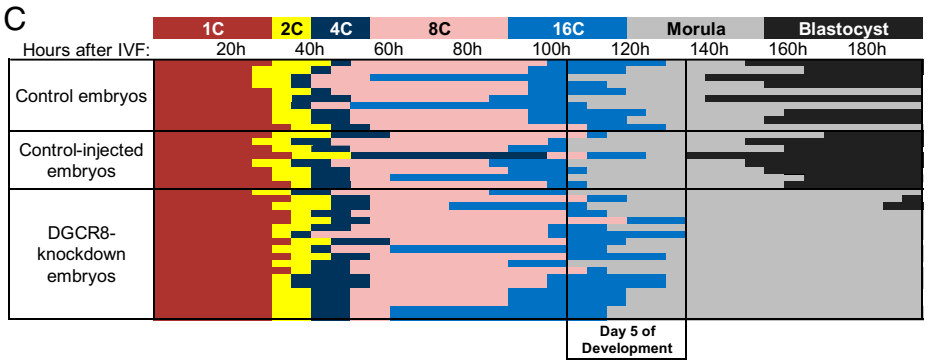


Fig. 3. Loss of embryonically expressed canonical miRNAs causes developmental arrest at morula stage. (*A*) Percentage of embryos that developed to blastocyst in control embryos (CO), control-injected embryos (CI), embryos injected with *DGCR8*-targeting siRNA and MO (KD), embryos injected with *DGCR8*-targeting siRNA only (siRNA only), and embryos injected with DGCR8-targeting MO only (MO only). Eleven biological replicates were used, with at least 50 embryos per group. Data are presented as mean ± SEM *$P < 0.01$, **$P < 0.00001$. (*B*) Time-lapse data of control embryos, control-injected embryos, or *DGCR8*-KD embryos cultured in Esco Medical Miri time-lapse incubator for 8 d after IVF. Percentage of embryos that cleaved to each stage of the total embryos cultured. Average time of cleavage of the embryos that developed to that particular stage presented as mean ± SEM. (*C*) Time-lapse data of control embryos, control-injected embryos, or *DGCR8*-KD embryos that formed a morula by the end of day 5 cultured in Esco Medical Miri time-lapse incubator for 8 d after IVF. Colors indicate cleavage stage. Change of colors from one to the next indicates cleavage. The *x* axis is time in hours after IVF. Data were collected during six time-lapse runs.

in each group were used for analysis (*SI Appendix*, Fig. S4*A*). Samples were mapped to the most recent bovine genome and >90% of reads mapped in each sample (*SI Appendix*, Fig. S4*B*). PCA showed differences in expression between day 3 and day 5 samples (Fig. 4*A*). Whereas day 3 samples were mostly similar, regardless of treatment, day 5 DGCR8-KD embryos, which undergo developmental arrest at day 5, showed, as anticipated, a unique expression profile compared with control groups.

To identify the number of transcripts whose relative abundance was affected by loss of embryonic miRNAs, differential expression analysis between groups was conducted using a false-discovery rate (FDR) $P < 0.05$, fold-change > 2, and average RPKM > 0.4 in at least one group (Dataset S3). There were very few differences between control and control-injected groups, with only 6 up-regulated genes and 5 down-regulated genes in day 3 control-injected embryos compared with controls, and only 2 up-regulated genes and 40 down-regulated genes in day 5 control-injected embryos compared with controls (Fig. 4*B* and *SI Appendix*, Fig. S4 *C* and *D*). The low number of differentially expressed genes between control and control-injected embryos provided confidence for comparing both control groups against DGCR8-KD embryos (Fig. 4*B*). Compared with control groups, there were 121 differentially expressed genes in day 3 DGCR8-KD embryos, nearly all of which (112 of 121) were up-regulated in the DGCR8-KD embryos (Fig. 4*C*). An increase in transcript abundance in DGCR8-KD embryos was anticipated given that miRNAs negatively regulate transcript abundance. Up-regulated genes were enriched for pathways related to acute phase response, endodermal cell differentiation, cell-substrate junction assembly, and cell redox homeostasis (Dataset S1). By day 5 of development, there were 494 differentially expressed genes between DGCR8-KD embryos and control groups, 141 up-regulated in the DGCR8-KD embryos, and 353 down-regulated (Fig. 4*D*). The up-regulated genes showed enrichment for protein-related processes, including ubiquitination and endoplasmic reticulum function (*SI Appendix*, Table S1). The down-regulated genes showed enrichment for redox processes and response to reactive oxygen species, spreading of epidermal cells and cell response to growth factors, immune response, proteolysis, neural tube closure, positive regulation of ERK1/2 cascade, and most notably, negative regulation of apoptotic processes (*SI Appendix*, Table S1). *DGCR8* was, expectedly, one of the down-regulated genes (*SI Appendix*, Fig. S5).

