Dynamics of global transcriptome in bovine matured oocytes and preimplantation embryos

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Global activation of the embryonic genome is the most critical event in early mammalian development. After fertilization, a rich supply of maternal proteins and RNAs support development whereas a number of zygotic and embryonic genes are expressed in a stage-specific manner leading to embryonic genome activation (EGA). However, the identities of embryonic genes expressed and the mechanism(s) of EGA are poorly defined in the bovine. Using the Affymetrix bovine-specific DNA microarray as the biggest available array at present, we analyzed gene expression at two key stages of bovine development, matured oocytes (MII) and 8-cell-stage embryos, constituting the ultimate reservoir for life and a stage during which EGA takes place, respectively. Key genes in regulation of transcription, chromatin-structure cell adhesion, and signal transduction were up-regulated at the 8-cell stage as compared with 8-cell embryos treated with α-amanitin and MII. Genes controlling DNA methylation and metabolism were up-regulated in MII. These changes in gene expression, related to transcriptional machinery, chromatin structure, and the other cellular functions occurring during several cleavage stages, are expected to result in a unique chromatin structure capable of maintaining totipotency during embryogenesis and leading to differentiation during postimplantation development. Dramatic reprogramming of gene expression at the onset of development also has implications for cell plasticity in somatic cell nuclear transfer, genomic imprinting, and cancer.

gene expression | microarray

Early embryonic development is primarily reliant on maternal transcripts synthesized during gametogenesis. In mammals, the earliest stages of embryogenesis are regulated by maternally inherited components stored within the oocyte. After fertilization, the embryonic genome becomes transcriptionally active and begins to contribute to the early development process (1). Messages that preexist during embryogenesis are degraded after fertilization, and few new transcripts are produced. This is followed by *de novo* synthesis of embryonic mRNA at a species-specific cell stage. In mice, the major onset of transcription, embryonic genome activation (EGA), begins during the 2-cell stage; it begins during the 4-cell stage in humans, rats, and pigs, and during the 8-cell to 16-cell stage in cattle and sheep (2). However, a so-called "minor genome activation" is initiated as early as the 1-cell zygotic stage in bovine. From the zygotic stage to the blastocyst stage, expression of embryonic messages steadily increases during the progression of embryonic development (3). Upon egg activation and fertilization, maternal factors initiate developmental cascades of events that activate the embryonic developmental program (4). Even after the initiation of zygotic gene expression, maternal products continue to perform essential functions together with other maternal factors and through interactions with newly expressed zygotic products (5). After a common maternal and/or embryonic expression pattern, most genes are transcribed in a stage- and time-dependent manner (6). EGA is associated with the first differentiative events, successful embryo implantation, and fetal development. Regulation of bovine embryonic gene expression still remains an unsolved biological question. The identities of early expressed transcripts and proteins, and the mechanisms of EGA, are poorly defined.

In the current work, our aim is to better understand genome reprogramming during preimplantation development in bovine matured oocytes (MII), 8-cell-stage embryos, and 8-cell embryos treated with α-amanitin, a specific inhibitor of mRNA synthesis with bovine genome-specific high-resolution DNA microarrays (Affymetrix). Our results showed that hundreds of new messages are synthesized during early embryonic development, whereas a number of maternal messages still exist at least until the 8-cell stage. We also provide functional analyses of the expressed genes and confirmation of the DNA microarray experiments by real-time PCR for a subset of genes. The results presented here constitute a study of global transcription in bovine oocytes and early embryos by using the Affymetrix bovine-specific DNA microarray that is the biggest available array at present. These results can provide molecular biomarkers for development because embryonic mortality is the biggest limiting factor in animal reproduction and production. Furthermore, they may assist in increasing the efficiency of reprogramming in somatic cell nuclear transfer-derived embryogenesis and can serve as a basis for more hypotheses-driven research to elucidate the molecular biology of mammalian gametogenesis and embryogenesis.

Results

Transcriptome Analyses (Number of Genes Expressed). The Affymetrix GeneChip Bovine Genome Array contains 24,072 probe sets representing >23,000 transcripts, including assemblies from ≈19,000 UniGene clusters. The average percentage of probe sets that were called as present with an α value of 0.04, for MII, 8-cell embryos, and α-amanitin-treated 8-cell embryos were 50.3%, 52.7%, and 52.5%, respectively. To assess faithful amplification of original transcripts, we performed DNA microarray experiments using RNA samples from bovine fibroblast cells with one and two rounds of amplifications. The results showed that amplifications of messages with 1 versus 2 rounds were highly consistent with a correlation coefficiency of 0.93 (data not shown). Both the manufacturer's demonstrated linear amplification of messages with the same amplification kit as well as our preliminary data led us to conclude that the amplification before our array hybridization did not bias the ratio of individual mRNAs. We also confirmed DNA microarray results with real-time PCR. Smith *et al.* (7) recently

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Abbreviations: EGA, embryonic genome activation; MII, matured oocytes; NFYA, nuclear transcription factor Y-α; H2AFZ, H2A histone family, member Z; SMARCAL1, SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a-like 1; IGF2R, insulin-like growth factor 2 receptor; DNMT, DNA (cytosine 5) methyltransferase; STAT3, signal transducer and activator of transcription 3.

