Revealing the bovine embryo transcript profiles during early *in vivo* embryonic development

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Abstract

Gene expression profiling is proving to be a powerful approach for the identification of molecular mechanisms underlying complex cellular functions such as the dynamic early embryonic development. The objective of this study was to perform a transcript abundance profiling analysis of bovine early embryonic development *in vivo* using a bovine developmental array. The molecular description of the first week of life at the mRNA level is particularly challenging when considering the important fluctuations in RNA content that occur between developmental stages. Accounting for the different intrinsic RNA content between developmental stages was achieved by restricting the reaction time during the global amplification steps and by using spiked controls and reference samples. Analysis based on intensity values revealed that most of the transcripts on the array were present at some point during *in vivo* bovine early embryonic development, while the varying number of genes detected in each developmental stage confirmed the dynamic profile of gene expression occurring during embryonic development. Pair-wise comparison of gene expression showed a marked difference between oocytes and blastocysts profiles, and principal component analysis revealed that the majority of the transcripts could be regrouped into three main clusters representing distinct RNA abundance profiles. Overall, these data provide a detailed temporal profile of the abundance of mRNAs revealing the richness of signaling processes in early mammalian development. Results presented here provide better knowledge of bovine *in vivo* embryonic development and contribute to the progression of our current knowledge regarding the first week of life in mammals.

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Introduction

The first week of embryonic development is marked by a number of critical events. Maternal transcripts that support oocyte maturation and early embryonic development are synthesized and stored in the growing oocyte (Wassarman & Kinloch 1992). In the mouse, meiotic maturation and fertilization are known to trigger a rapid degradation of maternal transcripts (Piko & Clegg 1982, Bachvarova *et al.* 1985). The remaining maternal mRNAs sustain protein synthesis during the transcriptional silent phase until the newly formed embryonic genome is activated. In bovine embryos, the maternal to embryo transition (MET) occurs at the 8- to 16-cell stage (Memili *et al.* 1998). During the following stages, important morphological and functional changes occur to produce the blastocyst that is characterized by the presence of differentiated cellular structures namely the trophectoderm and the pluripotent cells of the inner cell

mass. All these steps are likely to be associated with marked changes in gene expression profiles, or more precisely RNA abundance, since prior to the MET the detected RNAs originate from the maternal stocks rather than being the product of immediate transcription. Moreover, the need to perform a descriptive RNA abundance profiling across bovine embryonic development is ambitious since the limited amount of starting material requires the use of an amplification method that often introduce biases that consequently prevent reliable comparison between developmental stages. Indeed, the pre-amplification procedures, either PCR or *in vitro* transcription based, have been developed to keep the relative abundance of the transcripts within a sample, but they have not been optimized for keeping the relative abundance of starting material between samples that show a wide range of starting material quantity. This is particularly important when comparing samples naturally containing different amounts of RNA such as

in the case of oocytes and/or early bovine embryos. Using an appropriate method that keeps the relative abundance of transcripts within and across developmental stages will provide better knowledge of the molecular mechanisms controlling *in vivo* embryonic development.

In the last years, numerous microarray profiling studies have been published regarding embryonic development, notably in the mouse (Hamatani *et al.* 2004a, 2004b, 2004c, Wang *et al.* 2004, Zeng *et al.* 2004, Zeng & Schultz 2005, Yoon *et al.* 2006, Su *et al.* 2007). However, the unavailability of adequate sequence data for numerous species has kept the microarray technology beyond the reach of investigators working on economically and scientifically important large domestic species such as cattle, pigs, and sheep. Possible solutions to this problem is either the use of cross-species hybridizations (Robert *et al.* 2000, Dalbies-Tran & Mermillod 2003, Adjaye *et al.* 2004, 2007, Vallee *et al.* 2008) or the fabrication of homemade cDNA arrays (Yao *et al.* 2004, Whitworth *et al.* 2005, Vallee *et al.* 2006, Dessie *et al.* 2007, Ghanem *et al.* 2007). The commercial arrays (Affymetrix) that have been made available for the cow greatly facilitate bovine gene expression profiling (Fair *et al.* 2007). However, even if large cohorts of genes are available on this bovine array, the gene list indicates a poor representation of known oocyte and early embryonic specific gene candidates. Similar findings were previously shown from a mouse embryo expressed sequence tag (EST) project (Ko *et al.* 2000) where a significant fraction of the genes expressed in early life were not represented in the collections of genes available on commercial arrays. Following this EST project, a mouse developmental array was developed (Carter *et al.* 2003) and was subsequently used for all transcript profiling analysis of oocytes, embryos, and stem cells (Hamatani *et al.* 2004a, 2004b, 2004c, Aiba *et al.* 2005, Vallee *et al.* 2008).