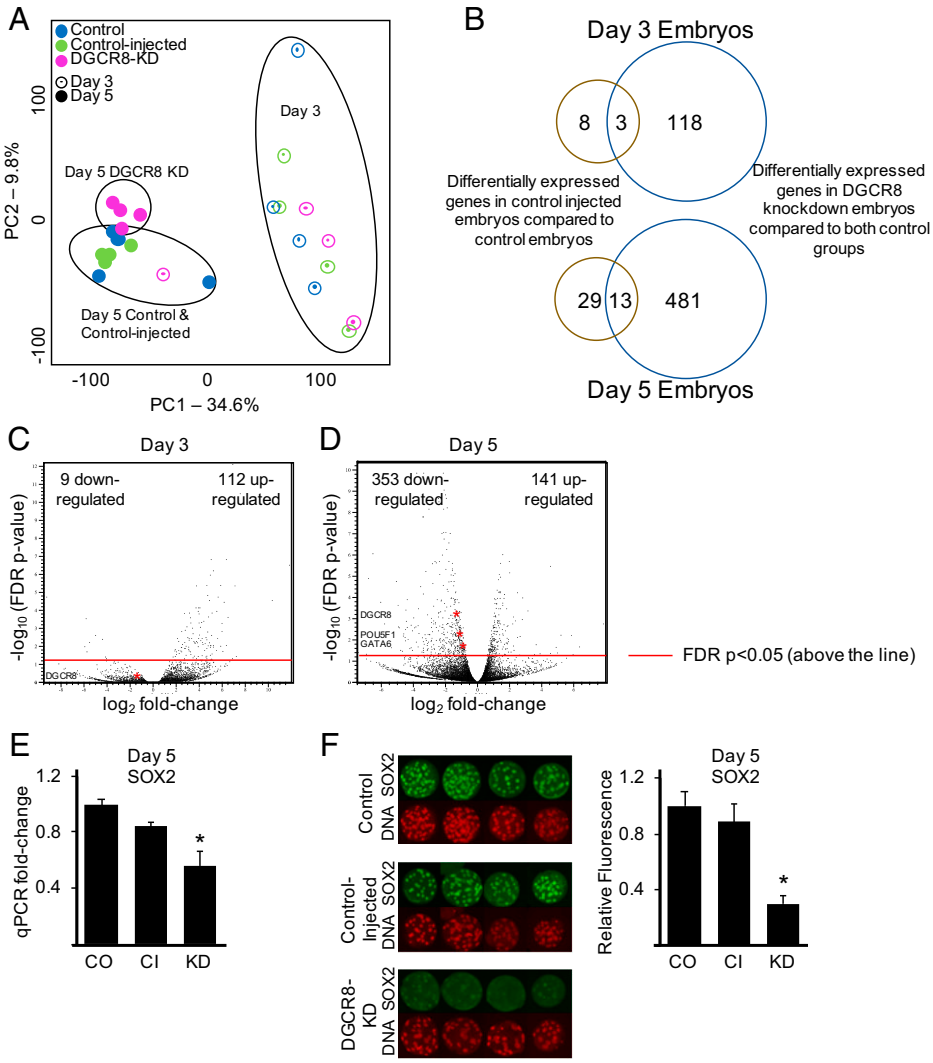


Fig. 4. DGCR8-knockdown alters global transcriptome of bovine embryos. (*A*) PCA graph of all 24 libraries. Samples group by day they were collected, although day 5 DGCR8-KD embryos tend to separate from day 5 control and control-injected embryos. PCA key (*Top Left*) indicates which color represents which treatment and which circle type represents which day samples were collected. (*B*) Venn diagram identifying overlap of differentially expressed genes in control-injected versus control embryos (yellow) and DGCR8-KD embryos versus both control groups combined (blue) on day 3 (*Upper*) and day 5 (*Lower*) of development. (*C*) Volcano plot of differentially expressed genes in day 3 DGCR8-KD embryos compared with controls. Each point represents a single gene, and points above the red line were considered significant (FDR $P < 0.05$). (*D*) Volcano plot of differentially expressed genes in day 5 *DGCR8*-KD embryos compared with controls. Each point represents a single gene and points above the red line were considered significant (FDR $P < 0.05$). Four biological replicates were used for developmental stage, and red stars represent notable genes listed to the left. (*E*) Relative *SOX2* mRNA levels measured by qPCR on day 5 of embryo development in control embryos (CO), control-injected embryos (CI), and embryos injected with *DGCR8*-targeting siRNA and MO (KD). Three biological replicates were used with 20 embryos each. (*F*) Relative SOX2 protein by immunocytochemistry in CO, CI, and KD embryos. Representative images (40X magnification) (*Left*) and relative fluorescent intensity (*Right*). Three biological replicates were used with 10 embryos each. *$P < 0.05$.

Additionally, a few known blastocyst markers were down-regulated in day 5 DGCR8-KD embryos compared with controls, most notably POU5F1 and GATA6 (Fig. 4*D* and *SI Appendix*, Figs. S6 and S7). SOX2 is a known marker of blastocyst development in bovine, although the gene is not included in the genome annotation. The location of the gene, however, is known, and the number of reads at that locus in DGCR8-KD embryos appeared lower than in control and control-injected embryos (*SI Appendix*, Fig. S8). Both qPCR and immunocytochemistry also showed a decrease in SOX2 in day 5 DGCR8-KD embryos compared with controls (Fig. 4 *E* and *F*). Differential expression of genes—such as POU5F1, GATA6, and SOX2—may underlie the developmental arrest observed in DGCR8-KD embryos.

Embryonic miRNAs Are Likely Not Involved in Maternal Transcript Degradation. Embryonic miRNAs are essential for degradation of maternal transcripts during EGA in nonmammalian species (21–23), and previous studies indicated a potential similar role for miRNAs during bovine EGA (33, 34). Our study indicated that 112 transcripts were up-regulated in day 3 embryos when embryonic miRNAs were eliminated. To determine the origin of the 112 up-regulated gene transcripts, RNA-seq data from a previous study (40) were run through the same pipeline as RNA-seq data in this study and used as a comparison. To determine which maternal transcripts are cleared by day 3 of development in bovine embryos, we included samples from MII oocytes, control day 3 embryos, and day 3 embryos treated with α-amanitin. Of the 112 up-regulated genes in day 3 DGCR8-KD embryos compared with controls, only 17 were considered maternal products that were cleared by day 3 of development, (Fig. 5). In contrast, 41 of the 112 up-regulated genes were considered to be embryonically expressed at EGA. It is therefore more likely that embryonic miRNAs are modulating embryonic gene expression during EGA, rather than playing a major role, if