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Table 1. Number of differentially expressed transcripts

$P < 0.01$ and fold change ≥ 2.0	8-Cell embryos vs. MII (n = 2,505)		8-Cell embryos vs. α-amanitin-treated 8-cell embryos (n = 1,811)		MII vs. α-amanitin-treated 8-cell embryos (n = 3,263)	
	8-Cell embryos (n = 1,413)	MII (n = 1,092)	8-Cell embryos (n = 1,490)	α-Amanitin-treated 8-cell embryos (n = 321)	MII (n = 1,263)	α-Amanitin-treated 8-cell embryos (n = 2,000)
Genes only	258	124	233	52	147	379
Transcribed locus-strongly similar	690	422	600	160	453	1,069
Transcribed locus-moderately similar	272	120	159	44	116	246
Transcribed locus-weakly similar	27	31	36	5	24	42
Transcribed locus only	286	395	462	60	523	264

n, number of transcripts.

showed linear amplifications of messages from even smaller amounts of mRNA, single bovine blastocysts.

To obtain a highly confident set of differentially expressed genes, we used a rigorous combination of *P* values ($P < 0.01$) and fold change values ($\geq$2.0). Although the cut-off value of $\geq$2.0 does not include genes with expression levels <2, which might have dramatic effects, we used <2.0 to minimize the noise. Significantly higher expression was detected for 2,505, 1,811, and 3,263 genes in 8-cell versus MII, in 8-cell versus α-amanitin-treated 8-cell, and in MII versus α-amanitin-treated 8-cell (Table 1). Of the 7,579 total significantly hybridized genes, only 1,193 corresponded to annotated genes, whereas the rest were transcribed loci with various levels of matches to known genes. Because annotation of the bovine genome has not been completed yet, we obtained our results as genes transcribed locus-strongly, locus-moderately, and locus-weakly similar and transcribed locus only with no match (Table 1). Biological and experimental replicates of the array hybridizations showed the reproducibility and reliability of the array data (Table 4, which is published as supporting information on the PNAS web site).

We confirmed microarray expression patterns of a panel of six genes by using real-time RT-PCR. These genes were nuclear transcription factor Y-α (NFYA; 140.8-fold), H2A histone family, member Z (H2AFZ; 98.9-fold), and SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a-like 1 (SMARCAL1; 7.1-fold), which all revealed increased expression in 8-cell embryos. Other genes whose transcripts were confirmed included insulin-like growth factor 2 receptor (IGF2R; 6.7-fold), DNA (cytosine 5) methyltransferase 1 (DNMT1; 4.3-fold), and signal transducer and activator of transcription 3 (acute-phase response factor) (STAT3; 4.2-fold), all of which revealed increased expression in MII according to the microarray data. In MII, transcripts of IGF2R, DNMT1, and STAT3 genes were detected 4.5-, 3.4-, and 2.16-fold higher than α-amanitin-treated 8-cell embryos, respectively. In the 8-cell embryos, transcripts of NFYA and H2AFZ genes were detected 37.9- and 125.9-fold higher than α-amanitin-treated 8-cell embryos, respectively (Fig. 1 *A–F*, which is published as supporting information on the PNAS web site). In α-amanitin-treated 8-cell embryos, transcripts of SMARCAL1 gene was detected 11.9-fold higher than in MII (Fig. 2 *A–F*, which is published as supporting information on the PNAS web site). These results showed faithful amplification of original transcripts in the oocytes and embryos and confirmed the gene expression levels detected by microarrays.

Functional Classification of Genes. We used NetAffx Analysis Center and CowBase at the AgBase database (www.agbase.msstate.edu) to achieve gene ontology analysis of significantly regulated genes whose expression was either up- and down-regulated in any one of the MII and 8-cell embryos. Categories within the mRNAs that increased or decreased at 2-fold or greater levels are shown in Fig. 3, which is published as supporting information on the PNAS web site. Regarding biological processes, MII had more metabolism-related genes up-regulated than physiological process (43% versus 30%, respectively). The 8-cell embryos showed a similar trend with 37% versus 17% for metabolism and physiological process, respectively (Fig. 3 *A1* and *B1*). Transcripts related to cellular components of MII were 47.5% for cell, and 38.3% for membrane, whereas the cellular components of the 8-cell embryos were 63.4% for cell and 27.2% for membrane (Fig. 3 *A2* and *B2*). In molecular functions analyses, MII and 8-cell embryos had similar levels of transcripts related to binding up-regulated (41% versus 42%, respectively). These two cell types also exhibited transcripts in diverse areas of other molecular functions (Fig. 3 *A3* and *B3*).