In this study, we present the transcript profiling of bovine *in vivo* early embryonic development on a bovine developmental array containing over one thousand transcripts derived from bovine oocyte and blastocyst subtracted libraries. Oocytes and embryos from three developmental stages (two-cell, eight-cell, and blastocyst) were used for this gene expression profiling. We chose to analyze embryos that developed *in vivo*, rather than *in vitro*, to study the molecular mechanisms involved during *in vivo* embryonic development and thus avoid the effect of culture conditions on gene expression. Results presented here will provide better knowledge of bovine *in vivo* embryonic development, and also be used for comparative analysis of early development in other mammalian species, including mouse and human. Therefore, it should contribute to the progression of our current knowledge regarding the earliest stages of mammalian life.

Results

Array design and fabrication

A detailed description of array design and fabrication of a preliminary version of this array (v.1.0) has been previously published (Sirard *et al.* 2005). The microarray platform used in this study (v.1.2) contains 1153 transcripts highly relevant to early embryonic development that were derived from four distinct subtracted libraries (the complete list is given in Supplementary Table 1, which can be viewed online at www.reproduction-online.org/supplemental/). The transcripts derived from the first subtracted library are preferentially expressed in germinal vesicle (GV) oocytes as the transcriptome was subtracted to a pool of somatic tissues (liver, kidney, spleen, muscle, and cumulus cells; Vallee *et al.* 2005). The second subtracted library consists of transcripts preferentially found in GV oocytes as the maternal transcriptome was subtracted using the RNA content of day 8 *in vitro* produced blastocysts. The third subtracted library is the reverse experiment (day 8 blastocysts – GV oocytes) producing a gene collection containing candidates expressed following the MET. Finally, the fourth subtracted library is from day 8 *in vitro* produced blastocysts subtracted using a sample made from a pool of somatic tissues, which produces a gene collection enriched in candidates related to the specific nature of the blastocyst. The number of transcripts derived from each subtracted library found on the microarray slide is presented in Table 1. Each clone was sequenced and spotted in four replicates on the array. Furthermore, multiple controls that can be used as positive or negative control spots were added in a randomized fashion on the array. They consist of *GFP* cDNA fragment, Alien 1, Alien 2, and *Arabidopsis thaliana* *CAB1* gene from SpotReport™ cDNA Array Validation System (Stratagene, La Jolla, CA, USA), ubiquitous genes (actin, tubulin, and ubiquitin), and finally, spotting solution controls and empty controls. In this experiment, Alien 1 and Alien 2 spots were used as negative controls and *GFP* and *CAB1* genes that were spiked in the samples prior to hybridization were used as exogenous positive controls for data normalization.

Table 1 Number of transcripts derived from each subtracted library.

Library #	1	2	3	4
Library name ^a	GV oocytes	GV oocytes	Blastocysts	Blastocysts
	Somatic tissues	Blastocysts	GV oocytes	Somatic tissues
Number of transcripts	403	283	206	261

^aFor each library, transcripts derived from that library are preferentially expressed in the stage represented in italics. The subtraction procedure was performed with the stage below.

Figure 1 aRNA amplification yield. The average yield of aRNA obtained for each developmental stage after two rounds of linear amplification. Total RNA from three pools of ten oocytes or embryos was used for amplification using the RiboAmp HS RNA Amplification kit (Molecular Devices Corporation, Sunnyvale, CA, USA).

Validation of linear RNA amplification

Pools of ten oocytes or embryos were used for amplification, and, as shown in Fig. 1, the amount of amplified RNA (aRNA) generated from the restricted amplification reaction is different for each developmental stage, which is in corroboration with the natural RNA contents between these stages. During the amplification, the incubation period was limited to avoid a plateau phase, typical of any amplification method, which would hinder the capacity to perform across stage comparisons. Determination of the optimal incubation time was done by performing a time course that followed the reaction kinetics (Gilbert *et al.* 2009). To further validate that no distortion had occurred during the amplification reaction, quantitative PCRs were performed with four different genes from unamplified, first and second round amplification samples for all developmental stages (Fig. 2).

