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MicroRNAomic Analysis of Spent Media from Slow and Fast-Growing Bovine Embryos Reveal Distinct Differences

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Simple Summary: Embryos release microRNAs (miRNAs) into their surrounding spent media during early development. Gene expression is expected to vary between embryos of good and poor developmental potential, therefore identifying miRNAs unique to good quality embryos can aid in embryo selection. This study aimed to characterize miRNA expression in the spent media of embryos identified as slow versus fast growing, as developmental timing may be indicative of embryo quality. Distinct miRNA populations were detected in the spent media conditioned with bovine embryos growing at different developmental rates at the two-cell, eight-cell, and blastocyst stage in-vitro. The results highlight novel and non-invasive miRNA biomarkers of early embryo development.

Abstract: In bovine embryos, microRNA (miRNA) expression has been profiled at each stage of early development in-vitro. miRNAomic analysis of spent media has the potential to reveal characteristics of embryo health, however, applications are limited without categorizing miRNA profiles by embryo quality. Time-lapse imaging has shown the timing of embryo development in-vitro may be indicative of their developmental potential. The aim of the study was to profile miRNAs in the spent media of slow and fast-growing bovine embryos throughout the pre-implantation period. Bovine cumulus-oocyte-complexes were aspirated from ovaries, fertilized, and cultured to blastocyst stage of development. At 2-cell, 8-cell, and blastocyst stage, each microdrop of 30 presumptive-zygotes were classified as slow or fast-growing based on the percentage of embryos that had reached the desired morphological stage. Following hybridization on a GeneChip miRNA 4.0 array, comparative analysis was conducted between spent media of slow and fast-growing embryos. In total, 34 differentially expressed miRNAs were identified between the comparison groups, with 14 of the miRNAs detected in the 2-cell samples, 7 miRNAs detected in the 8-cell samples, and 12 miRNAs detected in the blastocyst samples. The results demonstrate distinct miRNAs populations can be identified between slow and fast-growing embryos, highlighting novel biomarkers of developmental potential at each stage of pre-implantation development.

Keywords: miRNA; spent media; bovine; IVF; biomarker; embryonic health; preimplantation; embryo development

Introduction

It is well established that embryos of differing developmental potential have different genomic, proteomic, and metabolomic profiles [1]. These variations in expression are detectable intracellularly, and more recently, in the spent culture media of IVF systems [1]. Analysis of small molecules found in the spent media (SM) has the possibility of revealing biomarkers related to intrinsic embryo physiology. Strong evidence suggests that array-based metabolomics and miRNAomic analysis are good candidates for adjunct assessment methods of embryo quality. Metabolomics and miRNAomic

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profiling of embryo SM have revealed distinct signatures between embryos of different morphological appearance [2], chromosomal status [3], sex [4], and implantation outcome [5].

Advancements in time-lapse imaging have also revealed the importance of morpho-kinetic assessment in assessing embryo quality [6]. This relatively new technique has shown the timing of the onset and duration of key morphological events, such as cleavage, compaction, and blastocyst formation, may indicate normal and aberrant embryo development. Since in-vivo embryos develop faster than their in-vitro counterparts, it is commonly accepted that faster developing in-vitro embryos are healthier [7]. In fact, research has shown in-vitro embryos that cleave earlier have higher blastocyst rates. Timing may be indicative of stress experienced by an embryo. The absence or low levels of stress factors such as reactive oxygen species may mean embryos can develop faster as less time can be spent initiating repair pathways [7].

However, other groups have presented a counter idea that the slower-growing embryo has more time to correctly initiate and choreograph the events of embryogenesis. Research by Market-Velker and colleagues compared slow-growing (SG) and fast-growing (FG) embryos, with in-vivo embryos, on factors such as methylation status, expression of imprinted genes, embryo cell number, and morphology [8]. Their findings showed that SG embryos were most similar to in-vivo embryos on all parameters measured [7]. Genomic imprinting and expression of metabolic markers in SG embryos closely mirrored those of in-vivo embryos. Market-Velker and colleagues postulate FG embryos may transition too rapidly during the first few embryonic stages causing an inability to maintain epigenetic information [8]. Thus, it appears embryos grow within a time range, whereby anything too slow may indicate a pathological condition, while too fast may signify an embryo erroneously rewired to move onto the next developmental stage.