Embryonic Versus Maternal: Comparative Transcriptomes of 8-Cell Embryos and MII. Comparative analyses of gene expression between 8-cell embryos and MII showed that 258 gene transcripts were increased by 2-fold or greater in 8-cell embryos as compared with MII (Table 1). On the other hand, 124 gene transcripts were increased by 2-fold or greater in MII as compared with 8-cell embryos (Table 1). Selected transcripts of genes of 8-cell embryos versus MII, and MII versus 8-cell embryos, included in each gene ontology category are shown in Table 2 and Table 5, which is published as supporting information on the PNAS web site, respectively. Of the genes that exhibited increased expression in 8-cell embryos as compared with the MII, 41% fell in the 2- to 5-fold range, 36% in the 5- to 15-fold range, and 23% in the 15-fold and greater range. We detected interesting transcripts expressed at significantly different levels only at the 8-cell stage (Table 2). These mRNAs include regulators of transcription such as NFYA and upstream regulatory factor 2 (USF2); transcripts with roles in cell adhesion such as desmocolin 2 (DSC2) and collagen, type XII, α-1 (COL12A1); signal transducers such as prostaglandin E receptor 4 (subtype EP4) (PTGER4) and β-adrenergic receptor kinase 1 (ADRBK1); transporters including cellular retinoic acid-binding protein 1 (CRABP1); metabolism-related gene transcripts such as salidase 3 (NEU3); and immune response-related messages such as chemokine (C-X-C motif) ligand 6 (CXCL6). Of the genes that showed increased expression in MII, 75% were in the 2- to 5-fold range, 24% in 5- to 15-fold ranges, and 1% in the 15-fold and greater range. Several of the maternal transcripts also were detected at the 8-cell stage (Table 5). Significantly higher levels of transcripts in the MII were in the broad functional classes of regulation of transcription, DNA methylation, cell adhesion, apoptosis/cell death, protein folding, electron transport, metabolism, and immune response. These classes represent a rich supply of maternal contribution to embryo development.

Transcriptomes of 8-Cell Embryos Versus 8-Cell Embryos Treated with α-Amanitin. Inhibition of transcription by using α-amanitin during early embryogenesis until the 8-cell stage provided the means to

Table 2. Expression of selective genes for equal or >2-fold differences in 8-cell embryos when compared with MII