Microarray reproducibility

Reproducibility of microarray hybridization was verified as a quality assessment by calculating the correlation coefficients of signal intensities between replicated experiments in a pair-wise manner (Fig. 3A). The excellent mean correlation (0.98) found between replicated experiments is adding confidence in the validity of the experiment. Furthermore, an unsupervised hierarchical clustering using all genes found on the array clustered all replicates alongside their appropriate developmental stage (Fig. 3B). The clustering dendrogram (Fig. 3B) reveals that the two- and eight-cell embryos are subgrouped together. By contrast, the GV oocyte and blastocyst are independently significantly different from the other developmental stages.

Analysis of RNA abundance profiles in bovine *in vivo* embryos

To perform the analysis of gene expression in bovine *in vivo* embryos, all microarray data were uploaded in the NIA array analysis tool and a threshold was established according to the plot of error function to determine which candidates were generating signals significantly above background e.g. considered as present. Genes with a mean log signal intensity above the establish threshold (log 2.5) were considered as expressed, whereas the genes with signal intensities below the threshold were designated as 'absent'. This analysis based on intensity values revealed that the majority of the genes found on our bovine developmental array are expressed at some point during *in vivo*

Figure 2 Efficiency assessment of the amplification procedure in preserving the relative RNA content between developmental stages. Quantification by RT-PCR of four genes in bovine oocytes and *in vivo* embryos. Cyclin B1 (CCNB1), H3 histone, family 3A (H3F3A), lactate dehydrogenase B (LDHB), and NACHT leucine-rich repeat and PYD containing 9 (NLRP9). Reactions were performed with unamplified (gray line), first (pink line) and second round (blue line) amplification cDNA samples ($n=3$). Shown is the relative mRNA abundance (mean $\pm$ S.E.M.).

Figure 3 Microarray reproducibility. (A) Correlation coefficients of signal intensity between replicated experiments in a pair-wise manner. (B) Hierarchical clustering of replicated experiments. Colors correspond to relative RNA abundance for the detected genes, each of which is represented by one horizontal bar.

embryonic development. The number of genes detected varies between each developmental stage, confirming the dynamic profile of gene expression observed during embryonic development (Table 2).

Comparative transcript abundance profiling

To obtain a perspective on RNA abundance fluctuations, we performed a pair-wise comparison of gene expression microarray data for all developmental stages (Fig. 4). Expectedly, the pair that showed the biggest difference in the nature and abundance of transcripts was GV oocytes and blastocysts. As shown in the log ratio plot, 776 clones were overexpressed in GV oocytes and 52 were overexpressed in blastocysts (Fig. 4). Genes overexpressed in GV oocytes were predominantly derived from subtracted libraries 1 and 2, whereas genes overexpressed in blastocysts were exclusively derived from subtracted libraries 3 and 4. The complete list of genes overexpressed in GV

oocytes compared with two-cell, eight-cell, and blastocysts can be viewed in Supplementary Table 1. Furthermore, the comparison of two- and eight-cell embryos revealed no major difference between those two developmental stages (Fig. 4), which support the fact that they cluster together in the hierarchical clustering analysis (Fig. 3).

Maternal versus embryonic transcripts

Principal component analysis of our microarray data revealed that the majority of the genes expressed during the bovine first week of embryonic *in vivo* development are grouped in three main clusters corresponding to three general trends of abundance patterns (Fig. 5 and Supplementary Table 1). Additionally, classification of genes according to the Gene Ontology biological process category allowed the functional classification of genes making up the three main general trends (Khatri *et al.* 2002). The first two clusters present a profile

Table 2 Genes detected in each developmental stage.

Stage	Genes detected ^a	(%) ^b
Oocyte	1137	98.61
Two-cell	1038	90.03
Eight-cell	1055	91.50
Blastocyst	981	85.08

^aClones with a mean log signal intensity above the calculated background threshold (log 2.5) were considered as expressed.

^bPercentage of genes expressed on total number of transcripts on the array.

characteristic of maternal genes (Fig. 5A and B). The last cluster, corresponding to a maternal-to-embryonic expression pattern, regroups 10% of the genes in our microarray data (Fig. 5C).