To date, few studies have profiled SM conditioned with SG and FG embryos. One study that did so examined the metabolites present in the SM cultured with SG and FG embryos. Using NMR microscopy, Perkel and Madan were able to detect distinct metabolic signatures in the SM between SG and FG embryos at the 2-cell, 8-cell, 16-cell, and blastocyst stage of development [9]. Specifically, their data showed distinct differences between media cultured with the 4-cell SG and FG embryos for pyruvate, and at the 16-cell stage for acetate, tryptophan, leucine/isoleucine, valine, and histidine. Four-cell SG embryos had higher consumption of pyruvate, while 16-cell SG embryos released more acetate into the media, in comparison to their FG counterparts [9]. Acetate is produced when there is an over-abundance of acetyl-CoA, such as in situations where the Krebs cycle or electron-transport chain is malfunctioning. Since both processes lie within the mitochondria, this observation suggests SG embryos exhibit some level of mitochondrial dysfunction resulting in a metabolic disturbance.

Embryonic mitochondrial biogenesis analysis conducted in our lab revealed metabolic distress may be related to mitochondrial dysfunction. In this study, GLYCOX and OXPHOS gene expression were examined in SG and FG embryos at the 2-cell, 8-cell, morula, and blastocyst stage of development [10]. GLYCOX and OXPHOS genes regulate pathways in embryo energy production, whereby OXPHOS is a mitochondrial-dependent pathway and dominates in early embryo development, GLYCOX dominates in late embryo development [10]. Data indicates SG embryos had higher expression of both OXPHOS and GLYCOX genes at all time-stages in comparison to FG embryos. This over-expression may serve as a compensatory mechanism for mitochondrial dysfunction occurring in SG embryos. Overall, data from our lab suggest that metabolic assays are sensitive in detecting differences between embryos growing at different rates [10].

Recent data from our lab has also shown the miRNA expression in the SM is different between SG and FG embryos. In this preliminary experiment, 6 candidate miRNAs, miR-196a, miR-181a, miR-155-5p, miR-148a, miR-302c, miR-370 and snRNA U6, were examined in the SM conditioned with SG and FG embryos at the 2-cell, 8-cell, 16-cell stage, and blastocyst-stage [11]. MiR-181a, miR-148a, miR-155-5p and snRNA U6 had increased expression in SG embryos in comparison to FG embryos [11]. This preliminary study shows SM profiling is sensitive in detecting miRNA differences between embryos growing at different developmental rates.

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Therefore, the objective of the present study was to globally profile, using a heterologous miRNA microarray, the miRNAs expression in the SM of SG and FG embryos at the 2-cell, 8-cell, and blastocyst stage of development.

Materials and Methods

Ethics

All experiments were conducted in accordance with the requirements of the Animals for Research Act of Ontario, and the Canadian Council for Animal Care.

Chemicals

All chemicals were attained from Sigma-Aldrich, Oakville, ON, Canada, unless stated otherwise.