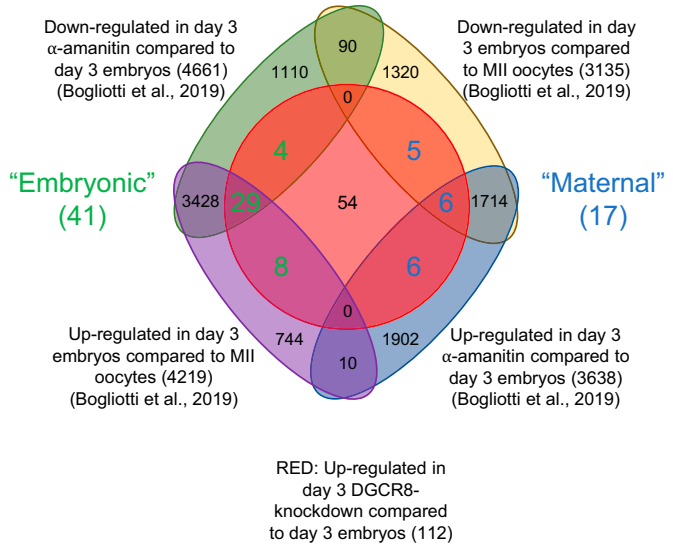


Fig. 5. Venn diagram analysis of gene expression in day 3 DGCR8-KD embryos. Venn diagram representing overlap of up-regulated genes in day 3 DGCR8-KD embryos (red) with up-regulated genes in day 3 α-amanitin–treated embryos compared with controls (blue), down-regulated genes in day 3 embryos compared with MII oocytes (yellow), day 3 α-amanitin–sensitive genes (green), and up-regulated genes in day 3 embryos compared with MII oocytes (purple). Overlap with up-regulated genes in day 3 embryos compared with MII oocytes and down-regulated genes in day 3 α-amanitin compared with controls indicates "embryonic" origin, while overlap with down-regulated genes in day 3 embryos compared with MII oocytes and up-regulated genes in day 3 α-amanitin compared with controls indicates "maternal" origin. See Bogliotti et al. (40).

any, in degradation of maternal transcripts leading up to and during EGA in bovine preimplantation development.

Differentially Expressed Transcripts Are Putative Targets of Embryonic miRNAs. To ascertain whether loss of embryonic miRNAs was linked to increased transcript abundance observed in day 3 and day 5 DGCR8-KD embryos, miRNAs identified in small RNA-seq experiments were analyzed using TargetScan for potential targets (41). Because there was no overlap of up-regulated genes between day 3 and day 5 of DGCR8-KD embryos, target prediction was independently performed on day 3 or day 5 miRNAs and predicted targets were compared with day 3 or day 5 up-regulated genes, respectively. To limit the number of targets, only miRNAs with an average expression >15 RPM were used for target prediction, and only targets with a cumulative weighted context ++ score <−0.35 were considered against up-regulated genes. There were 26 highly expressed miRNAs representing 22 miRNA families in day 3 embryos used for target prediction, which together, had 2,588 predicted targets. From these targets, there was overlap of 11 of the 101 genes up-regulated in day 3 DGCR8-KD embryos (11 of the initial 112 were unannotated and therefore cannot be compared as targets) (*SI Appendix*, Table S2). For day 5 analysis, there were 31 highly expressed miRNAs representing 26 miRNA families. From these miRNAs, a total of 2,788 targets were predicted, which matched to 30 of the 127 up-regulated genes in day 5 DGCR8-KD embryos (14 of the initial 141 unannotated, and therefore cannot be compared as targets) (*SI Appendix*, Table S3). Interestingly, 10 of the overlapping targets were predicted by the miR-17-5p/20/93/106 family, from miR-17-5p and miR-93. Together, these comparisons show loss of embryonic miRNAs is likely to contribute, at least in part, to the increased abundance for some transcripts in DGCR8-KD embryos. Lack of sufficient prediction technologies or indirect effects of the loss of miRNAs in bovine embryos could account for the remainder of the observed changes.

A similar analysis was performed to ascertain a potential role for the loss of embryonic miRNAs in the observed developmental arrest of DGCR8-KD embryos; miRNAs that were highly expressed in the blastocyst were used for target prediction. Targets of the analyzed miRNAs were then used for gene ontology enrichment analysis, to determine if there were certain cellular functions or pathways that could be most affected by loss of these miRNAs. Additionally, because there were so many uniquely expressed miRNAs in the blastocyst stage, stringency was increased to a weighted context ++ score <−0.7. Using these parameters, 935 unique targets were identified by 73 miRNAs. Gene ontology for these targets showed enrichment ($P < 0.05$) for 54 terms, the most significant being RNA-polymerase II transcription, cell cycle and cell maturation, and most notably, stem cell differentiation (*SI Appendix*, Table S4). These results implicate the embryonic miRNAs in bovine blastocyst formation and survival.

Discussion

By targeting *DGCR8* using a combined siRNA/MO approach that not only results in decreased *DGCR8* mRNA and DGCR8 protein levels but also abolishes expression of miRNAs, we report here that embryonic miRNAs are required for successful development to the blastocyst stage in cattle with developmental arrest at the morula stage. This finding is in contrast to mouse, where embryos depleted of embryonic-DGCR8 develop at essentially a normal incidence to the blastocyst stage in vitro (24), and could even survive to gastrulation in vivo (29). Although biogenesis of some miRNAs does not require DGCR8, these miRNAs are expressed at very low levels in embryonic stem cells, and are therefore unlikely to play a significant role in regulation of gene expression during preimplantation development (42).

Although the primary function of DGCR8 is miRNA biogenesis, DGCR8 is involved in other processes [e.g., mediating UV-induced DNA damage repair (43), regulating mRNAs, snoRNAs, and long intergenic non-coding RNAs (lincRNA) (44)]. In addition, DGCR8 can associate with other nucleases besides Drosha to affect cleavage of double-stranded RNAs (45). It is formally possible that our observations are not solely miRNA-mediated and due to an alternative function of DGCR8. This possibility is most unlikely because comparison of highly expressed embryonic miRNAs with up-regulated mRNA transcripts in DGCR8-KD embryos shows enrichment for seed-sequence matches.

Previous studies have employed small RNA-seq to identify small RNAs in bovine oocytes and embryos (46, 47). In contrast to the present study, these studies examined only a few developmental stages. Furthermore, these studies used methods that included adapter-ligation steps, which can introduce biases. We used a ligation-free library preparation method that, although shown to be less efficient, is more accurate in correctly detecting known spike-in miRNAs (39). We find that <1% of reads mapped to the *B. taurus* miRBase in nearly all of our samples, consistent with the lower efficiency of library preparation, but high accuracy in expression patterns when compared with qPCR results. Although miRNAs are present across all stages, the greatest increase in uniquely expressed miRNAs is after EGA, with the highest level of expression in the blastocyst, a finding consistent with what is observed in mice (27). This increase in embryonic miRNAs is also mirrored by increased *DGCR8* transcript abundance after EGA. Thus, embryonic expression of miRNAs after EGA is likely evolutionarily

conserved in mammals. Although miRNAs are present in mammalian oocytes, they do not repress their mRNA targets, and in particular, do not lead to mRNA degradation (24, 25). This lack of function is likely a consequence that miRNA concentration is not sufficient to target mRNAs and the large number of maternal mRNAs present in full-grown oocytes (26). The reductive cleavage divisions that occur during preimplantation development, coupled with embryonic miRNA expression, presumably results in an increase in miRNA concentration sufficient to target mRNAs. Consistent with this proposal is that embryonic miRNAs can target and decrease transcript abundance in bovine preimplantation embryos as early as the 8-cell/16-cell stage and through to the blastocyst stage (33–35). This proposal is also consistent with our data indicating that miRNAs do not play a role in clearing maternal mRNAs prior to EGA, noting that in zebrafish and frogs, embryonic miRNAs are essential to clear maternal mRNAs (22, 23). Our data suggest that such a role is absent for embryonic miRNAs in degradation of maternal mRNAs during bovine preimplantation development.