Validation of microarray data by quantitative RT-PCR

Finally, confirmation of the normalized microarray data was performed by a quantitative RT-PCR analysis of four transcripts. The selection of these candidates used for validation was based on the clustering analysis described previously (Fig. 5). Two transcripts with a maternal profile, *NLRP9* and *CCNB1* (Fig. 5A and B), and two transcripts with a maternal-to-embryonic profile, *H3F3A* and *LDHB* (Fig. 5C), were chosen as representative of the major expression profiles observed. Aliquots of *in vivo* embryos unamplified RNA were used for the PCR amplifications and detailed information regarding the primers is presented in Table 3. Comparing the profiles generated from the PCR amplifications to the one produced by the microarray data allowed validation of our normalized microarray data. The results shown in Fig. 6 confirm the profiles of all candidates detected by microarray. Although no significant difference was observed between the developmental stages for *H3F3A*,

the expression profile obtained from the quantitative RT-PCR was similar to the one obtained from the microarray data (Fig. 6).

Discussion

The main objective of this study was to perform a transcript abundance profiling analysis of bovine early embryonic development *in vivo* using a bovine developmental array. The molecular description of the first week of life at the mRNAs level is particularly challenging when considering the important fluctuations in RNA content that occur between developmental stages. Since embryos contain very little RNA for evaluation, a modified linear amplification method was used to produce aRNA in sufficient amount to perform microarray hybridizations. During the amplification, the incubation period was limited to avoid a plateau phase, typical of any amplification method, which would hinder the capacity to perform across stage comparisons. As shown, the abundance patterns of the unamplified samples throughout development are conserved after the first round of amplification and most importantly after the second round of amplification. Therefore, the modified amplification method used in this study preserved the relative RNA content between samples, which is essential to allow the production of physiologically relevant developmental stage profiles. Additionally, a key step to keep the relative initial RNA contents between samples was done during data normalization. Indeed, an equal amount of aRNA was used to prepare all microarray probes in order to avoid the bias caused by the high variability, which characterizes low intensity signals that would have been generated in a much larger proportion with the low RNA content stages. This aRNA equalization alleviates the natural RNA content differences between developmental

Figure 4 Mean pair-wise comparison. Scatter plots representing gene expression between each pair of developmental stages. Horizontal axis represents the average log intensity of clones, whereas a vertical axis represents the log ratio of signal intensity for each clone between one stage and another stage. Colored spots (red and green) represent clones that passed the FDR 10%. Red spots represent array features with higher expression at the stage indicated on that row, whereas green spots represent array features with lower expression. The number of clones overexpressed is indicated in red and the number of clones underexpressed is indicated in green besides each scatter plot.

Figure 5 Clustering analysis. The NIA array analysis tool was used to perform a principal component analysis (three principal components, 1.5-fold change, and 0.7 correlation threshold) using all genes on the array. The vast majority of present transcripts (99%) fall within one of these clusters. Maternal profile (A and B) and maternal-to-embryonic profile (C) are observed. Gray lines represent transcript intensities, and the red line is the average intensity of the cluster.

stages that were preserved during the pre-amplification step. In order to take this into account, following the amplification reaction, an equal amount of an *in vitro* produced RNA coding for GFP was spiked in every sample. Since the concentration of aRNA differed between the samples, the volume taken from each

samples for hybridization proportionally differed. The signals generated by the GFP spikes were representative of the RNA yields for each developmental stage and were used to correct the data to the initial natural relative abundance between developmental stages. Failing to do so would have involved the comparison of different amount of embryos at each developmental stage, which would have compromised the capacity to perform across stage analyses. The validation of the entire data normalization procedure was performed with quality assessment of the microarray data and quantitative PCR profiles.

Unsupervised hierarchical clustering independently clustered all the replicates by their appropriate developmental stage with minimal branch tree distances. With the exception of a few genes, most of the genes behaved similarly across the three replicates for each developmental stage. The wider range of RNA abundance among replicates for some genes may reflect the asynchrony of embryo development *in vivo*. The clustering of the two- and eight-cell embryos from the other stages is consistent with the fact that there is very little transcription before the eight-cell stage (Memili *et al.* 1998). Although the depletion of maternal mRNA either through translation or RNA decay begins during oocyte maturation, destruction of these mRNAs is typically not complete until the 8- to 16-cell stages. Thus, most of the transcripts present are maternally derived. By contrast, transcripts present at the blastocyst stage are, for the most part, newly synthesized and form a distinct population compared with the one found in GV oocytes. Although the onset of embryonic transcription occurs at different stages depending on the species (Telford *et al.* 1990), independent studies performing hierarchical clustering of microarray experiments on mouse pre-implantation embryos also clustered developmental stages according to MET; prior to embryonic genome activation, during major reprogramming, or after embryonic genome activation (Hamatani *et al.* 2004a, 2004b, 2004c, Zeng *et al.* 2004). The concordance of the hierarchical cluster

Table 3 Primers used for validation by quantitative PCR.

