Sex determines the expression level of one third of the actively expressed genes in bovine blastocysts

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Although genetically identical for autosomal Chrs (Chr), male and female preimplantation embryos could display sex-specific transcriptional regulation. To illustrate sex-specific differences at the mRNA level, we compared gene-expression patterns between male and female blastocysts by DNA microarray comparison of nine groups of 60 bovine in vitro-produced blastocysts of each sex. Almost one-third of the transcripts detected showed sexual dimorphism (2,921 transcripts; false-discovery rate, $P < 0.05$), suggesting that in the absence of hormonal influences, the sex Chrs impose an extensive transcriptional regulation upon autosomal genes. Six genes were analyzed by qPCR in in vivo-derived embryos, which displayed similar sexual dimorphism. Ontology analysis suggested a higher global transcriptional level in females and a more active protein metabolism in males. A gene homolog to an X-linked gene involved in network interactions during spliceosome assembly was found in the Y-Chr. Most of the X-linked-expressed transcripts (88.5%) were up-regulated in females, but most of them (70%) exhibited fold-changes lower than 1.6, suggesting that X-Chr inactivation is partially achieved at the blastocyst stage. Almost half of the transcripts up-regulated in female embryos exhibiting more than 1.6-fold change were present in the X-Chr and eight of them were selected to determine a putative paternal imprinting by gene expression comparison with parthenogenetic embryos. Five (*BEX*, *CAPN6*, *BEX2*, *SRPX2*, and *UBE2A*) exhibited a higher expression in females than in parthenotes, suggesting that they are predominantly expressed by the paternal inherited X-Chr and that imprinting may increase the transcriptional skew caused by double X-Chr dosage.

gender | preimplantation | microarray | imprinting | X-inactivation

In mammals, sexual dimorphism is mostly attributable to sex-related hormonal differences in fetal and adult tissues; however, this may not be the sole determinant. Before gonad differentiation occurs, male and female preimplantation embryos display phenotypic differences that can only be attributed to the different sex Chr dosage. Although male and female blastocysts carry the same autosomal DNA, gender-specific transcription or translation occurs. At these early stages, sex Chrs modulate the genome machinery leading to differences in epigenetic status (1) and expression level of both X-linked (2, 3) and autosomal genes (1, 4, 5). These molecular events are reflected in phenotypic differences reported under some culture conditions, including differences in speed of embryo development, survival after vitrification, cell number at the blastocyst stage, and metabolism. In particular, glucose metabolism is thought to differ between male and female embryos (6), which may lead to a skewing in sex ratio because of preferential loss of embryos of one sex occurring both in vitro (7, 8) and in vivo (9, 10).

In a genomic context, transcriptional analyses during preimplantation development provide a useful tool to study hormone-independent sexual dimorphism phenomena. Both sex Chrs encode transcripts, which not only can have a direct effect upon phenotypic differences (i.e., *G6PD* and *HPRT*) but also can modulate the expression of autosomal genes (1). In adult tissues, one of the X-Chrs is inactivated, but some genes can escape from the X-Chr

inactivation process and be expressed biallelically. This situation is especially common during preimplantation development, when X inactivation is a reversible dynamic process (11) and may lead to an up-regulation of X-linked genes in female embryos (2, 3). On the other hand, male embryos only contain the maternally inherited X-Chr; thus, an X-linked gene up-regulation in females may also occur as a result of an imprinting mechanism leading to a total or partial maternal allele transcriptional repression (11).

Global gene expression analyses in preimplantation embryos are scarce, mainly because of the technical difficulties in obtaining the necessary large number of embryos per group. To our knowledge, there is only one report on global differences in gene expression in male and female blastocysts, which used a transgenic mouse model to obtain the biological material (12). Sex-sorted semen constitutes a powerful tool for these studies, as it can provide a large number of embryos of known sex in species with a longer preimplantation period, such as bovine. In this study, we aimed to analyze preimplantation sexual dimorphism mechanisms at the transcriptional level by microarray gene-expression profiling of bovine blastocysts produced in vitro. We also confirmed the findings for in vivo-derived embryos, performed ontology analysis, reported a previously unreported Y-linked transcript, and determined the putative imprinting of eight X-linked genes up-regulated in female embryos.