A blastocyst is comprised of an outer layer of trophectoderm cells, which gives rise to the placenta, and an inner group of pluripotent inner cell mass (ICM) cells, which gives rise to the embryo proper and extraembryonic tissues. Blastocyst formation is well characterized in cattle with several key transcription factors involved in ICM and trophectoderm formation: namely, POU5F1 (48), NANOG, GATA6, HNFA, SOX17, and CDX2 (49). We find that POU5F1 and GATA6 are down-regulated in day 5 DGCR8-KD embryos. Our results also indicate that SOX2, another key ICM marker for pluripotency in bovine blastocysts (50), is down-regulated in DGCR8-KD embryos. Taken together, these results suggest the developmental arrest at the morula stage in DGCR8-KD embryos could be due to loss of key transcription factors required for blastocyst formation, likely an indirect effect of the loss of embryonic miRNAs. Two other noteworthy down-regulated genes in day 5 DGCR8-KD embryos are CAPN2, which when deleted in mouse embryos results in arrest at the morula stage (51), and SPIC, which is expressed in and required for ICM formation in mouse (52). Also, inhibiting miR-130b function decreases the incidence of morula and blastocyst formation in bovine (36). We find that miR-130b is significantly up-regulated during the morula and blastocysts transition, consistent with a role for miR-130b in the transition and why DGCR8-KD embryos arrest at the morula stage. Finally, a recent study documented an important role of embryonic miR-378a-3p on bovine blastocyst formation and hatching (53).

In conclusion, embryonic miRNAs are required for bovine preimplantation embryo development to the blastocyst stage. Embryonic miRNAs likely regulate embryonic transcripts, beginning at EGA, and most extensively during the morula-to-blastocyst transition.

Materials and Methods

Materials were purchased from Sigma-Aldrich, unless otherwise stated.

Generation of In Vitro Embryos. For all experiments, ovaries were collected at Cargill Meat Solutions, a commercial slaughterhouse in Fresno, California, and transported to the laboratory in a sterile, warm saline solution (37 °C, 8.5 g/L NaCl). A range of ∼40 to 200 ovaries were used for each experiment, depending on the number of oocytes and embryos required. Follicles 2 to 8 mm in diameter were aspirated using a 21-gauge needle, to collect an average of five to eight cumulus-oocyte complexes (COCs) per ovary. COCs were washed multiple times in a collection medium (60% M199, 40% H-SOF, 2% fetal bovine serum [FBS]), and matured for 20 to 22 h at 38.5 °C and 5% CO_2 in M199 medium supplemented with 10% FBS (HyClone), 0.1 mM alanyl-glutamine, 0.2 mM sodium pyruvate, 5 μg/mL gentamicin (Gibco), 50 ng/mL human epidermal growth factor, 50 ng/mL ovine follicle-stimulating hormone (FSH; NHPP), 3 μg/mL bovine luteinizing hormone (LH; Sioux Biochemical), and 0.1 mM cysteamine hydrochloride, or in a commercial bovine oocyte maturation medium (BO-IVM, IVF Biosciences). Matured oocytes were used for IVF using frozen semen, prepared using Tyrode's albumin lactate pyruvate-based medium (54). Semen from a single *B. taurus* bull with proven fertility was provided by Semex. After 6 h (microinjected embryo experiments) or 16 h (nonmicroinjected embryo experiments), IVF embryos were denuded from cumulus cells by vortexing for 5 min in H-SOF and cultured up to 8 d in either KSOMaa medium (Life Global) supplemented with BSA (4 mg/mL) or in commercial in vitro culture medium (BO-IVC, IVF Biosciences) at 38.5 °C and 5% CO_2, 5% O_2, and 0% N_2. Culture medium was supplemented with 5% FBS after 3 d in culture. For α-amanitin treatment groups, IVF embryos were cultured for 56 h in the same culture medium as controls, but supplemented with 50 μg/mL α-amanitin. For time-lapse experiments, embryos were cultured in an Esco Medical Miri Time Lapse incubator (Esco Technologies), with images taken every 5 min.

Collection of Oocytes and Embryos. For molecular analyses, oocytes and embryos were washed twice through H-SOF and twice through PBS with 0.1% PVA, placed in a PCR tube with minimal media, snap frozen in liquid nitrogen, and stored at −80 °C until required. Germinal vesicle (GV) oocytes were denuded and collected immediately after aspiration from ovaries, MII oocytes were denuded and collected after 24 h of maturation, and zygotes (1C) were denuded and collected 16 h after IVF. The other stages were collected at the following times post-IVF: 2C and 2α embryos at 30 h, 4C and 4α embryos at 44 h, 8C and 8α embryos at 56 h, 16C embryos at 72 h, M at 120 h, and BL at 168 h.