Gene name	Gene symbol	Primer sequences	Amplicon size (bp)	Annealing temperature (°C)	Melting temperature (°C)	Accession number
Cyclin B1	CCNB1	Up: 5'-GATCAGCACTCTAGCACAGC-3' Low: 5'-AAAGGATAAGTAAAAAGAACTTCAAC-3'	181	52	81	NM_001045872
H3 histone, family 3A	H3F3A	Up: 5'-CATGGCTCGTACAAAGCAGA-3' Low: 5'-AGACGCTGGAAGGGAAGTTT-3'	213	55	86	NM_001014389
Lactate dehydrogenase B	LDHB	Up: 5'-AAGTGGATTACCAAGCACCCT-3'	181	59	84	NM_174100
NACHT, leucine-rich repeat and PYD containing 9	NLRP9	Low: 5'-AGAAACGCCTGCCACATTCCT-3' Up: 5'-TACGATGCTGGCACCAACAGT-3' Low: 5'-AGACTGACCAAGGCTTCACACA-3'	215	58	88	NM_001024664

Figure 6 Microarray validation by quantitative PCR. Quantification of cyclin B1 (*CCNB1*), H3 histone, family 3A (*H3F3A*), lactate dehydrogenase B (*LDHB*), and NACHT leucine-rich repeat and PYD containing 9 (*NLRP9*) mRNA levels in bovine oocytes and embryos during *in vivo* embryonic development. Reactions were performed with unamplified cDNA samples ($n=3$). Shown is the relative mRNA abundance (mean $\pm$ S.E.M). Different letters indicate a significant difference of relative mRNA abundance ($P<0.05$).

analysis with previous results found on a different species lends confidence to the reliability of the conclusions drawn from our transcript profiling of prehatching bovine embryos.

When pair-wise comparison of genes present across all developmental stages was performed, the pair that showed the biggest difference was GV oocytes and blastocysts. Known oocyte-specific genes are included in the list of genes overexpressed in oocytes such as, *ZP1*, *ZP2*, *ZP3*, and *FIGLA* (Philpott *et al.* 1987, Liang *et al.* 1990, 1997, Epifano *et al.* 1995). Other genes that were previously identified in our laboratory and in others as preferentially expressed in oocytes were also present in this list such as: *BTG4*, *HMG2*, and *PAIP1* (Vigneault *et al.* 2004, Pennetier *et al.* 2005, Vallee *et al.* 2005). The particular expression profiles of these genes suggest a possible implication in early bovine development and embryonic genome activation. The similar profiles observed between the two- and eight-cell stages are probably due in part by the lack of genes derived from these two developmental stages found on the array but also it reflects the fact that major maternal mRNA depletion events occur before the two-cell stage, as seen in the mouse (Piko & Clegg 1982). Following this major wave of mRNA decay, the expression profiles seem to maintain a certain stability prior to embryonic genome activation occurring at the 8- to 16-cell stages (Memili *et al.* 1998).

Three general trends of abundance patterns among genes expressed during the bovine first week of embryonic *in vivo* development were uncovered by principal component analysis of our microarray data. The first two trends are characteristics of maternal genes and thus suggest an important role for these transcripts

during early embryonic development. Functional classification revealed that regulation of transcription and cell cycle are the biological processes overrepresented in both of these clusters. The first cluster represents genes that are rapidly degraded following fertilization (such as *NLRP9*, *FIGLA*, *ZP2*, *ZP3*, and *ZP4*) and the second cluster represents progressively degrading transcripts (such as *CCNB1*, *HMG2*, *PTTG1*, and *BTG4*). The profiles observed for *NLRP9* and *HMG2* obtained using microarrays are similar to previously reported expression profiles during bovine *in vitro* embryonic development obtained by PCR (Vigneault *et al.* 2004, Dalbies-Tran *et al.* 2005). In the last cluster, corresponding to a maternal-to-embryonic expression pattern, some transcripts are equally abundant in oocytes and blastocysts (such as *H3F3A* and *EEF1A1*) while others are more abundant in blastocysts than in oocytes (such as *LDHB* and *HIST4A4*). Functional classification revealed that translation and oxidation reduction are the biological processes represented in this last cluster. Genes found in the later cluster present a pattern characterized by a rapid decrease in maternal mRNAs after fertilization, followed by an increase in expression after embryonic genome activation, suggesting a constant role for these factors during embryonic development. Finally, a validation of the normalized microarray data by quantitative RT-PCR confirmed the profiles of four selected transcripts with either maternal or maternal-to-embryonic profiles. Moreover, this validation provided the confirmation that our microarray normalization strategy used to correct for the different amounts of RNA found in each developmental stage was successful and did not create any distortion.