Oocyte Collection and In-Vitro Production of Bovine Embryos

Bovine ovaries were collected from a local abattoir (Cargill Canada, Guelph, Ontario) and transported to the laboratory in a thermo flask under phosphate buffered saline (NaCl, 136.9 mM; Na2HPO4, 8.1 mM; KCL, 1.47 mM; KH2PO4, 1.19 mM; MgCl2.6H2O, 0.49 mM) at a temperature of 35-36°C. Follicles ranging from 4mm-8mm were aspirated using an 18G vacutainer needle and were suspended in HEPES-buffered Hams F-10, supplemented with 2% donor calf serum (PAA Laboratories Inc., ON, Canada). Cumulus oocyte complexes (COCs) were washed twice with 3ml synthetic S-IVM (Sigma-Aldrich) and washed once with 3mL S-IVM supplemented with 0.5 g/ml of follicle stimulating hormone, 1 g/ml of luteinizing hormone and 1 g/ml of estradiol (Sigma-Aldrich). Approximately, groups of 15-20 COCs with homogenous cytoplasm and 4-5 layers of granulosa cells were matured in 80µl drops of S-IVM under a layer of silicone oil for 22-24 hours at 38.5°C in an atmosphere of 5% Co_2 with 100% humidity. After maturation, the COCs were washed twice with 3ml HEPES buffered Tyrode's albumin-lactate-pyruvate medium (HEPES/Sperm TALP) supplemented with 15% BSA (0.0084 mg/ml final; fatty acid free, Sigma-Aldrich) and washed twice with 3mL IVF-TALP (IVF-TALP consisting of Tyrode's solution, supplemented with 15% BSA and 2 mg/ml heparin (Sigma-Aldrich)). Approximately 20 COCs were placed in 80µl drops of IVF-TALP under a layer of silicone oil. Frozen thawed bovine sperm was prepared using the swim-up technique. Thawed sperm was placed in HEPES/Sperm TALP and incubated for 45 minutes at 38.5°C in an atmosphere of 5% CO2 with 100% humidity prior to centrifugation at 200g for 7 minutes. The COCs and sperm were co-incubated at a final concentration of 1.0×10^6 at 38.5°C in 5% Co_2 with maximum humidity. At 18 hour post fertilization (hpf), the presumptive zygotes (PZ) were denuded by gentle vortexing for 90 seconds, followed by washing twice with 3ml HEPES/Sperm TALP, and once with in-vitro culture (IVC) media ($CaCl_2 \bullet 2H_2O$, 1.17 mM; KCL, 7.16 mM; KH_2PO_4, 1.19 mM; $MgCl_2 \bullet 6H_2O$, 0.49 mM; NaCl, 107.7 mM; $NaHCO_3$, 25.07 mM, Na lactate (60% syrup), 3.3 mM; ChemiconMillipore, Billerica, MA, USA) supplemented with 50µL of 100x non-essential amino acids (glycine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, L-serine; all 0.2 mM final), 100µL 50x essential amino acids (L-arginine hydrochloride, 0.6 mM final; L-cysteine, 0.1 mM final; L-histidine hydrochlorideH_2O, 0.2 mM final; L-isoleucine, 0.4 mM final; L-leucine, 0.4 mM final; L-lysine hydrochloride, 0.4 mM final; L-methionine, 0.1 mM final; L-phenylalanine, 0.2 mM final; L-threonine, 0.4 mM final; L-tyrosine, 0.2 mM final; L-tryptophan, 0.05 mM final; L-valine, 0.4 mM final), 25µL of sodium pyruvate (0.00886 mg/ml final), 2.5µL of gentamicin (25 mg/ml final; all from Invitrogen, Burlington, ON, Canada), and 280µl of 15% BSA (0.0084 mg/ml final). Approximately 30 PZ with homogenous cytoplasm were cultured in 30µl of IVC media under silicone oil at 38.5°C in an atmosphere of 5% CO_2, 5% O_2, 90% N_2. Each 3.5 ml dish contained 6 micro-drops of IVC media (each with a volume of 30 microliters), whereby the PZ were cultured.

Collection of Spent In-Vitro Culture Media Conditioned with SG and FG Embryos

On day 0 of culture, each of the 6 micro drops containing 30 PZ was assigned group numbers ranging from 1-6. This made it possible to follow the same cohort of embryos throughout the pre-

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implantation period while collecting SM at specific time points corresponding to the 2-cell, 8-cell, and blastocyst-stage of development. Microdrops were classified as a SG or FG group at each time point, based on the percentage of embryos that have reached the desired morphological stage at a given time point. At 18-30 hpf, microdrops were considered SG if the cohort had <50% reach the 2-cell-stage and FG if the cohort had ≥ 50% reach the 2-cell stage. Once the groups were designated SG or FG, the embryos were placed into fresh microdrops of IVC media, retaining their original group number, and placed in the incubator for another 30 hours to reach the 8-cell stage. Approximately 25µl of conditioned media from each microdrop from 2-cell SG and FG groups were collected, pooled, and placed in separate Eppendorf tubes. At the 8-cell stage, the cohort of embryos was assessed for 8-cell rate formation and cohorts were considered SG if the 8-cell rate was <50% and FG if the 8-cell rate was ≥ 50%. After designation, the embryos were placed into a fresh microdrop of IVC media, retaining their original group number, and placed in the incubator for another 72 hours to allow for blastocyst formation. Approximately 25µl of conditioned media from each microdrop from 8-cell SG and FG groups were collected, pooled, and placed in separate Eppendorf tubes. At the blastocyst stage, the cohort of embryos was assessed for a final time for blastocyst rate formation and cohorts were considered SG if blastocyst rates were <20% and FG if blastocyst rates were ≥ 20%. After designation, the embryos were taken out of the drop and the SG and FG embryos were placed in separate Eppendorf tubes and flashed frozen. Approximately 25µl of conditioned media from each microdrop from SG and FG groups were collected, pooled, and placed separately in Eppendorf tubes. Multiple IVF runs were completed until 1100µl of SM was collected for each developmental stage: 2-cell SG/2-cell FG, 8-cell SG/8-cell FG, and blastocyst SG/blastocyst SG.