Small RNA-Seq. Four replicates of 20 oocytes or embryos from each developmental stage were collected for small RNA-seq libraries (44 samples total). Total RNA was isolated using Quick-RNA MicroPrep kit (Zymo Research), including DNase treatment. All 7 μL of total RNA was then used for library preparation using the SMARTer small RNA (smRNA)-Seq Kit for Illumina (Clontech Laboratories) according to the manufacturer's instructions. The SMARTer smRNA-Seq Kit for Illumina is designed for small RNA input (1 ng) and uses polyadenylation of RNAs for an oligo(dT) primer, followed by a template switching technology in order to have ligation-free adapter and index incorporation to avoid biases associated with adapter ligation (39). After library preparation, the NucleoSpin Gel and PCR Clean-Up kit (Macherey-Nagel) was used for library purification according to the manufacturer's protocol. cDNA library concentration and quality were determined using the Qubit system with dsDNA HS reagents and the Aligent Bioanalyzer system with HS DNA Assay reagents, respectively. Size selection for lengths between 148 bp and 185 bp (adapter size of 153 bp) of each library was performed with the BluePippin System (Sage Science) using 3% Agarose Gel Cassettes (Sage Science). Quantification of size-selected libraries was again performed using the Qubit system with dsDNA HS reagents and the Aligent Bioanalyzer system with HS DNA Assay reagents. Libraries were pooled and Illumina sequencing performed using NextSeq SE 75 base pairs (Illumina). Samples were sequenced to get at least 30 million reads per stage (from the four replicates). Raw reads (55) were demultiplexed, imported in CLC Genomics Workbench (CLC Bio), and trimmed by removal the first three bases at 5′ end and the adapter sequence at 3′ end, followed by removal of poly-A tailing. Reads were mapped to the ARS-UCD *B. taurus* reference genome (annotation 1.2.97) using the RNA-Seq tool, and mapped reads were normalized to RPM. Reads were categorized by biotype for comparison of small RNA populations across stages. Additionally, small RNAs were extracted and combined using the "Extract and Count" tool in CLC Genomics Workbench (CLC Bio), and subsequently mapped to miRBase v22.1 using the "Annotate and Merge" tool. miRNA reads were normalized to RPM by dividing by the total number of raw reads in each sample multiplied by 10^6. Only miRNAs that were present in at least three of the four replicates were considered present in a stage of development. Stages were then compared for differentially expressed miRNAs. miRNAs were considered significantly different if P value was <0.05.

Knockdown of DGCR8. For microinjection experiments, embryos were denuded 6 h after IVF, and injected in a medium of H-SOF supplemented with 10% FBS.

About 7 pl of an siRNA (25 μM) plus MO (1 mM) mixture was injected into each embryo. Groups of ∼30 embryos were injected at one time, with up to three groups (∼90) in each replicate for both *DGCR8* and control/scrambled. Injection mixtures were either *DGCR8*-targeting siRNA and *DGCR8*-targeting MO (KD) or control/scrambled siRNA and control/scrambled MO (control injected, CI). In some experiments, DGCR8-targeting siRNA (siRNA only) or *DGCR8*-targeting MO (MO only) was injected alone to serve as additional controls for development profiling or for validation of mRNA knockdown. Sequences of siRNAs and MO were as follows:

DGCR8-targeting siRNA 5′ GCUCAACUUCUACGGAGCUUCUCUU (Invitrogen),
DGCR8-targeting MO 5′-GCTCCCACATGTCTCCATAGTACAG (Gene-Tools),
Control/scramble siRNA 5′ GGUGACUUUGUCGAACAAAUU (Invitrogen),
Control/scramble MO 5′-CCTCTTACCTCAGTTACAATTTATA (Gene-Tools).

After injection, embryos were again washed through H-SOF, and cultured as described above.

DGCR8 Rescue cRNA Generation, Injection, and Analysis. For the rescue experiments a Flag-tagged *DCGR8* coding region (CDS) that was mutated to be resistant to both the *DGCR8* targeting MO and siRNA (*SI Appendix*, Fig. S9) was subcloned into a pIVT vector (utilizing SbfI and KpnI cutting sites); the cDNA was purchased from Genewiz. The resulting pIVT-flag-DGCR8 construct was linearized by NdeI and used as template for in vitro transcription using the T7 mScriptTM Standard mRNA Production System (CELL SCRIPT) according to the manufacturer's instructions. Rescue embryos were microinjected as described above with final concentration of 25 μM siRNA, 1 mM MO, and 0.25 μg/μL *DGCR8* cRNA. Embryos were analyzed for development to the two-cell stage (30 h) and eight-cell stage (54 h).

Reverse-Transcription qPCR. For profiling of *DGCR8* across development, four replicates of 10 oocytes or embryos per sample were collected at each developmental stage. Total RNA was isolated using PicoPure RNA Isolation Kit (Arcturus), including a DNase treatment. Prior to RNA isolation, each sample was spiked with 8 μL of 250 fg/μL *HCRED1* cRNA, used as an exogenous control. To confirm *DGCR8* KD, 3 replicates of 20 embryos per sample were collected on day 2 and day 5 of development. For SOX2 qPCR, 3 replicates of 20 embryos per sample were collected on day 5 of development. Total RNA was isolated from samples using Quick-RNA MicroPrepkit (Zymo Research), including DNase treatment and 25-μL elution volume. Ten microliters of total RNA was then used for reverse transcription. SuperScript II Reverse Transcriptase (1 μL; Invitrogen), RNaseOUT (1 μL; Invitrogen), anchored oligo$(dT)_{20}$ primers (0.5 μL at 50 μM; Integrated DNA Technologies), and random hexamer primers (0.5 μL at 50 μM; Integrated DNA Technologies) were used for reverse transcription according the manufacturer's protocol. Reaction conditions were as follows: 25 °C for 10 min, 42 °C for 50 min, 70 °C for 15 min, and 4 °C hold. cDNA was stored at −20 °C until required.

Quantitative real-time PCR was performed in a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) with a final reaction volume of 20 μL, containing PowerUp SYBR Green Master Mix (10 μL) (Applied Biosystems), forward and reverse primers (0.25 μL each at 100 μM; Integrated DNA Technologies), nuclease-free water (4.5 μL; Invitrogen), and cDNA diluted 1:5 (5 μL). Reactions were performed on the QuantStudio system using "standard cycling mode" reaction, as follows: 50 °C for 2 min, 95 °C for 2 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min, followed by 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. Target genes and controls were run in duplicate or triplicate. Relative abundance was calculated using the $2^{-\Delta\Delta Ct}$ method and analyzed using Student's *t* test (56, 57). Either *HCRED1* abundance (profiling across stages) or bovine *GAPDH* (knockdown confirmation) were used as controls.

