IN THIS ISSUE, PSYCHOLOGY
Correction for the “In This Issue” summary entitled “Universal displays of pride and shame,” which appeared in issue 33, August 19, 2008, of Proc Natl Acad Sci USA (105:11587–11588).

The authors note that the figure is copyrighted by Bob Willingham and is reprinted with permission. The online version has been corrected. The figure and its corrected legend appear below.

Blind athletes (Right) show pride in victory like sighted athletes (Left). [Reproduced with permission (Copyright 2004, Bob Willingham).]

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PERSPECTIVE

The authors note that a reference was inadvertently omitted from their article. On page 4607, right column, in Conclusion: How Do New Gene Clusters Form?, line 17, the reference callout “(109–111)” should instead read “(109–111, 113).” The added reference appears below.


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DEVELOPMENTAL BIOLOGY

The authors note that due to a printer’s error, the affiliation information for some authors appeared incorrectly. The correct affiliation for V. Havlicek and U. Besenfelder is “Reproduction Centre-Wieselburg, University of Veterinary Medicine, 1210 Vienna, Austria”; and the correct affiliation for H. Lehrach and J. Adjaye is “Department of Vertebrate Genomics, Max Planck Institute for Molecular Genetics, 14195 Berlin, Germany.”

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APPLIED BIOLOGICAL SCIENCES

The authors note that the author name Christian Steinckluler should have appeared as Christian Steinckluler. The author line has been corrected online. The corrected author line appears below.

Claudia Colussi, Chiara Mozzetta, Aymone Gurtner, Barbara Illi, Jessica Rosati, Stefania Straino, Gianluca Ragone, Mario Pescatori, Germana Zaccagnini, Annalisia Antonini, Giulia Minetti, Fabio Martelli, Giulia Piaggio, Paola Gallinari, Christian Steinckluler, Emilio Clementi, Carmela Dell’Aversana, Lucia Altucci, Antonello Mai, Maurizio C. Capogrossi, Pier Lorenzo Puri, and Carlo Gaetano

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IMMUNOLOGY

The authors note that due to a printer’s error, in the Abstract, beginning on line 6, “TDCs include two conventional dendritic cell (DC) subtypes, CD8loSirp+ and CD8hiSirp−, which have different origins. We found that the CD8+Sirp− DCs represent a conventional DC subset that originates from the blood and migrates into the thymus” should instead read: “TDCs include two conventional dendritic cell (DC) subtypes, CD8loSirp+ and CD8hiSirp−, which have different origins. We found that the CD8+Sirp− DCs represent a conventional DC subset that originates from the blood and migrates into the thymus.”

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Genome-wide expression profiling reveals distinct clusters of transcriptional regulation during bovine preimplantation development in vivo


1Institute of Farm Animal Genetics, Friedrich Loeffler Institute, Mariensee, 21355 Neustadt, Germany; 2Reproduction Centre-Wiesbaden, University of Veterinary Medicine, 1210 Vienna, Austria; and 3Department of Vertebrate Genomics, Max Planck Institute for Molecular Genetics, 14195 Berlin, Germany

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Bovine embryos can be generated by in vitro fertilization or ovarian nuclear transfer; however, these differ from their in vivo counterparts in many aspects and exhibit a higher proportion of developmental abnormalities. Here, we determined for the first time the transcriptomes of bovine metaphase II oocytes and all stages of preimplantation embryos developing in vivo up to the blastocyst using the Affymetrix GeneChip Bovine Genome Array which examines approximately 23,000 transcripts. The data show that bovine oocytes and embryos transcribed a significantly higher number of genes than somatic cells. Several hundred genes were transcribed well before the 8-cell stage, at which the major activation of the bovine genome expression occurs. Importantly, stage-specific expression patterns in 2-cell, 4-cell, and 8-cell stages, and in morulae and blastocysts, were detected, indicating dynamic changes in the embryonic transcriptome and in groups of transiently active genes. Pathway analysis revealed >120 biochemical pathways that are operative in early preimplantation bovine development. Significant differences were observed between the mRNA expression profiles of in vivo and in vitro matured oocytes, highlighting the need to include in vivo derived oocytes/embryos in studies evaluating assisted reproductive techniques. This study provides the first comprehensive analysis of gene expression and transcriptome dynamics of in vivo developing bovine embryos and will serve as a basis for improving assisted reproductive technology.