miRNA Extraction

miRNA extraction was isolated from spent and unconditioned IVC media using an RNeasy mini kit (Qiagen, Hilden, Germany) as downstream array analysis required total RNA sample input. Briefly, 350µL of spent and plain IVC media was aliquoted to a 2.5 mL Eppendorf tube and equal volumes of QIAzol lysis reagent were added, vortexed for 20 seconds, and placed on the benchtop at room temperature for 10 minutes. This was followed by the addition of 350µL of chloroform and incubated for 2 minutes at room temperature, prior to centrifugation at 12g (15,000 RPM) at 4°C for 15 minutes. After, the supernatant was placed into the RNeasy MinElute spin column for total RNA separation. Once all the supernatant was processed, washing steps using buffer RWT, buffer RPE, and 80% ethanol, as per manufacturer protocol, was performed. The RNA was eluted using 30µL of RNAse-free water and immediately stored at –80°C prior to microarray analysis. In total, 3 biological replicates of pooled SM from 2-cell SG/2-cell FG, 8-cell SG/8-cell FG, and blastocyst SG/blastocyst FG groups and plain media was processed and prepared for microarray analysis.

miRNA Microarray Hybridization

Microarray processing was all conducted by our collogues at Genome Quebec (McGill University, Montreal Quebec). Briefly, microarray profiling was conducted using the Affymetrix GeneChip miRNA 4.0 assay (Affymetrix, Santa Clara, CA, USA), according to manufacturer's instructions and as described previously by Reza et al., 2018 [12]. Briefly, each sample of RNA was labelled using the FlashTag Biotin RNA Labelling Kit (Genisphere, Hatfield, PA, USA), quantified, fractionated, and hybridized to the miRNA microarray. The protocol is as follows: labelled RNA is heated to 99°C for 5 minutes, then heated at 45°C for 5 minutes, prior to hybridization via constant agitation at 60rpm for 16 hours at 48°C on an Affymetrix 450 Fluidics Station. The microarray chip is washed and stained with Genechip Fluidics Station 450, prior to being scanned with the use of an Affymetrix GCS 3,000 scanner and computed using the Affymetrix Genechip command console software.

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Statistical Analysis

For Genechip microarray analysis, CEL files were imported in the Transcriptome Analysis Console® 4.0.2.15 (TAC) software in RMA+DMG (all organisms) mode. Comparative analysis was carried out between SM samples 2-cell SG/2-cell FG, 8-cell SG/8-cell FG, and blastocyst SG/blastocyst FG and control (unconditioned media) using fold-change and independent T-test, in which the null hypothesis was that no difference exists between the 2 groups. Probes were differentially expressed at a fold-change of ≤ -2 or ≥ 2 (p-value < 0.05), where probe-sets were considered expressed if ≥ 50% of samples have a detectable above background (DABG) values below DABG threshold of < 0.05 and a false discovery rate (FDR) < 0.05. All statistical tests and visualization of differentially expressed genes were done using TAC software (version 4.0.2.15).

mRNA Target Pathway Prediction of Differentially Expressed miRNAs

Functional analysis of differentially expressed miRNAs (DEM) detected between 2-cell SG/2-cell FG, 8-cell SG/8-cell FG, and blastocyst SG/blastocyst FG SM conditions was performed using TargetScan Human 7.2 (http://www.targetscan.org/vert_72/) under Cow annotation, to construct a gene-list from the DEM. Genes with a cumulative context score of <-0.5 were included in the list. From the gene-list, gene-set enrichment analysis (GSEA) was conducted using DAVID 6.8 (https://david.ncifcrf.gov/) with the option gene-ontology: biological processes. Pathways with a p-value of <0.05 were considered significantly enriched.