Primer sequences were as follows:

HCRED1 fwd 5′-GCCCGGCTTCCACTTCA,
HCRED1 rev 5′-GGCCTCGTACAGCTCGAAGTA,
DGCR8 fwd 5′-TCATCAACCCCAACGGGAAG,
DGCR8 rev 5′-TCACTTGGGTTCTCGCACTC,
GAPDH fwd 5′-TTCAACGGCACAGTCAAGG,
GAPDH rev 5′-ACATACTCAGCACCAGCATCAC,
SOX2 fwd 5′-CATTAACGGCACACTGCCCC,
SOX2 rev 5′-TGAAAATGTCTCCCCCGCCC,

miRNA Reverse-Transcription qPCR. For miRNA knockdown validation and small RNA-seq validation, four replicates of 20 oocytes or embryos per sample were collected on day 5 of development (knockdown validation), or at the MII stage, day 2, day 3, and day 5 of development, as well as day 3 after treatment with α-amanitin (RNA-seq validation). Total RNA was isolated from samples using Quick-RNA MicroPrepkit (Zymo Research), including DNase treatment and 25-μL elution volume. Next, 6.5 μL of total RNA was used for reverse transcription using the miRCURY LNA RT Kit (Qiagen), which included an external small RNA spike-in, UniSp6. This particular kit was chosen because the reverse-transcription approach mirrors the approach used to construct the cDNA libraries in the SMARTer smRNA-Seq Kit for Illumina that was used for small RNA-seq. qRT-PCR was then performed using the miRCURY LNA SYBR Green PCR Kit (Qiagen) and miRCURY LNA miRNA PCR Assay primers for four miRNAs, the control spike-in, UniSp6, and small, noncoding RNA U6 as a negative control for knockdown validation (Qiagen). Expression of miRNAs was then determined using the ΔΔ-CT method with the external spike-in as the background expression. Primer sequences were chosen to match the bovine miRNA sequence exactly, and were as follows:

mml-miR-371-3p 5′AAGUGCCGCCAUGUUUUGAGUGU,
ssc-miR-7 5′UGGAAGACUAGUGAUUUUGUUGUU,
bta-miR-1434-3p 5′GAAGAAAUCUAAGGUCUGAGG,
mmu-miR-202-5p 5′UUCCUAUGCAUAUACUUCUUU.

Immunocytochemistry. For validation of DGCR8 KD, 3 replicates of day 5 embryos were fixed in groups of 20 in 4% paraformaldehyde for 10 min at room temperature, permeabilized with 1% Triton X-100 in D-PBS solution for 30 min, then blocked in D-PBS containing 0.1% Tween 20, 1% BSA, and 10% Normal Donkey Serum (Gemini Bio) for 2 h. Embryos were then incubated overnight at 4 °C with primary antibody and incubated the next day for 1 h with the appropriate secondary antibody. DNA was stained with 10 μg/mL Hoechst 33342, and the embryos were mounted on a coverslip in ProLong Gold Antifade Mountant solution (Life Technologies). Fluorescence was detected on a Leica TCS SP laser-scanning confocal microscope and optical sections images were acquired for each embryo. Using NIH ImageJ software, all sections were combined by maximum projection. Nuclear signal was measured in several places, and mean intensity was calculated. The cytoplasmic signal was then measured in several places, and the mean of these intensities was subtracted from the nuclear signal mean. One-way ANOVA was used to determine significance. The following antibodies and dilutions were used:

Anti-DGCR8 rabbit polyclonal antibody (Proteintech #25835-1-AP, 1:300),
Anti-SOX2 goat polyclonal antibody (Santa Cruz #17320; 1:300),
Donkey anti-Rabbit IgG secondary antibody Alexa Fluor 488 (Invitrogen #A21206; 1:500),
Donkey anti-Goat IgG secondary antibody Alexa Fluor 488 (Invitrogen #A11055; 1:500).

RNA-Seq of DGCR8-KD Embryos. For mRNA libraries, 4 replicates of 20 embryos per sample were collected on day 3 and day 5 of development, representing 8-cell/16-cell and morula-stage embryos, respectively, for control embryos, control-injected embryos, or DGCR8-KD embryos. Day 3 was chosen to capture expression at the beginning of major EGA, and day 5 chosen to capture expression prior to the observed developmental arrest in DGCR8-KD embryos. Total RNA was isolated from samples using Quick-RNA MicroPrepkit (Zymo Research), including DNase treatment and 10-μL elution volume. Five micrliters of total RNA was used for cDNA preparation using the Ovation RNA-Seq System V2 (NuGEN) according to the manufacturer's instructions. cDNA samples were quantified using a Qubit system with dsDNA BR reagents. Two micrograms cDNA in 100 μL low TE buffer was used for sonication, using the following parameters: nine cycles of 30 s on high and 90 s off, spinning down samples every three cycles. After confirming cDNA was sonicated to the right size range for library preparation (∼74 to 400 bp), 1 μg sonicated cDNA was used for library preparation using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England BioLabs). Library concentration and quality were determined using the Qubit system with dsDNA HS reagents and the Aligent Bioanalyzer system with HS DNA Assay reagents, respectively. Libraries were pooled, and sequencing was performed using NextSeq SE 75 base pairs (Illumina). Samples were sequenced to have at least 11 million reads. Reads from each sample (58) were

trimmed (first 9 bp removed) and mapped to the ARS-UCD *B. taurus* reference genome (annotation 1.2.97) using the RNA-Seq tool in CLC Genomics Workbench (CLC Bio, which calculated gene-expression levels as RPKM. Differential expression analysis was then run to compare groups (control vs. control-injected vs. *DGCR8*-KD) using the Differential Expression tool in CLC Genomics Workbench. This tool uses multifactorial statistics based on a negative binomial generalized linear model. Additionally, differentially expressed genes between control-injected and control-uninjected embryos were compared with differentially expressed genes between *DGCR8*-KD embryos and both control-injected and control-uninjected embryos in day 3 and day 5 embryos. Genes were considered significantly different if FDR $P < 0.05$, fold-change > 2, and an average RPKM >0.4 in at least one of the compared groups. The National Center for Biotechnology Information DAVID Functional Annotation was used to identify overrepresented functional categories within the differentially expressed transcripts, using *B. taurus* genes for the background (59). Processes were only considered if $P < 0.05$.