In conclusion, the study presented herein provides a profile of a unique collection of genes having their RNA present during bovine prehatching embryonic development *in vivo*. The strategy used in this study to correct for the varying amount of RNA found in each developmental stage, that is the use of a modified RNA amplification technique combined to a microarray normalization procedure, will provide a more accurate outlook of the transcript abundance profiling during *in vivo* bovine embryonic development. However, since the microarray platform used in this study covers only a portion of the genome, we are currently working on the enrichment of our bovine development microarray platform specially to include genes derived from each developmental stage. Nevertheless, results obtained in this study have proven that microarray is a powerful tool for the study of gene expression profiling during embryonic development. The major strength of this experiment relies on the fact that *in vivo* embryonic development was studied with a developmental array enriched with transcripts expressed during early embryonic development. This *in vivo* embryonic development profiling will serve as a reference for future experiments and will enable comparisons between IVF protocols, developmental stages, and even between laboratories. Finally, a better comprehension of bovine early embryonic development *in vivo* will contribute to the progression of our current knowledge regarding the first week of life in mammals.

Materials and Methods

Oocytes and embryos collection

Holstein and Simmental heifers were used for the oocytes and embryos collection. For the collection of GV oocytes, cumulus-oocyte complexes from 3 to 6 mm follicles were obtained by follicle liquid aspiration from ovaries as previously described (Vigneault *et al.* 2004). The oocytes were mechanically denuded by vortexing and washed several times in PBS solution to prevent cumulus cell contamination. For the collection of *in vivo* two- and eight-cell embryos, animals were stimulated by application of Stimufol (FSH:LH=5:1) twice a day at a dosage of 1.0 ml for a period of four consecutive days. Following the stimulation, animals were artificially inseminated using bull spermatozoa. The embryo recovery was done by Besenfelder *et al.* (2001) and performed as described previously. Briefly, tubal endoscopic flushing was performed by accessing the oviducts using a glass capillary that was replaced by a silicon-covered flushing metal tube. Forty to sixty ml PBS plus 1% FCS were flushed through the oviducts and collected via an embryo flushing catheter in an embryo filter (Em Con, no. 04135; Immuno Systems Inc., Spring Valley, WI, USA). A further 300–400 ml was used for additional flushing of the uterine horns. To get two-cell stage embryos, donor animals were flushed 48 h post-insemination, while eight-cell stage embryos were obtained by flushing at 72 h post-insemination. For the collection of blastocysts,

donor cows were prepared according to the following protocol. A total of 320 mg p-FSH (Folltropin; Bioniche Animal Health, Montréal, Quebec, Canada) was given using eight i.m. injections in decreasing concentrations over a 5-day period. Content of dominant follicles (>6 mm) was aspirated using an ultrasound guided system 36–48 h before the first FSH injection, which is any time between day 6 and day 12 of the estrous cycle. A prostaglandin injection (500 µg estrumate; Schering, Montréal, Quebec, Canada) was given to induce the luteolysis of the corpus luteum at the eight p-FSH injections. Another prostaglandin injection was given 12 h after the first one. The cow was inseminated with frozen semen 12 and 24 h after the first signs of estrus. The embryos were recovered from the uterine horns 7 days after the first signs of estrus by flushing the uterine horns with a PBS solution. Once recovered, morphological good quality GV oocytes, two-cell embryos, eight-cell embryos, and blastocysts were washed twice in PBS, frozen in Cryotubes in groups of 10, and stored at –80 °C until RNA isolation. To limit the possibility of individual effect, pools of ten specimens repeated thrice for each developmental stage for a total of 120 embryos coming from 23 heifers and four Holstein bulls were used for array analysis. Animals were cared for according to the recommended code of practice following the guidelines of the Canadian Council on Animal Care (1993).