Results

Differentially Expressed miRNAs between 2-Cell SG vs. 2-Cell FG, 8-Cell SG vs. 8-Cell FG, and Blastocyst SG vs. Blastocyst FG SM

Overall, 34 DEM were identified between the 3 SM comparison groups, in which 14 miRNAs belonged to 2-cell SG vs. 2-cell FG, 7 miRNAs were detected between 8-cell SG vs. 8-cell FG, and 13 miRNAs were differentially expressed between the blastocyst SG and blastocyst FG groups. Of the 14 DEM detected between 2-cell SG and 2-cell FG, 12 miRNAs and 2 miRNAs were upregulated and downregulated (Table 1), respectively, in 2-cell SG SM in comparison to 2-cell FG SM. For 8-cell SG and 8-cell FG, 6 miRNAs and 1 miRNA were upregulated and downregulated (Table 2) respectively, in 8-cell SG SM in comparison to 8-cell FG SM. Of the 13 DEM detected between blastocyst SG and blastocyst FG SM, 9 miRNAs and 4 miRNAs were upregulated and downregulated (Table 3) in blastocyst SG SM in comparison to blastocyst FG SM, respectively. It appears that SG embryos are releasing, rather than up-taking, miRNAs in their environment. Differences in miRNA expression in the SM seem to be highest at the early and late stages of pre-implantation development between SG and FG embryos.

Table 1. DEM between 2-Cell SG SM vs. 2-Cell FG SM: downregulated (red) and upregulated (green). The majority of miRNAs were upregulated in 2-cell SG SM in comparison to 2-Cell FG SM.

miRNAs	Fold-Change	P-Value
bta-miR-455-3p	-2.83	1.19E-06
bta-miR-628	-2.07	0.0104
bta-miR-2359	2.02	0.0113
bta-miR-2412	2.03	0.0025
bta-miR-2452	2.68	0.0006
bta-miR-2325a	3.12	0.003
bta-miR-1343-5p	3.16	0.0184
bta-miR-2421	4.04	0.0002
bta-miR-2434	6.56	0.0005
bta-miR-2393	13.36	0.0004
bta-miR-2444	17.58	0.0005
bta-miR-2361	41.56	0.001
bta-miR-3613	47.62	0.0002

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bta-miR-2325c	58.04	0.0003
bta-miR-2359	2.03	0.0025

Table 2. DEM between 8-Cell SG SM vs. 8-Cell FG SM. The majority of miRNAs were upregulated in 8-cell SG SM in comparison to 8-Cell FG SM.

miRNAs	Fold-Change	P-Value
bta-miR-3613b	-6.1	9.48E-06
bta-miR-1343-5p	2.14	0.0149
bta-miR-450b	3.04	0.0002
bta-miR-2487	3.23	6.61E-06
bta-miR-2885	4.09	1.48E-07
bta-miR-1281	4.27	0.0033
bta-miR-760-5p	4.55	0.0001

Table 3. DEM between blastocyst SG SM vs. blastocyst FG SM. The majority of miRNAs were upregulated in SG SM in comparison to FG SM.

miRNAs	Fold-Change	P-Value
bta-miR-450b	-2.71	0.0002
bta-miR-760-5p	-2.48	0.0076
bta-miR-2296	-2.34	0.0004
bta-miR-6535	-2.11	0.007
bta-let-7b	2.05	1.26E-05
bta-miR-2402	2.18	1.66E-05
bta-miR-23a	2.34	5.21E-07
bta-miR-23b-3p	2.45	3.90E-06
bta-miR-17-5p	2.47	0.0102
bta-miR-2898	2.51	0.0046
bta-miR-615	2.66	0.0035
bta-miR-320a	2.79	1.45E-08
bta-miR-24-3p	2.83	8.65E-07

Interestingly, the majority of miRNAs detected in SM were expressed in a stage-specific manner, with only 3 miRNAs being co-detected in more than one SM condition. Bta-miR-1343-5p was upregulated in the SM of both 2-cell and 8-cell SG, in comparison to their 2-cell and 8-cell FG counterparts. Bta-miR-450b and bta-miR-760-5p were detected in both 8-cell and blastocyst SM. However, the expression of the two miRNAs differed between the two conditions. Both miRNAs were upregulated in the SM of 8-cell SG embryos, while the two miRNAs were downregulated in SM cultured with SG blastocyst. It should be noted that no miRNAs were consistently differentially expressed across all three SM groups.