Accession no.	Gene name	Symbol	Fold change
Regulation of transcription			
NM_001014956	Nuclear transcription factor Y-α	NFYA	140.8*
NM_174809	H2A histone family, member Z	H2AFZ	98.9
XM_585780	Inhibitor of DNA-binding 1, dominant-negative helix–loop–helix protein	ID1	16.6
NM_001024570	Similar to MGC2941 protein	MGC2941	16.5
NM_001001162	Upstream stimulatory factor 2	USF2	16.1*
NM_175050	v-myb myeloblastosis viral oncogene homolog (avian)	MYB	6.5
NM_177432	Interferon responsive factor 1	IRF1	5.8
NM_174428	Polymerase (DNA-directed), δ2, regulatory subunit 50 kDa	POLD2	5.3
NM_001015527	General transcription factor IIF, polypeptide 1	GTF2F1	4
NM_174402	Nuclear receptor subfamily 2, group F, member 1	NR2F2	3.8
XM_612996	Aryl hydrocarbon receptor	AHR	3.7
NM_173999	B cell translocation gene 1, anti proliferative	BTG1	3.1
NM_001034598	Nuclear transcription factor Y-γ	NFYC	2.2
Chromatin modification			
NM_176666	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a-like 1	SMARCAL1	7.1
Cell adhesion			
XM_587512	Desmocollin 2	DSC2	318.5*
XM_611630	Collagen, type XII, α 1	COL12A1	12.7*
Apoptosis/cell death			
	CD97 antigen	CD97	2.5
XM_581509	Tumor necrosis factor receptor superfamily, member 5	TNFRSF5	9.6
NM_173902	Clusterin	CLU	8.7
NM_001024482	Abl-philin 2, isoform 2	ZDHHC16	5.8
NM_174637	Catenin, β-like 1	CTNNBL1	5.8
XM_589701	Deoxyribonuclease II, lysosomal	DNASE2	5.2
NM_001014941	B cell receptor-associated protein 31	BCAP31	4.1
Antiapoptosis			
NM_174721	Presenilin 1	PSEN1	4.1
Protein folding			
NM_174550/ NM_203322	Heat shock 70-kDa protein 1/heat shock 70-kDa protein 2	HSPA1A/ HSPA1B	6.1
XM_867578	Heat shock 70-kDa protein 5	grp78	5.9
BT021599	T-complex protein 1, γ subunit (TCP-1-γ) (CCT-γ)	CCT3	3.2
NM_174589	Prostaglandin E receptor 4 (subtype EP4)	PTGER4	352.1*
NM_174710	Adrenergic, β, receptor kinase 1	ADRBK1	61.5†
Signal transduction			
NM_181025	Prostaglandin F receptor (FP)	PTGFR	24.5†
NM_174231	Adrenergic, β-2, receptor, surface	ADRB2	12.8*
NM_181021	Guanine nucleotide-binding protein (G protein), α-stimulating activity polypeptide 1	GNAS1	10.6
NM_174649	Protein kinase, cAMP-dependent, regulatory, type II, β	PRKAR2B	7.4*
NM_174410	Purinergic receptor P2Y, G protein-coupled, 1	P2RY1	7.1†
NM_181028	Cellular retinoic acid-binding protein 1	CRABP1	147.8*
XM_583977	Solute carrier family 2 (facilitated glucose/fructose transporter), member 5	SLC2A5	28.2*
NM_174657	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3	SLC25A3	11.6‡
NM_001033616	$GABA_A$ receptor-associated protein	GABARAP	8.3
NM_174602	Solute carrier family 2 (facilitated glucose transporter), member 1	SLC2A1	5.7
NM_174729	Voltage-dependent anion channel 3	VDAC3	5.2
NM_174223	ATP-binding cassette, subfamily C (CFTR/MRP), member 1	ABCC1	4.4
NM_174022	Clathrin, light polypeptide (Lca)	CLTA	3.6
NM_174282	Coatomer protein complex, subunit ζ 1	COPZ1	3.5
XM_613433	Chloride intracellular channel 4	CLIC4	3.4
Electron transport			
NM_181014	Monoamine oxidase A	MAOA	16.1
NM_174630	Ubiquinol-cytochrome *c* reductase core protein II	UQCRC2	9.6
NM_174208	Thioredoxin 2	TXN2	8.5
NM_175783	NADH dehydrogenase (ubiquinone) 1 β subcomplex 9, 22 kDa	NDUFB9	5.5
XM_618577	Succinate dehydrogenase complex, subunit B, iron sulfur (Ip)	SDHB	5.1
NM_174813	Ubiquinol-cytochrome *c* reductase, Rieske iron-sulfur polypeptide 1	UQCRFS1	4.9
NM_176675	Cytochrome *c* oxidase subunit VIb	COX6B	4.9
NM_001014897	Thioredoxin-like 4B	TXNL4B	3.1
Metabolism			
NM_174122	Sialidase 3 (membrane sialidase)	NEU3	27.2*
NM_174746	Acetyl-coenzyme A synthetase 2 (AMP-forming)-like	ACAS2L	8.8*
NM_174100	Lactate dehydrogenase B	LDHB	5.1
NM_174762	UDP glycosyltransferase 1 family, polypeptide A4	UGT1A4	4.5*
NM_177521	Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1	SULT1A1	4.0*
NM_001012668	Hexokinase 1	HK1	3.1
NM_177502	Aminotransferase 1, glutamic-oxaloacetic transaminase 1, soluble	GOT1	3.1
NM_001007815	Microsomal glutathione *S*-transferase 1	MGST1	2.7
NM_001024475	Phosphatidylserine decarboxylase	PISD	2.6*
NM_173990	*S*-adenosylmethionine decarboxylase 1	AMD1	2.4
NM_205813	Phosphoribosylaminoimidazole carboxylase	PAICS	2.0
Immune response			
NM_174300	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	CXCL6	182.2*
NM_174091	Interleukin 18 (interferon-γ-inducing factor)	IL18	56.7*
NM_174008	CD14 antigen	CD14	35.1*
NM_175700	Chemokine (C-X-C motif) ligand 1	CXCL1	16.8*
NM_174092	Interleukin 1, α	IL1A	14.6‡
Cell cycle			
NM_001015665	Cyclin E2	CCNE2	34.1*
NM_174016	Cell division cycle 2, G^1 to S and G^2 to M	CDC2	7.7
NM_175793	Mitogen-activated protein kinase 1	MAPK1	5.5

*Not detected in MII.
†Not detected in MII but detected in 8-cell α-amanitin-treated embryos.
‡Not detected in 8-cell α-amanitin-treated embryos but detected in MII.

analyze transcripts synthesized only during embryogenesis. A total of 233 gene transcripts were increased by 2-fold and greater in 8-cell embryos (Table 1). Of the genes that exhibited increased expression in 8-cell embryos, 36% of them fell in the 2- to 5-fold range, 40% of them in the 5- to 15-fold range, and 24% in the 15-fold and greater range (Table 3). The results of data analyses of genes expressed at the 8-cell stage as compared with the 8-cell α-amanitin-treated embryos confirmed the embryonic gene expres-

Table 3. Expression of selective genes for >2-fold differences in 8-cell embryos when compared with α-amanitin-treated 8-cell embryos