Early mammalian embryogenesis critically depends on a tightly controlled, well orchestrated program of gene expression. Fertilization and the early cleavage stages are associated with massive epigenetic reprogramming of the paternal and maternal chromatin and initiated by maternal RNA and proteins accumulated during oogenesis and the final stages of oocyte maturation (1). Large scale synthesis of messenger RNA from the diploid embryonic genome is initiated at a species-specific time point. This occurs in murine embryos at the end of the first cell cycle, in human embryos at the 4-cell stage and in bovine embryos at the 8-cell stage (2–4). The characterization of normal cell cycle, in human embryos at the 4-cell stage and in bovine embryos at the 8-cell stage (Fig. 1 A), reflecting the transition from maternal genome to embryonic genome transcription. The highest number of differentially regulated genes was found between 4-cell and 8-cell stage (Fig. 1 A and B), demonstrating the major onset of embryonic genome activation (EGA) at the 8-cell stage (3, 4). Amongst the several hundred transcripts identified, genes previously known to be embryonically expressed include DNMT3A, claudin (CLDN), tight junction protein 3 (ZO3), and p53 tumor suppressor phosphoprotein (TP53) (17, 18).

The fact that complex changes were observed in the transcriptome during the first cleavage stages indicates that many transcriptional adjustments take place before major EGA (p-cell


The authors declare no conflict of interest.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE12327).

1W.A.K. and S.S. contributed equally to this work.

2To whom correspondence should be addressed. Email: heiner.niemann@fli.bund.de.

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values; Fig. 1C, Fig. S1). In our dataset, 2,473 genes were significantly up-regulated (at least 2-fold) in 8-cell embryos, morulae, and blastocysts, compared to 2-cell and/or 4-cell stages. These can be classified as genes involved in EGA. Altogether 321 genes (13%) were up-regulated in the 2-cell stage and 285 genes (11.5%) were up-regulated in the 4-cell stage compared to zygotes; 197 (8%) genes were common to both of these two sets and remained up-regulated until the blastocyst stage.

A group of 48 genes were up-regulated in the 2-cell stage, down regulated in the 4-cell stage, and then up-regulated again remaining highly transcribed to the blastocyst stage. Among these were IL18 and tumor protein, translationally-controlled 1 (TPT1); the latter is one of the most abundantly expressed genes of embryonic genome activation seems to be evolutionarily conserved (18).

Another interesting pattern was seen in 22 genes (Fig. S1, Table S1) for which transcript levels peaked at the 2-cell stage. These 2-cell-specific genes, along with the 48 genes mentioned above, make a set of 70 genes, which may be important factors for the onset of embryonic genome activation. Of these 70 genes, 49 were previously unknown. The known genes include cysteine-rich, angiogenic inducer, 61 (Cyr61) which is an important determinant of the genetic reprogramming that occurs in meiosis prior to fertilization. The 2-cell stage was chosen as the reference stage due to the distinct cellular characteristics of 2-cell embryos (19) and carboxypeptidase E (CPE), which is involved in the biosynthesis of peptide hormones and neurotransmitters known to activate mitogen-activated protein kinase (20).

In total, approximately 350 significantly expressed genes could be identified which are transcribed in a stage-specific pattern before the major onset of EGA which occurs at the 8-cell stage. This implies specific functions of these genes during early cleavage stages.

**Confirmation of Array Data by Real Time RT-PCR.** Two independent RT-PCR analyses of the amplified RNA (aRNA) verified that the array data accurately reflect the relative abundance of selected amplified transcripts in the samples (Fig. S2).

To obtain independent verification, individual embryos were collected at each stage and analyzed by real time PCR. These were compared with published expression profiles of genes, known to be important for mouse, human, and bovine development (Fig. S3). BMP15, KIT, GDF9, STAT3, C-MOS and ZP2, 3, 4 are oocyte markers (2, 18, 21–25). The array analysis of bovine embryos revealed expression of ZP2, 3, 4, GDF9, BMP15, cadherin (CDH3), but also OCT4 (POU5F1), CLDN, ZO3, and TP53 (Fig. S3a).

The maternal expression profiles of DNMT1, ZP2, and GDF9 and the embryonic expression profiles of GLUT8, DNMT3A and VDAC2 were confirmed by real time RT-PCR from single embryos (n = 6). Genes with stage-specific expression (SPP1, BMPRIB, SUBT1H1) or with expression before EGA (POLR1C, CTSH, CD48) by the Affymetrix array analysis, were checked by real time RT-PCR (Fig. 2).

In control experiments for the RT-PCR, reverse transcription of oocyte RNAs was primed with either poly(T) oligos or random hexamers. Identical results were obtained irrespective of the primers used, indicating that the modifications of the Poly(A) tails in oocytes did not affect amplification and hybridization under our experimental conditions (data not shown).