Predictions of miRNA-mRNA Targets for Differentially Expressed miRNAs Detected between 2-Cell SG vs. 2-Cell FG, 8-Cell SG vs. 8-Cell FG, and Blastocyst SG vs. Blastocyst FG SM

With regards to 2-cell SG vs. 2-cell FG miRNAs, bta-miR-2361 did not have any predicted targets that met the cumulated weighted score cutoff and thus were excluded from the analysis. From the remaining 13 miRNAs differentially expressed, a total of 635 mRNAs were predicted (Supplemental Table S1). 137 mRNAs were significantly enriched across 35 biological pathways (Table 4). Moving forward to 8-cell SG vs. 8-cell FG miRNAs, bta-miR-2487 was not found on the TargetScan database and thus was excluded from the analysis. From the remaining 6 DEM, a total of 579 mRNAs were predicted (Supplemental Table S2). 141 mRNAs were significantly enriched across 36 different biological processes in DAVID (Table 5). Lastly, a total of 837 mRNA targets were predicted for the 12 miRNAs differentially expressed between blastocyst SG vs. blastocyst FG condition (Supplemental Table S3). When inputted into DAVID, 239 mRNAs were enriched across 76 different biological processes (Table 6). Due to the significant number of biological processes enriched in each

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comparison group, only the top 5 pathways with the most genes enriched were featured on the tables. Overall, the majority of the predicted targets of DEM across the 3 conditions clustered around biological processes controlling transcription and proliferation.

Table 4. Top 5 enriched biological pathways of predicted genes regulated by miRNAs differentially expressed in 2-cell SG SM vs. 2-Cell FG SM.

GO Term	Genes	P-Value
Positive regulation of transcription from RNA polymerase II promoter	33	3.05E-04
Negative regulation of transcription from RNA polymerase II promoter	26	4.65E-04
Negative regulation of transcription, DNA-templated	14	0.03335617
Negative regulation of cell proliferation	13	0.02255407
Spermatogenesis	12	0.01965493

Table 5. Top 5 enriched biological pathways of predicted genes regulated by miRNAs differentially expressed in 8-cell SG SM vs 8-Cell FG SM.

GO Term	Genes	P-Value
Positive regulation of transcription from RNA polymerase II promoter	33	1.86E-04
Intracellular signal transduction	14	0.045985
Negative regulation of cell proliferation	13	0.01862582
Protein transport	11	0.03235415
Positive regulation of ERK1 and ERK2 cascade	10	0.01051371

Table 6. Top 5 enriched biological pathways of predicted genes regulated by miRNAs differentially expressed in blastocyst SG SM vs blastocyst FG SM.

GO Term	Genes	P-Value
Positive regulation of transcription from RNA polymerase II promoter	36	0.0078327
Regulation of transcription from RNA polymerase II promoter	26	1.16E-05
Positive regulation of cell proliferation	20	0.0062254
Small GTPase mediated signal transduction	19	8.66E-04
Positive regulation of transcription, DNA-templated	18	0.0236799

Cross-referencing the enriched genes from each comparison group with their respective miRNA-mRNA gene-list allowed for the identification of miRNAs whose gene targets were enriched in DAVID. Out of the 14 miRNAs differentially expressed between 2-cell SG vs. 2-cell FG SM, bta-miR-1343-5p and bta-miR-2443 gene targets were highly represented in DAVID. Of the 7 miRNAs detected between 8-cell SG vs. 8-cell FG SM, bta-miR-1343-5p and bta-miR-2885 gene targets had the highest representation in GSEA. Among the 12 miRNAs detected between blastocyst SG vs. blastocyst FG SM, the gene targets of bta-miR-6535 were overly represented in DAVID.