Accession no.	Gene name	Symbol	Fold change
Regulation of transcription			
XM_585780	Inhibitor of DNA-binding 1, dominant-negative helix–loop–helix protein	ID1	18.1
BT020733	RNA-binding motif protein 14	RBM14	11.9
BT021849	Similar to MGC2941 protein	MGC2941	6.1
Cell adhesion			
M81190	Desmocollin 2	DSC2	222.5*
L33774	Desmocollin 3	DSC3	45.7*
NM_173999	B cell translocation gene 1, antiproliferative	BTG1	20.5
NM_174367	Integrin, α V (vitronectin receptor, α polypeptide, antigen CD51)	ITGAV	4.9
NM_176872	Thrombospondin 2	THBS2	3.2*
Apoptosis/Cell death			
NM_001024482	Abl-philin 2, isoform 2	ZDHHC16	4.1
Protein folding			
NM_174096.2	J domain-containing protein 1	JDP1	13.3†
NM_001014869	Peptidylprolyl isomerase-like 1	PPIL1	4.3
Transport			
AY821681	Cellular retinoic acid-binding protein 1	CRABP1	220.1*
XM_588265	Solute carrier family 38, member 2	SLC38A2	124.9*
NM_174657	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3	SLC25A3	31.6†
NM_174782	Solute carrier family 12 (sodium/potassium/chloride transporters), member 2	SLC12A2	5.3
NM_175796	ATP synthase, H + transporting, mitochondrial F1 complex, β polypeptide	ATP5B	3.6
NM_174689	ADP-ribosylation factor 2	ARF2	3.3
NM_001034048	$GABA_A$ receptor-associated protein	GABARAP	2.5
NM_174602	Solute carrier family 2 (facilitated glucose transporter), member 1	SLC2A1	2.1
BT020993	Ubiquinol-cytochrome *c* reductase core protein II	UQCRC2	12.7
NM_176675	Cytochrome *c* oxidase subunit VIb	COX6B	9.0
NM_181014	Monoamine oxidase A	MAOA	7.6
NM_174813	Ubiquinol-cytochrome *c* reductase, Rieske iron-sulfur polypeptide 1	UQCRFS1	5.8
NM_174208	Thioredoxin 2	TXN2	4.5
NM_174179	Succinate dehydrogenase complex, subunit D, integral membrane protein	SDHD	2.3
NM_173968	Thioredoxin	TXN	2.1
Metabolism			
NM_001007818	Purine nucleoside phosphorylase	NP	148.3†
NM_174444	Prostaglandin I2 (prostacyclin) synthase	PTGIS	19.5*
NM_177485	Aminomethyltransferase (glycine cleavage system protein T)	AMT	9.2
BT021838	Lysophospholipase 3 (lysosomal phospholipase A2)	LYPLA3	4.1
NM_001007815	Microsomal glutathione *S*-transferase 1	MGST1	3.8
NM_175796	ATP synthase, H + transporting, mitochondrial F1 complex, β polypeptide	ATP5B	3.5
NM_177512	Glycoprotein-4-β-galactosyltransferase 2	B4GALT1	2.9
NM_174175	Seryl-tRNA synthetase	SARS	2.2
NM_174644	Isocitrate dehydrogenase 3 (NAD+) α	IDH3A	2.2
NM_173977	Aconitase 2, mitochondrial	ACO2	2.1
Immune response			
NM_174300	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	CXCL6	181.8*
NM_174091	Interleukin 18 (interferon-γ-inducing factor)	IL18	33.5*
NM_175700	Chemokine (C-X-C motif) ligand 1	CXCL1	32.6*
NM_174092	Interleukin 1, α	IL1A	13.3†
NM_174252	Complement component 4-binding protein, α	C4BPA	10.2*
NM_174008	CD14 antigen	CD14	9.8*
XM_581509	Tumor necrosis factor receptor superfamily, member 5	TNFRSF5	5.4

*Not detected in MII and α-amanitin-treated 8-cell embryos.
†Not detected in α-amanitin-treated 8-cell embryos.

sion that we detected in the 8-cell embryos as compared with MII (Table 2). For example, the cell adhesion-related gene desmocolin 2 and transport-related cellular retinoic acid-binding protein 1 also were detectable at higher fold rates in the 8-cell embryos as compared with their α-amanitin-treated counterparts. Interestingly, a metabolism-related gene, purine nucleoside phosphorylase (NP), was detected to be 149-fold higher in the 8-cell embryos and not detectable in the α-amanitin-treated embryos.

Transcriptomes of MII Versus 8-Cell Embryos Treated with α-Amanitin. We determined maternal transcripts that were degraded or used up between the MII and 8-cell stage by using α-amanitin during early embryogenesis. Total of 147 gene transcripts were increased by 2-fold and greater in 8-cell embryos. Of the genes that exhibited increased expression in MII, 84% fell in the 2- to 5-fold range, 14% in the 5- to 15-fold range, and 2% in the 15-fold and greater range. Selected transcripts of the genes detected in the MII as compared with α-amanitin-treated 8-cell embryos are presented in several gene ontology categories (Table 6, which is published as supporting information on the PNAS web site).