RNA extraction and amplification

Total RNA was extracted from three pools of each developmental stage using either the Picopure RNA extraction kit (Molecular Devices Corporation, Sunnyvale, CA, USA) or the Absolutely RNA Microprep Kit (Stratagene) according to the manufacturer's instructions. Extensive validations revealed the similarity between RNA extracted from both kits (I Gilbert, S Scantland, I Dufort, M-A Sirard & C Robert 2007, personal communication). Next, a DNase treatment was included for the removal of genomic DNA contamination. RNA was precipitated using sodium acetate (0.1 V), isopropanol (1.5 V), and linear acrylamide (20 µg/ml) (Ambion, Austin, TX, USA) as a carrier. RNA was resuspended in 11 µl water and stored at –80 °C. Total RNA was amplified by two rounds of linear amplification using the RiboAmp HS RNA Amplification kit (Molecular Devices Corporation) according to the manufacturer's instructions except the *in vitro* transcription lasted 80 min in the first round and 180 min in the second round to avoid the plateau phase of the reactions as determined by a time course analysis of the kinetics of the *in vitro* transcription reactions (Gilbert, 2009). During the second round of amplification, UTP amino-allyl (Ambion) were incorporated. The aRNA was quantified with the Nanodrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA).

Probe labeling and microarray hybridization

For every developmental stage, 2.5 µg amplified amino-allyl modified RNA from each sample was used for labeling with Alexa fluor 647 (Molecular Probes, Burlington, Canada) according to the manufacturer's protocol. The labeled RNA

was purified using the Picopure RNA extraction kit (Molecular Devices Corporation) and resuspended in 15 μ l elution buffer. Each probe was quantified using the Nanodrop ND-1000 (NanoDrop Technologies). A universal reference was used as a standard for our microarray experiments. This universal reference pool, composed of aRNA from GV oocytes and blastocysts obtained through the modified amplification protocol described above and DNA from a subtracted library obtained from a subtraction of bovine GV oocytes and somatic tissues (Vallee *et al.* 2005), was labeled with Alexa fluor 555 (Molecular Probes). Three microgram aliquots of the labeled reference were used in every hybridization.

As positive controls, GFP fragment (Vigneault *et al.* 2004) and *CAB1* gene from *A. thaliana* included in the SpotReport cDNA Array Validation System (Stratagene) were also amplified and labeled. To account for the equalization of the natural RNA content differences between developmental stages when using equal amount of aRNA for microarray probes, 100 ng GFP amino-allyl modified aRNA was added to each sample following the second round of amplification prior to labeling with Alexa fluor 647 (Molecular Probes). For the *CAB1* control, 15 ng DNA labeled with Alexa fluor 555 and 647 (Molecular Probes) were added to the probes prior to hybridization.

Hybridizations were performed in triplicate on our bovine developmental microarray slide (v.1.2) containing 1153 transcripts derived from subtracted libraries (Sirard *et al.* 2005). Hybridizations were performed using the Slidehyb buffer #1 (Ambion) at 50 °C for 18 h in the SlideBooster (Advalytix, San Francisco, CA, USA). Slides were then washed twice with 2 $\times$ SSC–0.5% SDS at 50 °C for 15 min and twice with 0.5 SSC–0.5% SDS at 50 °C for 15 min. Next, the slides were dipped thrice in 1 $\times$ SSC and thrice in H₂O. Finally, the slides were dried by centrifugation at room temperature at 1200 g for 5 min.

Microarray image processing

Slides were scanned using the VersArray ChipReader System (Bio-Rad) and visualized with the ChipReader software (Media Cybernetics, San Diego, CA, USA). Microarray image processing was performed with the ArrayPro Analyzer software (Media Cybernetics). Microarray experiments presented in this study adhere to the standards proposed by the Microarray Gene Expression Data Society. Raw and normalized data are stored in the public ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MEXP-2075.

Microarray data analysis

A three-step strategy was used to normalize our microarray data. First, in order to remove any spatial effect on the array, an ANOVA analysis was performed on each array, by computing and subtracting any column effect (four columns of subarrays on each array), any row effect (considering only two rows of subarrays: the top 16 and the bottom 16 subarrays), and any column/row interaction. Then, in order to account for hybridization efficiency between arrays, an ANOVA analysis