Discussion

To the best of our knowledge, this study was the first to globally profile miRNAs in the SM conditioned with embryos growing at different developmental rates. Our results indicate distinct miRNA populations can be identified between SG and FG embryos. More importantly, these unique miRNA signatures can be detected at the early, mid, and late stages of pre-implantation embryo development. Our results suggest SG embryos, at all conditions examined (2-cell SM, 8-cell SM, and blastocyst SM), preferentially release miRNAs into the extracellular environment. Although no other study has reported this, metabolomics studies have detected distinct metabolites in the SM conditioned with embryos differing in developmental rate and viability. These studies suggest lower quality embryos are metabolically more active than their higher quality counterparts. Since

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metabolism is influenced by the genes expressed within the cell, it can be postulated that increases in embryonic metabolism are preceded by higher genetic activity. Perhaps, miRNAs are used within the embryo to initiate and modulate gene expression influencing metabolic turnover. Evidence does suggest extracellular miRNA population serves as a good indicator of intracellular miRNA expression. Thus, our result indicates increases in metabolic activity in non-viable and SG embryos may also be driven by increases in miRNA expression, which are detectable in SM.

Across the 3 conditions examined, only the miRNAs detected between blastocyst SG vs. blastocyst FG SM had some previous annotation in literature. Specifically, miR-320a and miR-24-3p, which were detected to be upregulated in blastocyst SG SM, have also been cited in previous SM studies. According to Kropp and Khatib, miR-24-3p was one of the five miRNAs they detected to be upregulated in SM conditioned with degenerate blastocyst [2]. In a subsequent supplementation study, miR-24 was added to the plain media of morula stage embryos. Supplementation resulted in a 44-fold increase in expression of miR-24 in blastocyst cultured with miR-24 and a 27.3% decrease in blastocyst rates [2]. Kropp and Khatib postulate miR-24 influenced the expression of CDKN1b, which is a cell cycle regulator. Despite the differences in the classification of non-viable embryos, whereby we used developmental rate and Kropp and Khatib examined arrested/degenerate embryos, both studies indicate miR-24 may serve as a biomarker of embryo viability at the blastocyst stage of development.

Another miRNA identified in this study and previously annotated in literature was miR-320a. A recent study by Berkhout and colleagues suggest that miR-320a is a pre-implantation marker secreted by embryos. Specifically, the researchers profiled the miRNome of SM conditioned with embryos either scoring low or high in morphological scores [13]. It was determined that miR-320a was secreted by higher quality embryos. Subsequently, miR-320a was supplemented in the culture media of human embryonic stem cells. Berkhout and colleagues reported miR-320a was able to stimulate the migration of decidualized human embryonic stem cells, with downstream transcriptome analysis revealing miR-320a modulates genes regulating cell adhesion and cytoskeleton organization [13]. Interestingly, our study found miR-320a to be upregulated in SM conditioned with SG embryos. Capalbo and colleagues have postulated embryos release miRNAs into the extracellular environment as a means of paracrine communication with endometrial tissue [5]. Thus, the findings in our study suggest miR-320a may serve to inhibit implantation as it was found to be released by SG embryos. Although consensus about the role of miR-320a is mixed, it should be noted that our study and the one conducted by Berkhout and colleagues were done in different species and culture conditions were not identical. Thus, inter-species differences and environmental conditions may have influenced the findings of both studies. Perhaps, miR-320a may have both inhibitory and stimulatory effects on implantation as miRNAs have various targets within the genome.

Aside from miR-320a and miR-24, miR-615 and miR-17 have also been previously annotated in literature, albeit in cancer-related studies. Specifically, miR-615 has been characterized to play a role in angiogenic events influencing tumorigenesis. Icli and colleagues demonstrated that miR-615 has anti-angiogenic effects, whereby expression of the miRNA significantly inhibited endothelial cell proliferation and migration [14]. Similarly, miR-17 has been cited in literature to have anti-oncogenic effects. Hossain and colleagues reported miR-17 transfection in breast cancer tissue resulted in the translational repression of the breast cancer associated gene *A1B1* [15]. The subsequent downregulation of *A1B1* decreased breast cancer proliferation. Thus, it seems the miRNAs upregulated in SG embryos at the blastocyst stage have roles in cancer development. Cancer and embryogenesis rely on similar pathways for growth and development. Therefore, it is interesting to see that cancer-related miRNAs are detectable in media conditioned with embryos growing at different rates. Perhaps, these anti-proliferative miRNAs in cancer, serve to inhibit growth and development in an embryo.