**Cluster Profiles of Transcriptomes.** Clusters with similar expression profiles were grouped for a global view of preimplantation transcriptome dynamics (Fig. 3, Table S2). The 4,173 maternal transcripts could be grouped as high, medium, or rare transcript abundance in oocytes. These maternal transcript clusters were
characterized by a drop of transcription levels between the 4- and 8-cell stages. A second group of genes showing embryonic transcription includes three clusters representing 3,505 genes (Fig. 3). Significantly increased transcript levels were found from the 4-cell to 8-cell stage onwards, indicating genes (ID2, ZO3, CLDN4, TP53, etc.) transcribed during major embryonic activation.

In addition, stage-specific clusters were detected. The clusters of 8-cell enriched transcripts and reduced transcript prevalence in 8-cell stage are depicted (Fig. 3C). The stage-specific clusters of zygotes, 2–4-cell embryos, morulae, and blastocysts, are given in Table S2.

A novel finding is the rapid degradation of maternal messenger RNAs that occurs after the 4-cell stage concomitantly with the major onset of embryonic transcription at the 8-cell stage (Fig. 3A), instead of the previously assumed gradual degradation of maternal messenger RNAs (26, 27). This indicates that maternal RNA is actively degraded in a coordinated manner and that degradation and genome activation are tightly linked.

**Oocyte Enriched Genes.** A set of 429 genes were found to be at least 2-fold significantly regulated in in vivo oocytes, with respect to all other stages. Of this set, 35 genes were up-regulated only in oocytes (for example: PGF, RAD52, CST6, CPA1, POLR2J2). Some of these genes are depicted in Fig. 4A, which shows a decreasing expression pattern during preimplantation development (Table S3).

![Fig. 2. Stage-specific onset of gene expression before major genome activation. Array data, white columns; Real Time RT-PCR with in vitro embryos, black columns; Real Time RT-PCR with in vivo derived embryos, shaded columns.](image1)

![Fig. 3. Identification of new expression profiles by cluster analysis (grouping of genes by various algorithms (f.ex. K-means) according to their similarity in expression profiles across a set of experiments or samples). The expression graphs were generated by the K-Means clustering algorithm. (Top row) Three clusters showing maternal expression profiles and high, medium, or low expression at zygote stage. (Middle row) Three clusters with embryonic expression profiles and high, medium, or low expression at the blastocyst stage. (Bottom row) Two clusters with 8-cell-specific up and down-regulated transcript levels. Embryonic stages are depicted on the x axis.](image2)

![Fig. 4. Identification of oocyte enriched genes and of genes differentially expressed between in vivo and in vitro produced embryos. (A) Oocyte enriched genes. (B) Genes differentially expressed between in vivo and in vitro oocytes.](image3)
Comparison of the Transcriptomic Profile in Vivo and in Vitro Matured Bovine Oocytes. In vivo derived oocytes, more genes were detected than in their in vitro derived counterparts (12,049 vs. 11,332 transcripts) and 1,799 transcripts were significantly differentially regulated by 2-fold between the two types of oocytes. Of these, the majority of the genes (1,267) were up-regulated in in vivo derived oocytes compared to in vitro cultured oocytes (532). For example, NOLA2, SDHB, TKT, DDX39 and MPV17 were up-regulated, while MYB, AQP3, ORP150, BMP4 and STAT3 were less abundant in in vivo derived oocytes than in their in vitro matured counterparts (Fig. 4B). This study gave similar results to a previously published Affymetrix bovine chip analysis which identified 821 transcripts that were differentially expressed between in vivo and in vitro matured bovine oocytes (28).

Pathways Operative during Preimplantation Development. Pathway analysis delineated essential signaling and metabolic pathways in embryonic development. MAPK, TGF-beta, insulin signaling, and metabolic processes such as glycolysis and oxidative phosphorylation were functional and components of these pathways were subject to significant changes in transcript levels during preimplantation development (Fig. S4). It is noteworthy that the most prominent differences occurred between the 4-cell and 8-cell stages, reflecting an early transition from maternal to embryonic gene expression.

Discussion

Here, we provide the transcriptomes of in vivo grown oocytes and early embryonic stages from the bovine species (Bos taurus). These data are an important step toward an unbiased view of gene transcription and regulation during early mammalian development. Most studies on embryonic transcriptomes have been focused on the mouse (29–32), which shows peculiarities of preimplantation development potentially limiting its utility as a model for human embryogenesis. Previous studies have used bovine embryos from in vitro sources and/or smaller arrays, which yield fragmentary pictures of the transcriptional activity in early embryos (for review see ref. 33).