was performed on all the arrays using only the reference samples. An array effect was then estimated and removed from the expression of both the reference and the developmental stage expressions. Finally, in order to take into consideration the difference in RNA quantities found naturally in each developmental stage as seen in Fig. 1, the spiked-in GFP controls were used for normalization. Prior to labeling, each sample was spiked with equal amount of GFP (100 ng). Then, for each probe, an equal amount of aRNA (2.5 μ g) was used for hybridization. This allowed optimal hybridization, but consequently varied the quantity of embryos and GFP used for each developmental stage. The signals generated by the GFP spikes were representative of the RNA yields for each developmental stage. By normalizing our data with the signals generated by the GFP, we were able to correct the data to the natural relative RNA abundance between developmental stages. This consequently transformed the dataset on each array to account for the natural differences in RNA content that characterized the bovine early developmental stages while maintaining optimal hybridization conditions (Fig. 1). The normalized data were next uploaded in the NIA array analysis tool for analysis (Sharov *et al.* 2005). First background threshold was determined according to the plot of error function (s.d. (=square root of the error variance), versus expression level (log intensity)). Clones with a mean log signal intensity above the calculated background threshold (log 2.5) were considered as present. Next, a one-way ANOVA (default parameters, except FDR<0.1) was conducted. Further data processing including correlation coefficient, hierarchical clustering, and mean pair-wise comparison were also performed through NIA microarray analysis tool.

DNA sequencing and clones identification

DNA sequencing and clones identification were performed as previously described (Sirard *et al.* 2005). Briefly, the resulting sequence traces were visualized with the online freeware Chromas 1.45 (<http://www.technelysium.com.au/chromas.html>) and uploaded into a cDNA Library Manager program (coded by Genome Canada Bioinformatics) that automates and facilitates sequence analysis and clone identification (Vallee *et al.* 2005). For clone identification, sequence traces were uploaded into the cDNA Library Manager, trimmed (Phred software, Seattle, WA, USA) and compared against a locally installed GenBank database (Basic Local Alignment Search Tool (BLAST) <http://www.ncbi.nlm.nih.gov/blast/>).

Quantitative PCR

Primer sequences are shown in Table 3. For each gene examined, a representative standard curve, consisting of PCR products purified with the QIAquick PCR Purification Kit (Qiagen) and quantified with a Nanodrop ND-1000 (NanoDrop Technologies) was included in the run. The standard curve consisted of four dilutions of the purified PCR products (diluted from 0.1 pg to 0.1 fg). Quantitative PCR was executed on a LightCycler apparatus using SYBR green incorporation (Roche Diagnostics). Aliquots of the same total RNA used for microarray hybridizations were reverse transcribed using

4U of Sensiscript Reverse Transcriptase (Qiagen) in a reaction containing 1×RT buffer, 0.5 mM dNTP, 10U RNase inhibitor (Promega) and 10 µM random decamers (Ambion). The RNA was incubated with random decamers 5 min at 65 °C then placed on ice. The other components were added to the reaction and incubated 10 min at 25 °C to allow priming of the random decamers and then 1 h at 37 °C. Each PCR contained cDNA corresponding to 1/20 embryo and a reaction mixture consisting of 0.25 mM of each primer, 3 mM MgCl₂ in 1× SYBR green mix containing dNTPs, FastStart Taq DNA polymerase enzyme, and buffer (Roche). The PCR conditions used for all genes were as follows: denaturing cycle of 10 min at 95 °C; 50 PCR cycles (denaturing: 95 °C for 5 s; annealing: specific temperature, see Table 3, for 5 s; extension: 72 °C for 20 s); followed by a melting cycle. The DNA quantification was performed using LightCycler Software Version 3.5 with comparison with the standard curve. A one-way ANOVA and Tukey's test were performed using SAS Software Version 9.1 to determinate the statistically significant differences in mRNA levels between each developmental stage. The statistical significance was set at $P < 0.05$. The PCR products specificity was confirmed by analysis of the melting curve given by the Lightcycler software. The products were then electrophoresed on a 1% agarose gel and were sequenced to confirm that the proper product was amplified.

Declaration of interest

The authors declare there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

M V was involved in the conceptualization of this project, produced a subtracted library, analyzed the results, and wrote the manuscript. I D carried out the RNA extraction and amplification, labeling and hybridization of the microarray. S D performed the quantitative RT-PCR and analyzed the results. A L designed and carried out the microarray data normalization. C G produced three subtracted libraries and designed and produced the bovine developmental microarray slide (v.1.2). I G designed the protocols used for the RNA amplification procedure. C R was involved in the conceptualization and realization of this project, and critically reviewed the manuscript for important intellectual content. M A S was involved in the conceptualization of this project and the preparation of the manuscript, and provided mentorship. All authors read and approved the final manuscript.

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