Target Prediction of miRNAs. To identify potential targets, miRNAs were entered into TargetScan (41). TargetScan uses miRBase as its miRNA database, and predicts targets based on seed sequences. These seed sequences were also confirmed for target prediction. Predicted targets with cumulative weighted context ++ score <-0.35 (41) were then compared with differentially expressed genes in *DGCR8*-KD embryos identified by RNA-seq.

Data, Materials, and Software Availability. The datasets supporting the results of this article have been deposited in publicly accessible databases and are available in the Sequence Read Archive (SRA) database, https://www.ncbi.nlm.nih.gov/sra [accession nos. SRA PRJNA600523 (55) for *B. taurus* small RNA sequencing of preimplantation oocytes and embryos and SRA PRJNA600538 (58) for RNA-seq of *B. taurus DGCR8*-KD embryos].

ACKNOWLEDGMENTS. Semen used in embryo production was provided by Semex. We thank Joseph Owen, Sadie Hennig, and Ramon Botigelli for their assistance with microinjection of embryos; Alma Islas-Trejo for her support with molecular biology; all laboratory members for their help with oocyte aspirations; and Jun Ma (University of Pennsylvania) for generating the *DGCR8* cRNA used for the rescue experiments. Funding for this project was provided by NIH Grants HD022681 (to R.M.S.) and HD095007 (to R.M.S. and P.J.R.).