In this study, special care was taken to obtain the natural transcriptome profile. Advanced ultrasound guided follicular aspiration and laparoscopic techniques were used to isolate oocytes and oviductal embryonic stages with minimal invasive-ness from a selected group of fertile female cattle. Embryos were frozen immediately after isolation from the reproductive tract to preserve the in vivo transcriptome as best as possible. The presence of >12,000 transcripts in each embryonic stage is significantly higher than the 8,000 transcribed genes typical of several somatic tissues from humans or mouse (32) and supports the notion that embryonic cells express a greater proportion of the genome than differentiated somatic cells, reflecting the uncommitted status of the pluripotent blastomeres. This dataset of expressed gene and transcription profiles can be used to assess the influence of culture conditions and handling in vitro. Bovine and human preimplantation development are similar with respect to the timing of epigenetic reprograming and activation of embryonic genome activation and the results of this study may be valuable for the evaluation of human embryos produced by various ARTs.

Several hundred genes are transcribed before the major onset of gene expression. Their low levels of expression may explain why their presence was not detected in previous, less sensitive analyses. Previous reports have alluded to minor bovine embryonic genome activation between the zygote and 4-cell stage (34–37). Our data show that this minor EGA occurs between the 2–4-cell stages. Among the genes which were up-regulated in 2–4-cell stages, seven transcripts (SARS, IL18, CRABP1, ACO2, TXN2, SLC38A2, SLC25A43) were previously identified as enriched in normal 8-cell bovine embryos relative to alpha-amanitin-treated 8-cell embryos (4).

An important novel finding of this study was the discovery of several genes expressed exclusively at only one stage of preimplantation development. The transient stage-exclusive expression suggests that these genes are critical only for specific stages of development. Stage-exclusive expression of the eukaryotic translation initiation factor eIF-4c has been reported in mouse embryos at the 2-cell stage, when murine embryonic genome activation occurs (38). The bovine orthologues genes of eukaryotic translation initiation factor, EIF2, EIF3, EIF4 and EIF5, became selectively up-regulated at the 8-cell stage, when the bovine embryonic genome is activated indicating a conserved role of this gene family in murine and bovine embryos.

Transcripts related to DNA methylation, histone modification, and chromatin remodeling could be clustered based on maximal or minimal expression at the 8-cell stage, reflecting critical molecular remodeling at this time point. These epigenetic modifications are key regulators of developmental processes during preimplantation development (39, 40) and spurious epi-genetic marks may have long-lasting consequences for the offspring. The identification of unique expression profiles for these groups of genes in embryos grown in vivo makes it feasible to investigate how in vitro culture conditions affect the expression of these critical remodelers of the embryonic genome and subsequently to optimize in vivo culture conditions.

The limitations of the data set presented in this study are (i) gene ontology information for the probe sets (each gene is represented as 11–22 probes) of the bovine Affymetrix chip is still incomplete and probes for some known developmentally important genes, such as telomerase (TERT) are lacking, (ii) the in vivo derived embryos were collected from superovulated donor cows because cleavage and blastocyst stage embryo collection from non-superovulated donor cows is not feasible. The viability of bovine blastocysts derived from superovulated donors is well established (41, 42) and forms the basis for the global embryo transfer industry with approximately 670,000 in vivo and approximately 290,000 in vitro derived bovine embryos transferred in 2006 (43).

In conclusion, this is the first comprehensive evaluation of transcriptome dynamics during bovine preimplantation development in vivo, it represents the natural physiological status as accurately as possible because oocytes and embryos were directly isolated from the reproductive tract of live donors, frozen, and processed for analysis. Results indicate a highly dynamic transcriptome both before and after the major embryonic genome activation and reveal the coordinated breakdown of maternal transcripts coinciding with major genome activation. The early expression of approximately 350 genes may explain the sensitivity of embryos to changes in their environment. These data can be used to assess the impact of various assisted reproductive techniques on the embryo and improve the safety of these techniques in both bovine and human reproduction.