Results

Microarray Overall Results and Validation. More than 1,000 blastocysts of known sex were produced in 12 independent experiments. The global gene-expression pattern of nine pools each of male and female bovine blastocysts ($n = 60$ blastocysts per pool) was compared with the GeneChip Bovine Genome Array. A total of 9,322 transcripts were present at the blastocyst stage. The total number of transcripts differing between male and female embryos is listed in Table 1 and Table S1. Hierarchical distribution clearly grouped the samples according to the sex, irrespective of the bull used (Fig. 1A and Fig. S1). Therefore, statistical analysis was performed by grouping the data from the nine arrays of each sex obtained from samples of the three different bulls. Principal component analysis demonstrated that the sexes clearly separated and, interestingly, within each sex, the three replicates from each bull clustered together (Fig. 1A and Fig. S1). The large sample size obtained after grouping the data allowed us to detect small

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Data deposition: Raw data from microarray experiments was submitted to the Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo>). The platform ID is GPL2112. The accession ID for the set of experiments described is GSE17921. A new 1382 pb cDNA was identified YZRSR2 (GQ426330).

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Table 1. Results of microarray analysis of transcripts differentially expressed between male (M) and female (F) bovine blastocysts

Comparison (# of samples)	Multiple test correction	Up-regulated genes in females	Up-regulated genes in males	Total	Fold-changes > 2 female/male
F vs. M(9 vs. 9)	None	1,667	2,089	3,745	53/2
	FDRP < 0.05	1,330	1,591	2,921	53/2
	FDR P < 0.01	897	914	1,811	53/2
	Bonferroni P < 0.05	290	92	382	45/2

Statistical analysis from comparison of nine pools each of male and female embryos.

absolute differences and after false-discovery rate (FDR) assessment of significance ($P < 0.05$) correction was applied to reduce the number of false-positives, a total of 2,921 transcripts differed between male and female blastocysts, which constitutes almost one-third of the transcripts actively expressed. The fold-change for most of the transcripts was below 2. For 55 transcripts, the fold-change was higher than 2, 53 of which were up-regulated in females. Transcripts up-regulated (with the higher values) in male and female bovine blastocysts are listed in [Tables S2](#) and [S3](#). A higher level of fold-changes of up-regulated transcripts was found in female than in male embryos.

Array validation by quantitative PCR (qPCR) was performed in embryos produced with unsorted sperm to confirm that observed sex-related transcript differences were not artifacts of the use of sorted sperm. Eight X-linked genes (*BEX1*, *CAPN6*, *FMR1NB*, *SAT1*, *BEX2*, *X24112*, *SRPX2*, and *UBE2A*), one transcript putatively present on both sex Chrs (*Y2467*), a previously unreported gene located on the Y-Chr (*YZRSR2*), and four autosomal genes (*GSTM3*, *PGRMC1*, *LAMA1*, and *DNMT3A*), together with two Y-linked genes with an X-linked homolog not present on the array were analyzed. Fold-change values obtained by qPCR were very similar to those obtained in the array (Table 2).

Fig. 1. Comparison of male and female bovine blastocysts. (A) Hierarchical clustering of the 382 differentially expressed transcripts (Bonferroni correction) between male and female bovine blastocysts, comparing three different bulls and the three pools of embryos derived from Y- and X-sorted semen from each bull. The color gradient determines normalized gene expression of all of the samples. The dendrogram on the left depicts the grouping of samples based on the similarity between them. Samples were clearly grouped according to sex (blue and red bars on *Left*). (B) Chr distribution for the total transcripts present (green bars) and up-regulated in male (blue bars) or female (red bars) embryos (FDR $P < 0.05$). Percentages for each Chr out of the total transcripts with a known Chr location (7,691, 1,287, and 1,065 transcripts for present and up-regulated in males and females, respectively). (C) The pie chart shows the percentage of expressed X-linked transcripts ($n = 218$) grouped according to the fold change (FDR $P < 0.05$ correction). From pink to red were up-regulated in females ($n = 193$), blue were up-regulated in males ($n = 3$), and green did not show differences ($n = 22$). The line chart shows the percentage of X-linked transcripts compared to the total up-regulated transcripts in females with a known location after FDR for four groups according to the fold-change.