1. V. Ambros, The functions of animal microRNAs. *Nature* **431**, 350–355 (2004).
2. Y. Lee *et al.*, MicroRNA genes are transcribed by RNA polymerase II. *EMBO J.* **23**, 4051–4060 (2004).
3. T. A. Nguyen *et al.*, Functional anatomy of the human microprocessor. *Cell* **161**, 1374–1387 (2015).
4. M. Ha, V. N. Kim, Regulation of microRNA biogenesis. *Nat. Rev. Mol. Cell Biol.* **15**, 509–524 (2014).
5. H. Zhang, F. A. Kolb, L. Jaskiewicz, E. Westhof, W. Filipowicz, Single processing center models for human Dicer and bacterial RNase III. *Cell* **118**, 57–68 (2004).
6. S. Iwasaki *et al.*, Hsc70/Hsp90 chaperone machinery mediates ATP-dependent RISC loading of small RNA duplexes. *Mol. Cell* **39**, 292–299 (2010).
7. D. P. Bartel, MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* **116**, 281–297 (2004).
8. R. C. Lee, R. L. Feinbaum, V. Ambros, The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* **75**, 843–854 (1993).
9. B. Wightman, I. Ha, G. Ruvkun, Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. *Cell* **75**, 855–862 (1993).
10. P. H. Olsen, V. Ambros, The lin-4 regulatory RNA controls developmental timing in *Caenorhabditis elegans* by blocking LIN-14 protein synthesis after the initiation of translation. *Dev. Biol.* **216**, 671–680 (1999).
11. K. Seggerson, L. Tang, E. G. Moss, Two genetic circuits repress the *Caenorhabditis elegans* heterochronic gene lin-28 after translation initiation. *Dev. Biol.* **243**, 215–225 (2002).
12. J. Rehwinkel, I. Behm-Ansmant, D. Gatfield, E. Izaurralde, A crucial role for GW182 and the DCP1:DCP2 decapping complex in miRNA-mediated gene silencing. *RNA* **11**, 1640–1647 (2005).
13. J. Liu *et al.*, A role for the P-body component GW182 in microRNA function. *Nat. Cell Biol.* **7**, 1261–1266 (2005).
14. R. S. Pillai *et al.*, Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science* **309**, 1573–1576 (2005).
15. R. S. Pillai, MicroRNA function: Multiple mechanisms for a tiny RNA? *RNA* **11**, 1753–1761 (2005).
16. P. Svoboda, V. Franke, R. M. Schultz, Sculpting the transcriptome during the oocyte-to-embryo transition in mouse. *Curr. Top. Dev. Biol.* **113**, 305–349 (2015).
17. P. Braude, V. Bolton, S. Moore, Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* **332**, 459–461 (1988).
18. N. A. Telford, A. J. Watson, G. A. Schultz, Transition from maternal to embryonic control in early mammalian development: A comparison of several species. *Mol. Reprod. Dev.* **26**, 90–100 (1990).
19. M. Misirlioglu *et al.*, Dynamics of global transcriptome in bovine matured oocytes and preimplantation embryos. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 18905–18910 (2006).
20. L. Li, P. Zheng, J. Dean, Maternal control of early mouse development. *Development* **137**, 859–870 (2010).
21. N. Bushati, A. Stark, J. Brennecke, S. M. Cohen, Temporal reciprocity of miRNAs and their targets during the maternal-to-zygotic transition in *Drosophila*. *Curr. Biol.* **18**, 501–506 (2008).
22. A. J. Giraldez *et al.*, Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. *Science* **312**, 75–79 (2006).
23. E. Lund, M. Liu, R. S. Hartley, M. D. Sheets, J. E. Dahlberg, Deadenylation of maternal mRNAs mediated by miR-427 in *Xenopus laevis* embryos. *RNA* **15**, 2351–2363 (2009).
24. N. Suh *et al.*, MicroRNA function is globally suppressed in mouse oocytes and early embryos. *Curr. Biol.* **20**, 271–277 (2010).
25. J. Ma *et al.*, MicroRNA activity is suppressed in mouse oocytes. *Curr. Biol.* **20**, 265–270 (2010).
26. S. Kataruka *et al.*, Low miRNA abundance disables microRNA pathway in mammalian oocytes. *Nucleic Acid Res.* **48**, 8050–8062 (2020).
27. Y. Ohnishi *et al.*, Small RNA class transition from siRNA/piRNA to miRNA during pre-implantation mouse development. *Nucleic Acids Res.* **38**, 5141–5151 (2010).
28. Q. Yang *et al.*, Highly sensitive sequencing reveals dynamic modifications and activities of small RNAs in mouse oocytes and early embryos. *Sci. Adv.* **2**, e1501482 (2016).
29. Y. Wang, R. Medvid, C. Melton, R. Jaenisch, R. Blelloch, DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal. *Nat. Genet.* **39**, 380–385 (2007).
30. D. Jukam, S. A. M. Shariati, J. M. Skotheim, Zygotic genome activation in vertebrates. *Dev. Cell* **42**, 316–332 (2017).
31. K. Whitworth *et al.*, Developmental expression of 2489 gene clusters during pig embryogenesis: An expressed sequence tag project. *Biol. Reprod.* **71**, 1230–1243 (2004). Correction in: *Biol. Reprod.* **71**, 2092 (2004).
32. E. C. Wright, B. J. Hale, C. X. Yang, J. G. Njoka, J. W. Ross, MicroRNA-21 and PDCD4 expression during in vitro oocyte maturation in pigs. *Reprod. Biol. Endocrinol.* **14**, 21 (2016).
33. S. K. Tripurani *et al.*, MicroRNA-212 post-transcriptionally regulates oocyte-specific basic-helix-loop-helix transcription factor, factor in the germline alpha (FIGLA), during bovine early embryogenesis. *PLoS One* **8**, e76114 (2013).
34. S. K. Tripurani, K. B. Lee, G. Wee, G. W. Smith, J. Yao, MicroRNA-196a regulates bovine newborn ovary homeobox gene (NOBOX) expression during early embryogenesis. *BMC Dev. Biol.* **11**, 25 (2011).
35. K. Goossens *et al.*, Regulatory microRNA network identification in bovine blastocyst development. *Stem Cells Dev.* **22**, 1907–1920 (2013).
36. P. B. Sinha *et al.*, MicroRNA-130b is involved in bovine granulosa and cumulus cells function, oocyte maturation and blastocyst formation. *J. Ovarian Res.* **10**, 37 (2017).
37. J. M. Cuthbert, S. J. Russell, I. A. Polejaeva, Q. Meng, K. L. White, A. D. Benninghoff, Comparing mRNA and sncRNA profiles during the maternal-to-embryonic transition in bovine IVF and scNT embryos†. *Biol. Reprod.* **105**, 1401–1415 (2021).
38. J. M. Cuthbert, S. J. Russell, I. A. Polejaeva, Q. Meng, K. L. White, A. D. Benninghoff, Dynamics of small non-coding RNAs in bovine scNT embryos through the maternal-to-embryonic transition†. *Biol. Reprod.* **105**, 918–933 (2021).
39. C. Wright *et al.*, Comprehensive assessment of multiple biases in small RNA sequencing reveals significant differences in the performance of widely used methods. *BMC Genomics* **20**, 513 (2019).
40. Y. S. Bogliotti *et al.*, Transcript profiling of bovine embryos implicates specific trasnсription factors in the maternal-to-embryo transition. *Biol. Reprod.* **102**, 671–679 (2020).
41. V. Agarwal, G. W. Bell, J. W. Nam, D. P. Bartel, Predicting effective microRNA target sites in mammalian mRNAs. *eLife* **4**, e05005 (2015).
42. J. E. Babiarz, J. G. Ruby, Y. Wang, D. P. Bartel, R. Blelloch, Mouse ES cells express endogenous shRNAs, siRNAs, and other Microprocessor-independent, Dicer-dependent small RNAs. *Genes Dev.* **22**, 2773–2785 (2008).
43. P. C. Calses *et al.*, DGCR8 mediates repair of UV-induced DNA damage independently of RNA processing. *Cell Rep.* **19**, 162–174 (2017).
44. S. Macias *et al.*, DGCR8 HITS-CLIP reveals novel functions for the microprocessor. *Nat. Struct. Mol. Biol.* **19**, 760–766 (2012).
45. S. Macias, R. A. Cordiner, P. Gautier, M. Plass, J. F. Cáceres, DGCR8 acts as an adaptor for the exosome complex to degrade double-stranded structured RNAs. *Mol. Cell* **60**, 873–885 (2015).
46. J. M. Cuthbert, S. J. Russell, K. L. White, A. D. Benninghoff, The maternal-to-zygotic transition in bovine in vitro-fertilized embryos is associated with marked changes in small non-coding RNAs. *Biol. Reprod.* **100**, 331–350 (2019).
47. G. C. Gilchrist, A. Tscherner, T. Nalpathamkalam, D. Merico, J. LaMarre, MicroRNA expression during bovine oocyte maturation and fertilization. *Int. J. Mol. Sci.* **17**, 396 (2016).
48. B. W. Daigneault, S. Rajput, G. W. Smith, P. J. Ross, Embryonic POU5F1 is required for expanded bovine blastocyst formation. *Sci. Rep.* **8**, 7753 (2018).
49. D. K. Berg *et al.*, Trophectoderm lineage determination in cattle. *Dev. Cell* **20**, 244–255 (2011).
50. M. D. Goissis, J. B. Cibelli, Functional characterization of SOX2 in bovine preimplantation embryos. *Biol. Reprod.* **90**, 30 (2014).
51. P. Dutt *et al.*, m-Calpain is required for preimplantation embryonic development in mice. *BMC Dev. Biol.* **6**, 3 (2006).
52. S. Kageyama, H. Liu, M. Nagata, F. Aoki, The role of ETS transcription factors in transcription and development of mouse preimplantation embryos. *Biochem. Biophys. Res. Commun.* **344**, 675–679 (2006).
53. K. C. Pavani *et al.*, Hatching is modulated by microRNA-378a-3p derived from extracellular vesicles secreted by blastocysts. *Proc. Natl. Acad. Sci. U.S.A.* **119**, e2122708119 (2022).
54. J. J. Parrish *et al.*, Bovine in vitro fertilization with frozen-thawed semen. *Theriogenology* **25**, 591–600 (1986).
55. E. P. Paulson, R. M. Schultz, P. J. Ross, Bos taurus small RNA sequencing of preimplantation oocytes and embryos. NCBI: SRA. https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA600523. Deposited 10 January 2020.
56. T. D. Schmittgen, K. J. Livak, Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc.* **3**, 1101–1108 (2008).
57. K. J. Livak, T. D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Δ Δ C(T)) Method. *Methods* **25**, 402–408 (2001).
58. E. P. Paulson, R. M. Schultz, P. J. Ross, RNA-sequencing of Bos taurus DGCR8-knockdown embryos. NCBI: SRA. https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA600538. Deposited 10 January 2020.
59. W. Huang, B. T. Sherman, R. A. Lempicki, Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* **4**, 44–57 (2009).