Materials and Methods

Isolation of in Vivo Preimplantation Stages from Synchronized Donors. The estrus cycles of donor animals from the experimental herd of the Institute for Farm Animal Genetics (Holstein-Friesian cows, 3–5 years of age) were synchronized with a gonadotropin treatment with a total dose of 500 IU porcine FSH (Pliuset) to induce a mild superovulatory response as described previously (44, 45). The animals were kept under identical conditions and were fed according to milk yields. In vivo matured oocytes were aspirated from the preovulatory follicles of 9 animals on day 0 (~ day of standing estrus) (46). For embryo collection, donor animals were inseminated with frozen/thawed semen samples from one Holstein-Friesian bull with proven fertility. Zygotes, 2-cell, 4-cell, and 8- to 16-cell stages were recovered by oviductal flushing using a minimally invasive endoscopic approach in 24 cows 1–4 days after artificial insemination (AI). These oviductal stages were collected in prewarmed PBS containing 0.5% BSA (45). Morulae
and blastocysts were collected by uterine flushing at day 7 or 8 after AI (7 animals) using routine nonsurgical procedures. All embryos were carefully evaluated under a stereomicroscope and only morphologically intact embryos meeting established criteria (47) were used. Embryos were pooled in groups of 5, in the case blastocysts, or 10 embryos for the other stages, washed in PBS + 0.1% PVA, frozen in a minimum amount of medium in siliconized 0.6 ml cups and stored at −80 °C. Typically, the time period between flushing and freezing was less than 30 min. Animal treatments were conducted according to the German animal welfare guidelines.

**In Vitro Maturation of Oocytes and In Vitro Production of Bovine Embryos.** Due to the limited numbers of in vivo developed embryonic stages, in vitro produced counterparts were used for optimization of the Real Time PCR assay and to verify the expression patterns of several genes.

Bovine oocytes and embryos were produced in vitro from slaughterhouse ovaries and preimplantation embryos described previously (48, 49). For fertilization, semen was used from the same bull that had been used for AI of the donor animals (see above). Metaphase II oocytes (24 h maturation), zygotes, 2-cell, 4-cell, 8-cell, and blastocysts were harvested 19 h, 37–39 h, 39–40 h, day 3, day 5, and day 7/8 after fertilization (day 0 = IVF). After washing three times in PBS containing 0.1% PVA the specimens were frozen at −80 °C in a minimal volume (5 μL) of PBS before RNA extraction.

**Sampling Strategy and mRNA Isolation for Array Hybridization.** Pools of 10 embryos were prepared for all stages except for the blastocyst stage when 5 were sufficient. Three pools each were prepared from in vivo metaphase II oocytes, zygotes, 8-cell embryos, morulae, and blastocysts, and two pools were prepared from 2-cell and 4-cell embryos, and independently processed for mRNA extraction. Poly(A)+ RNA was isolated from a Dynabeads mRNA Direct Kit (Invitrogen) according to the manufacturer’s instruction with some modifications. Briefly, embryo pools were lysed in 40 μL of lysis-binding buffer (100 mM Tris-HCl pH 8.0, 500 mM LiCl, 10 mM EDTA, 1% lithium dodecyl sulfate, 5 mM DTT) and incubated at room temperature for 10 min. Prewashed Dynabeads Oligo(dT)25 (5 μL) was added to the lysate followed by incubation for 5 min at room temperature on a shaker to allow binding of the poly(A)+ RNA to the beads. The beads were separated using a Dynal MPC-E-1 magnetic separation system (Invitrogen) followed by incubation in 4 μL of washing buffer A (10 mM Tris-HCl pH 8.0, 0.15 mM LiCl, 1 mM EDTA, 0.1% lithium dodecyl sulfate) and 2 times with washing buffer B (10 mM Tris-HCl, pH 8.0, 0.15 mM LiCl, 1 mM EDTA) at 65 °C for 3 min and the mRNA’s were used immediately as input for the first-strand cDNA synthesis using a T7 promoter-linked oligo(dT) primer following the standard protocol for the Affymetrix Two-Cycle cDNA Synthesis Kit (Affymetrix, Part. 900432). Protocols for the isolation and unbiased amplification of RNA from mammalian embryos have been published previously (22, 50). For this system, a optimized system for Affymetrix arrays was followed.

**Amplification, Labeling, and Hybridization to DNA Microarrays.** After second-strand cDNA synthesis, biotin-labeled cRNA was prepared in an in vitro transcription reaction using the GeneChip IVT Labeling Kit (Affymetrix). Ten μg of fragmented cRNA was used for hybridization on the GenChip Bovine Genome Array (Affymetrix, Part. 900432). The authors gratefully acknowledge the expert assistance of Klaus-Gerd Hadeler in ovum pick up and oviductal and uterine embryo flushings, the excellent help by Karin Korsawa and Erika Lemme during embryo collection and grading, as well as the support from Martin Zenke and Bernd Denecke (RWTH Aachen) who performed chip hybridization. This study was supported by a grant from the Deutsche Forschungsgemeinschaft (DFG Forschergruppe 478) to H.N. and J.A.