Table 2. Validation of array data by real-time qRT-PCR analysis

Gene	qPCR*	Array*	Statistical correction
<i>BEX1</i>	5.6	4.04	Bonferroni $P < 0.05$
<i>CAPN6</i>	4.13	4.07	Bonferroni $P < 0.05$
<i>GSTM3</i>	3.52	3.94	Bonferroni $P < 0.05$
<i>FMR1NB</i>	3.02	3.1	Bonferroni $P < 0.05$
<i>SAT1</i>	2.83	2.73	Bonferroni $P < 0.05$
<i>BEX2</i>	2.32	2.8	Bonferroni $P < 0.05$
<i>X24112</i>	2.28	2.63	Bonferroni $P < 0.05$
<i>SRPX2</i>	1.75	2.43	FDR $P < 0.01$
<i>PGRMC1</i>	1.52	1.62	Bonferroni $P < 0.05$
<i>UBE2A</i>	2.56	1.78	Bonferroni $P < 0.05$
<i>YZRSR2</i>	-1 [†]	-12.01	Bonferroni $P < 0.05$
<i>DDX3Y</i>	-1 [†]		Not in array
<i>EIF2S3Y</i>	-1 [†]		Not in array
<i>Y2467</i>	-5.38	-2.25	Bonferroni $P < 0.05$
<i>LAMA1</i>	-1.76	-1.93	Bonferroni $P < 0.05$
<i>DNMT3A</i>	-1.75	-1.19	FDR $P < 0.01$

*Expression fold-change of female versus male (positive values for up-regulated genes in females).

[†]Expressed only in males.

To determine whether the sex-related differences also occur for in vivo-derived embryos, the expression level of six genes was analyzed in in vivo-derived male or female embryos. Fold-change values obtained by qPCR were also similar to those obtained in the array (Fig. 2A).

Chr Distribution of Differentially Regulated Genes. Chr distribution comparison between expressed genes and up-regulated genes after FDR ($P < 0.05$) correction is shown in Fig. 1B. χ^2 analysis was performed to test for significant differences in Chr-location frequency between the three groups. The only Chr that displayed significant differences between up-regulated transcripts in males and females and expressed genes was the X-Chr, which accounted for 18.1% of the total up-regulated genes in females, whereas only 2.8% of the expressed transcripts were X-linked. Of the 218 X-linked transcripts expressed, 193 (88.5%) were up-regulated in females. Among them, only 10% exhibited a fold-change greater than 2, and most of them (70%) exhibited a fold-change lower than 1.66 (Fig. 1C, pie chart). Furthermore, X-linked genes accounted for almost half (47%) of the transcripts up-regulated in females that exhibit a fold-change higher than 1.66, whereas this percentage decreased (14%) in the groups with a lower fold change (Fig. 1C, line chart). The Chr distribution of the expressed X-linked genes along the X-Chr is shown in Fig. S2. Three regions, located at 0 to 3, 21 to 24, and 54 to 57 Mb account for 42.9% of the total transcripts up-regulated in females. No relation was found between fold-change and transcript location for the up-regulated X-linked transcripts in females and the 3 transcripts up-regulated in males and the 22 showing no sex-related differences were distributed in a similar way to the transcripts up-regulated in females. Some X-linked genes showing no sex-related dimorphism or up-regulation in males had a Y-Chr homolog, which suggests that the lack of sex-related differences may be caused by the transcription of both X- and Y-Chrs.

Gene Ontology. Gene ontology classification (FatiGo) was used for categorizing embryo expressed sequence tags of the gene ontology annotated genes present after FDR $P < 0.01$ correction (Fig. 3). Under the Molecular Function heading, nucleotide binding, DNA binding, nucleic acid binding, calcium ion binding, enzyme inhibitors, and transcription-factor activity were overrepresented among the up-regulated genes in female blastocysts, whereas hydrolase activity followed the opposite tendency. Biological Function analysis showed that transcription-related functions (regulation of transcription DNA-dependent, transcription, RNA

Fig. 2. Relative mRNA abundance. (A) Relative poly(A) mRNA abundance of six genes (four X-linked -*CAPN6*, *FMR1NB*, *SAT1* and *UBE2A*-, one Y-linked -*YZRSR2*-, and one autosomal -*DNMT3A*-) for male and female in vivo-derived blastocysts. (B) Relative poly(A) mRNA abundance of eight putative paternally expressed imprinted X-linked genes and one gene located on both X- and Y-Chrs (*Y2467*) for male (black bars), female (white bars), and parthenogenetic (dashed bars) in vitro blastocysts. Different letters indicate significant differences between groups based on one-way ANOVA ($P \leq 0.05$).

splicing, and RNA processing) were overrepresented in genes up-regulated in females, whereas translation, proteolysis, and protein transport followed the opposite tendency. In addition, signal transduction, cell differentiation, and multicellular organismal development were overrepresented in females, whereas metabolic process and cell cycle were in males. Finally, under Cellular Component, mitochondrion, mitochondrial inner membrane, and ribosome were overrepresented among the up-regulated genes in males, whereas cytoplasm, extracellular region, cytoskeleton, and cell projection were overrepresented in females.