Discussion

Global activation of the embryonic genome (EGA) is the most critical event of bovine preimplantation embryogenesis, which consists of more than just dividing the cytoplasm, changing length of cell cycles, and replicating DNA until the first cell differentiation occurs (3, 8–11). In fact, early embryogenesis is as important as the other phases of developmental biology because the embryo dies if the EGA fails to occur properly (12). Early embryonic development is a remarkable event in which dramatic reprogramming of gene expression takes place, and it is this reprogramming that sets the stage for later development during both pre- and postimplantation periods. Thus, better understanding of early embryonic development is relevant to understanding the events taking place during mammalian fetal development. During early bovine embryogenesis, maternal RNAs and proteins are degraded, and transcriptional and translational activation of the embryonic genome occurs simultaneously (3). Previous studies (13) show that EGA in bovine occurs as a minor genome activation starting as early as the 1-cell zygotic stage and continues until the 8- to 16-cell stage, at which time major genome activation takes place. Bovine mature oocytes arrested at the MII stage of metaphase contain a rich supply of RNA and proteins supporting embryonic development. However, embryonic transcription is essential for cell cleavage beyond the 8-cell stage (12). Despite this, both the identities of transcripts expressed during these critical stages of development and the mechanism of EGA are poorly defined.

To detect first transcripts of major EGA, we analyzed mid- to late 8-cell embryos. We both confirmed previously detected transcripts such as IGF2R and DNMT1 and identified previously uncharacterized transcripts such as regulator of chromatin-structure SWI/SNF and NFYA at the 8-cell stage (Table 2). *In vitro*-produced bovine blastocysts are known to have different mRNA expression patterns as compared with their *in vivo*-derived counterparts (14, 15). Although lower than *in vivo*-derived blastocysts, reasonable pregnancy and calving rates (35–45%) have been obtained by using *in vitro*-derived bovine blastocysts (7, 15). It might be that the *in vitro*-cultured embryos express different transcripts to deal with the suboptimal culture conditions and that the expression profiles do not necessarily indicate their abnormality. Analyses of gene expression in the 8-cell versus MII showed that there were 258 and 124 annotated genes up-regulated, respectively, with functions in diverse areas of cell biology such as regulation of transcription, chromatin modification, cell adhesion, apoptosis, signal transduction, transport, metabolism, and immune response (Table 2 and Fig. 3). Several transcripts of genes involved in transcription regulation were detected at the 8-cell stage in time for the major EGA (Table 2) (11, 10). NFYA, with a role in mRNA synthesis (16, 17), was

detectable at level 141-fold greater in 8-cell embryos as compared with MII. Expression level of the H2AFZ was 99-fold higher than that of MII (Table 2). The conserved histone variant H2AZ is a regulator of gene expression and silent heterochromatin (18). Bovine embryos exposed to α-amanitin cannot develop beyond the 8- to 16-cell stage (12), suggesting essential roles for a number of genes including these transcription factors during EGA and maternal-to-embryonic transition (MET). Analyses of expression patterns for genes IGF2R, DNMT1, STAT3, NFYA, H2AFZ, and SMARCAL1 with real-time PCR were consistent with the results obtained from the DNA microarrays (Fig. 1). Epigenetic mechanisms such as changes in chromatin structure and DNA methylation influence gene expression without changing the underlying DNA sequence. Chromatin structure changes during EGA (3, 8), and we detected SMARCAL1 at 7-fold higher levels in the 8-cell embryos as compared with MII (Table 2). As ATP-dependent chromatin-remodeling enzymes, mammalian SWI/SNF complexes are involved in nucleosome compaction influencing gene regulation, replication, and recombination and DNA repair (19, 20). These changes in gene expression, related transcriptional machinery, and chromatin structure, and the other cellular functions occurring during several cleavage stages, lead to a unique chromatin structure capable of maintaining totipotency during embryogenesis and leading to differentiation during postimplantation development (8).

DNA methylation represses gene expression in part by recruitment of the methyl-CpG-binding protein MeCP2, which in turn recruits a histone deacetylase activity (21, 22). Much of the bovine genome is demethylated after fertilization, reaching a low level at the 8-cell stage, and then remethylated by the blastocyst stage (23). DNMT1 is responsible for cytosine methylation in mammals and influences gene silencing. DNMT2 contains conserved methyltransferase motifs and might methylate DNA or bind to DNA causing a denaturant-resistant chromatin structure (22). DNMT1 and DNMT2 transcripts were 4- and 8-fold higher in the MII as compared with the 8-cell-stage embryos (Table 5). These results confirm previous findings and are consistent with the higher levels of DNA methylation in the MII than 8-cell embryos (23). It is possible that in bovine 8-cell embryos, DNA methylation is achieved mainly by the maternal or oocyte-specific DNA methyltransferase (DNMT1o), which comes to the nucleus at the 8-cell stage in the mouse (24). Genomic imprinting is another important determinant of proper development by monoallelic expression of genes that are regulated during germ-cell and embryo development (23). The genes encoding for insulin-like growth factor type 2 (IGF2) and its receptor (IGF2R) are reciprocally imprinted and expressed from the paternal and maternal genomes, respectively, in the fetal and adult mouse (25). These previous studies indicate that inactivation of imprinted genes occurs postfertilization (25). In the present study, IGF2R was expressed at $\approx$7-fold higher levels in MII as compared with 8-cell embryos, which are consistent with imprinting of the paternal genome at the time of fertilization (Table 5). One of the many other interesting genes whose transcripts were detected the 8-cell-stage embryo was desmocolin 2, which plays a role in cell adhesion (26) and was detected at 319-fold higher levels in 8-cell embryos than in the MII (Table 2).