Aside from previously annotated miRNAs, GSEA analysis also revealed novel stage-specific miRNA biomarkers. Bta-miR-1343 and bta-miR-2443 were miRNAs upregulated in SM cultured with 2-cell SG embryos. GSEA analysis indicated the gene targets of the two miRNAs had roles in

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regulating transcription and cell proliferation. Specifically, the majority of gene targets had biological implications pertaining to positive and negative regulation of transcription from RNA polymerase II promoter. This is an interesting finding as previous research suggests little to no transcription occurs at the 2-cell stage in bovine embryos. Prior to the 8-cell stage, the developing bovine embryo relies on parentally inherited transcripts (mRNA and miRNA) and proteins for survival. Therefore, it is unclear why the gene targets of miR-1343 and miR-2443 would cluster around promoting transcriptional events.

However, research by Vassena and colleagues does suggest embryos are capable of transcription prior to embryonic genome activation (EGA). Through genomic-wide transcript analysis of human oocytes and embryos, Vassena and colleagues detected a series of successive waves of embryonic transcriptional initiation events beginning as early as the 2-cell stage [16]. Therefore, their findings suggest transcriptional events in human embryos may begin as early as the 2-cell stage, and not at the 4-8 cell stage as previously thought. Although unexplored in bovine embryos, our results indicate transcriptional events may be occurring earlier than EGA. It can be hypothesized SG embryos may initiate transcriptional events earlier as a response to its delayed development. Early activation of the embryonic genome may serve as a repair mechanism to salvage an embryo during the pre-implantation period.

It should also be noted bta-miR-1343 and bta-miR-2443 had gene targets relating to spermatogenesis. Previous research profiling the origins of embryonic miRNAs has suggested the majority of miRNAs expressed prior to EGA are of maternal origin. However, researchers did discover that embryos can also inherit sperm-borne miRNAs. Although bta-miR-1343 and bta-miR-2443 have not been annotated in mammalian sperm, results from our study suggest that these miRNAs are of paternal origin capable of influencing transcriptional events in an embryo.

It should also be highlighted bta-miR-1343, bta-miR-760-5p, and bta-miR-450b were co-detected in more than one SM condition. Bta-miR-1343 was found to be upregulated in the SM of SG embryos at the 2-cell and 8-cell stage. With regards to bta-miR-760-5p and bta-miR-450bs, their expression was detected in media conditioned with the SG 8-cell and blastocyst embryos. Contrasting bta-miR-1343, both bta-miR-760-5p and bta-miR-450b were upregulated in 8-cell SG media, then were downregulated in blastocyst SG media. Although all 3 miRNAs have not been previously annotated in embryos or in SM, their consistent expression between 2-cell and 8-cell or 8-cell and blastocyst SM respectively, suggest they may have functional roles in normal and aberrant embryo development.

Conclusions

Overall, this study was the first to detect miRNA expression differences in the SM between SG and FG embryos with miRNAomic analysis. Across all 3 developmental stages examined (2-cell, 8-cell, and blastocyst), embryos with delayed development expressed more miRNAs in the SM, than those developing at a faster rate. It is postulated that the difference in miRNA expression is associated with increases in metabolic activity observable in non-viable embryos. Moreover, our findings also highlight novel miRNA biomarkers correlated with slow and fast-growing embryos at 2-cell, 8-cell, and blastocyst stage of development. Future research should focus on validating these miRNAs in the SM and within the embryo with subsequent gene expression studies to further elucidate the roles of these miRNAs in embryonic development.

Supplementary Materials: Table S1: Predicted mRNA targets of miRNAs differentially expressed between 2-cell SG, Table S2: Predicted mRNA targets of miRNAs differentially expressed between 8-cell SG SM vs. 8-Cell FG SM, Table S3: Predicted mRNA targets of miRNAs differentially expressed between blastocyst SG SM vs. blastocyst FG SM.

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Author Contributions: PD and PM conceived, developed, and planned the experiments. PD performed all the experiments and including sample preparation and data analysis. The manuscript was written by PD and SD, prepared by SD, with the support and guidance of PM. PM supervised the project.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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