Identification of a Previously Unreported Y-Linked Transcript. In the microarray there were three probes that were theoretically complementary to the X-linked *ZRSR2*; however, only one of the probes showed differential expression between male and female blastocysts, and it showed the highest difference among all of the genes. For this reason we designed primers specifically to amplify this sequence to sequence and clone the complete cDNA. A previously unreported 1382 pb cDNA was identified (GQ426330). The sequence [*YZRSR2*, Y-linked zinc finger (CCCH type), RNA-binding motif and serine/arginine rich 2 homologous] has 100% homology with a recently published BAC present in the bovine Y-Chr (AC216982.4) and is homologous to the X-linked gene *ZRSR2* (87% homology with the putative bovine gene -*XR_028361.2*- and 84% for human -*BC113480.1*-). The sequence contains an ORF encoding a protein of 446 amino acid residues. The transcript was found to be uniquely expressed in male embryos and tissues and was expressed from the beginning of embryonic genome activation (16-cell stage) onward and in all of the male tissues analyzed except the spleen (Tables S4 and S5).

Fig. 3. Fatigo-based comparative analysis of gene ontology for the family of genes exhibiting sex-related transcriptional differences based on (A) molecular function, (B) biological function, and (C) cellular component.

Putative Imprinted Gene Analysis. The expression of eight X-linked genes that were up-regulated in female blastocysts was analyzed in male and female blastocysts produced with unsorted semen and in parthenogenetic blastocysts. The expression level of five genes (*BEX1*, *CAPN6*, *BEX2*, *SRPX2*, and *UBE2A*) differed between parthenogenetic and female blastocysts, suggesting that the paternal allele is expressed at a higher level than the maternal (Fig. 2B). The expression level of one transcript present in both Y- and X-Chrs (*Y2467*) did not differ between parthenogenetic and female blastocysts. The presence of transcripts of these nine genes was also analyzed in denuded oocytes, preimplantation stage embryos, and adult tissues (Tables S4 and S5). All genes were present in oocytes and blastocysts, but the transcript presence in other stages differed among genes with two of them (*SAT1* and *UBE2A*) present in all stages. *CAPN6*, *SAT1*, and *UBE2A* were present in all tissues analyzed, whereas *Y2467* was not found in

adult tissues, *FMR1NB* was only expressed in testes, and *SRPX2* in kidney. The genes with a known location are shown in Fig. S2.

Discussion

Gene-expression variation may play a significant role in gender-specific early embryo development disparity through up- or down-regulating genes within physiological pathways. Herein we report extensive transcriptional differences occurring during pre-implantation and therefore not attributable to sex-specific hormonal actions. The transcription of genes located on the sex Chrs exerts direct or indirect effects on biological processes, which lead to sexual dimorphism. Genes on the Y-Chr will be only present in males, and X-Chr effects will be mediated by sex differences in the dose of X genes or their parental imprint. Almost half of the up-regulated transcripts in female embryos exhibiting a fold-change higher than 1.66 were X-linked, similar to the situation observed in mice (12). Dosage compensation by random X-Chr inactivation ensures an equal transcription level of X-linked genes for both sexes. Nevertheless, some genes may escape from X-Chr inactivation processes and be expressed from both X-Chrs, which cause an up-regulation in females. This escape is especially common during the preimplantation period (2, 3), when X inactivation is a reversible dynamic process (11) and leads to a phenotypic sexual dimorphism (7, 8). Although in vivo- and in vitro-produced embryos have been found to display the same transcriptional sexual dimorphism (12), sex-related differences may be exaggerated in in vitro-produced embryos compared with those derived in vivo, as X-Chr inactivation seems to be disrupted by some in vitro conditions (13, 14). However, the six genes (four X-linked, one Y-linked, and one autosomal) analyzed showed similar sex-related transcriptional differences for in vivo- or in vitro-derived blastocysts, suggesting that X-Chr inactivation is only partially achieved at the blastocyst stage in both in vivo and in vitro conditions, and that our in vitro conditions did not increase the sexual dimorphism. Sex-related differences are likely to be higher under sub-optimal conditions, which are known to alter sex ratio by preferential loss of embryos of one sex (7, 8). A similar situation occurs for other sex-related phenotypic differences, such as speed of development, which has been reported to be altered under some in vitro culture conditions, which lead to sex ratio distortion (15). However, under the conditions used in this study, no difference was observed in speed of development between male and female embryos (16), and the percentage of blastocysts at day 7 were similar between both groups (17).