Better understanding of EGA will contribute to our understanding of cell plasticity and nuclear reprogramming in somatic cell nuclear transfer experiments. Elucidation of EGA in normal embryos can shed light into what reprograms the differentiated donor cell into an undifferentiated state (27). Studies concerning EGA are most advanced in the mouse (8–11); however, it is important to determine whether this information obtained from one species is applicable to others because of significant species differences in developmental biology and EGA, i.e., embryonic development is much faster in the mouse than in the cow (5).

In addition, efficiency of producing a viable somatic cell nuclear transfer-derived animal is higher in bovine than in other species tested so far. Development to blastocyst stage occurs on days 4.5 and 7–8 for mouse and bovine embryos, respectively. It may be that the longer embryonic development in bovine provides an extra window of opportunity for the donor somatic nuclei to be reprogrammed more properly. Thus, bovine embryos provide an ideal opportunity to study fascinating dynamics of early embryonic development with a comparative functional genomics point of view. The data generated in this study fill a large gap in our knowledge about bovine EGA and pave the way for future studies aimed at understanding mechanisms of early bovine embryonic development by using hypothesis-driven research. As the bovine genome annotation becomes more complete, we expect to identify more genes unique to bovine. Further studies analyzing gene expression dynamics throughout the bovine preimplantation embryogenesis (i.e., from 1-cell to blastocyst stage) would define the full blueprint of early bovine development.

Methods

Detailed methods are provided in *Supporting Text*, which is published as supporting information on the PNAS web site.

***In Vitro* Maturation, Fertilization, and Culture and Sample Collection.** *In vitro* maturation, fertilization, and culture were performed as described (28). Briefly, oocytes with homogenous cytoplasm and several layers of cumulus cells were matured in tissue culture medium (TCM-199; GIBCO/Invitrogen) at 39°C for 24 h in a humidified incubator with 5% CO_2 in air. MII were fertilized with bull sperm from a bull of proven frozen-sperm fertility. After fertilization period, cumulus cells were removed, and presumptive zygotes were cultured in 50-μl drops of synthetic oviduct fluid (SOF; Specialty Media) covered with mineral oil 18 h postinsemination (hpi). For the α-amanitin treatment group, SOF medium also was supplemented with 50 μg/ml α-amanitin at the beginning of *in vitro* culture. On day 4, 10% FCS was added to each drop. MII were collected after a 24-h maturation period. They were fully stripped of cumulus cells by exposure to 0.1% hyaluronidase during high-speed vortexing for 5 min. MII without cumulus cells were selected for use in the present study. 8-Cell-stage embryos were collected 70–72 and 82–84 hpi. MII and 8-cell-stage embryos were washed three times in saline and then immediately frozen and stored at −80°C until use.

RNA Extraction and Linear Amplification. Two pools of MII, three pools of 8-cell embryos, and two pools of 8-cell α-amanitin-treated embryos from each stage were independently collected from separate sets of *in vitro*-produced bovine embryos. Three hundred MII and a range of 280–330 8-cell embryos per pool were collected to make the amount of total RNA recovered approximately equivalent among all stages. Total RNA was extracted from MII and embryos by using an RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. Quality of total RNA was analyzed with a Bioanalyzer (Agilent) by using an RNA 6000 Picochip kit. Total RNA ($\approx$5 ng/μl per replicate pool) was used for linear, two-cycle amplification by *in vitro* transcription following the manufacturer's set protocol (Affymetrix). The manufacturer's previous experiments using two rounds of amplification with small amounts of starting RNA have demonstrated a linear and faithful amplification.

These data were further supported by results of our preliminary experiments with one versus two rounds of amplifications, which showed consistent amplifications, and with real-time PCR on a panel of genes whose expression patterns were the same as in DNA microarrays (Figs. 1 and 2).

Microarray Target Preparation and Hybridization. By using the manufacturer's established protocol (Affymetrix), a total of 10 ng of RNA from each sample was used for single-stranded cDNA synthesis by using SuperScript II reverse transcriptase and T7-Oligo(dT) primer/Poly(A) controls (Affymetrix). The single-

stranded cDNA then was converted to double-stranded cDNA by using *Escherichia coli* DNA polymerase I (Affymetrix) for the first cycle. From template cDNA, biotin-labeled cRNA was prepared in an *in vitro*-transcription reaction by using the MEGAscript High-Yield transcription kit (Ambion). After *in vitro* transcription, 600 ng of cRNA from each replicate was used for the second-cycle first-strand cDNA synthesis. The first-strand cDNA from the second cycle then was converted to second-strand cDNA. From second-strand cDNA template, biotin-labeled cRNA was prepared in an *in vitro* transcription reaction by using GeneChip IVT labeling kit (Affymetrix) according to the manufacturer's instructions. Then 15 μg of biotin-labeled cRNA was fragmented in 1$\times$ fragmentation buffer solution provided with the GeneChip Sample Cleanup Module (Affymetrix) at 94°C for 35 min. A total of 10 μg of fragmented biotin-labeled cRNA per replicate in hybridization mixture then was hybridized to Bovine Midi_euk2v3 Genome Array from Affymetrix GeneChips and incubated overnight at 45°C in a rotating hybridization oven, all according to the manufacturer's instructions. The mixture was removed 16 h after hybridization, and, in several cycles, the chips were washed with nonstringent buffer and stained with streptavidin/phycoerythrin (SAPE) antibody solution according to the manufacturer's instructions (Affymetrix) by using an Affymetrix FS-450 fluidics station. The data were collected by using Affymetrix GeneChip Scanner 3300.

Analysis of Microarray Data. Microarray images were processed with the MAS 5.1 (Affymetrix) algorithm by using Bioconductor's *affy* module (29, 30). The data were analyzed by using HDBStat (31), quantile-quantile normalized, and then examined for outliers by using the Pearson correlation coefficient, an invariant gene set (32), and a deleted residuals approach (33). No obvious outliers were detected (data not shown). The pooled variance *t* test was used to test for differences between the 8-cell embryos, α-amanitin-treated 8-cell embryos, and MII samples. False discovery rates for the genes were calculated by using *t* test and *P* values (34). Fold changes were calculated based on the unadjusted data's means. Genes were annotated with NetAffx (35). Because of lack of genome annotation for the bovine array, ontological analysis (36) of the data were not possible. Genes in any single comparison with a $P < 0.01$, false discovery rates of $<20\%$, and a fold change in excess of 2.0 were considered to be significant and examined further.

Functional Classification of Differentially Expressed Genes. Significantly differentially expressed genes were analyzed further for significant functional classification by using the NetAffx analysis center (www.affymetrix.com/analysis/index.affx) and an AgBase database (www.agbase.msstate.edu). The AgBase is a web-accessible resource for functional analysis of agricultural plant and animal gene products. Goanna (define) tool takes a list of Ids as input, retrieves the sequence, and performs BlastP against a user-specified database to return annotated proteins with sequence similarity. GOSlimViewer takes the file from GORetriever as input and, by using a user-specified GOSlim, returns the data required to chart the data in Excel. GOSlim data are returned for each of the ontologies. Bovine genes were classified as annotated genes, strongly, moderately or weakly similar to known annotated genes in other species or with limited homology to other genes.

Gene Expression Analysis by Real-Time RT-PCR. Primers were designed (Primer Premier 5; Premier Biosoft International) (Table 7, which is published as supporting information on the PNAS web site) for six genes with different patterns of expression in DNA microarrays (NFYA, H2AFZ, SWI/SNF, SMARCAL1, IGF2R, DNMT1, and STAT3). Relative mRNA expression levels were determined by using the cDNA for quantitative real-time PCR amplification with SYBR Green I chemistry (Roche Applied Sciences) by using the LightCycler instrument (Roche Applied Sciences). GAPDH was the endogenous internal housekeeping gene that revealed less variability and better reproducibility. Specificity of all individual amplification reactions was confirmed by melting curve analysis. Real-time expression values were calculated by using the relative standard curve method. Standard curves were generated for each mRNA by using 10-fold serial dilutions for both the target of interest and the endogenous control (GAPDH) by measuring the cycle number at which exponential amplification occurred in a dilution series of samples. Values were normalized to the relative amounts of GAPDH mRNA, which were obtained from a similar standard curve. In real-time PCRs, the same initial amounts of target molecules were used, and the Cp values (10.80 ± 0.02) of GAPDH mRNA were constant in all samples. Statistical analysis of gene expression patterns was performed by using a relative expression software tool (REST, 384-beta version, May 2005), which is based on an efficiency-corrected mathematical model for data analysis. It calculates the relative expression ratio on the basis of the PCR efficiency (*E*) and crossing point deviation (ΔCP) of the investigated transcripts and on a newly developed randomization test macro. The mathematical model used is based on the PCR efficiencies and the crossing point deviation between the samples (28, 37). Differences at $P < 0.001$ were considered significant (Figs. 1 and 2).

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