Four-fifths of the up-regulated genes in female embryos and almost all of the up-regulated genes in male embryos were autosomal genes, indicating that sex-Chrs can exert an extensive regulation on the transcription of some autosomal genes, and therefore indirectly modulate sexual dimorphism. Gene ontology analysis was in agreement with observations previously reported. Among the cellular components, mitochondria and mitochondria inner membrane were overrepresented in the up-regulated genes in males, which is in agreement with the higher mtDNA copy number found in male embryos (1). Spliceosome, nucleotide, DNA, nucleic acid and calcium-ion binding, transcription factors activity, transcription and its regulation, and RNA splicing and processing were overrepresented among the up-regulated genes in females, which suggests a higher global transcriptional level for this sex; this is in accordance with the lower expression of de novo DNA methyltransferases (Dnmts) and lower methylation status reported in female embryos (1, 18) and stem cells (19). These differences in DNA-methylation regulation may account for the embryonic sex-specific susceptibility to a methyl-deficient maternal diet, and thus lead to gender-specific long-term effects in the offspring (20). The higher transcriptional level found in females is unlikely to be the direct consequence of the presence of an actively transcribed X-Chr in females instead of the Y-Chr, as X-Chr accounted for just 2.8% of the total expressed transcripts. How-

ever, it has been suggested that X-Chr encodes a modifier locus whose product represses de novo Dnmts, which could indirectly lead to these differences (19). In contrast, among the up-regulated genes in male blastocysts, ribosome, translation, proteolysis, and protein transport were overrepresented, suggesting a more active protein metabolism, which may be related with the differences in amino acid turnover recently found between male and female bovine blastocysts (21). Finally, cell differentiation, signal transduction, and development were overrepresented in females, which may suggest differences in developmental processes.

All these phenotypic sex-related differences cannot be attributable to sex-specific hormonal differences but to genomic actions, which are not only restricted to the preimplantation period, as sexual dimorphism phenomena independent of hormonal interaction have been reported in adult tissues. Thus, a large sex effect on gene expression (30% of expressed genes) and trans-regulation was described in mouse macrophages that were cultured 2 weeks *ex vivo*, and thus were not influenced by endogenous sex steroids (22). Furthermore, the chromosomal sex of muscle-derived stem cells influences their ability to promote skeletal muscle regeneration by differential transcription of genes related to cell stress response (23). Moreover, male and female cells, independent of hormonal influences, respond differently to stressors, and a possible sex-dependent gene regulatory mechanism was suggested to explain sexually dimorphic physiology and pathology (24). Interestingly, male and female embryos of different species (mice, bovine, and human) respond differentially to environmental stress (25). Collectively, these data suggest that a large degree of sexually dimorphic gene expression may be directly dependent on X- and Y-Chr dosage, rather than on the hormonal environment, and that cells differ and respond to stress innately according to sex, irrespective of their history of exposure to sex hormones.

The unique Y-linked transcript described here (*YZRSR2*) is located in the nonrecombining portion of the Y-Chr, which only maintains those genes responsible for large fitness effects, generally being restricted to those either required for male function or those that determine sex (26). In both humans and mice, *ZRSR2* has an autosomal homolog (*ZRSR1*) (27), which in mice is a paternally expressed imprinted gene that is silenced during oogenesis (28). The function of *YZRSR2* remains unclear, but it seems to have evolved from the X-linked gene *ZRSR2*, which encodes an essential splicing factor (29). Thus, *YZRSR2* may have a role in sex determination and sexual dimorphism by operating at the posttranscriptional level, although more studies would be needed to test this hypothesis. The Y-Chr is particularly important for the study of sex determination and fertility because of its rapid species-specific differential evolution and divergence (30). Unfortunately, it is the only Chr that remains unsequenced in the cattle genome project (31).

The higher expression of X-linked genes in female compared to male embryos may be caused by a preferential paternal allele expression (12), by a double allele expression, (2, 13, 14) or by a combination of both. In the first case, a lower expression for male and parthenogenetic embryos compared with their female counterparts is expected, as they lack a paternal X-Chr (12, 32). Five genes (*BEX1*, *CAPN6*, *BEX2*, *SRPX2*, and *UBE2A*) were found to be preferentially expressed by the paternal allele (Fig. 2B). Maternal allele expression level was lower, but not absent. Although autosomal and X-linked imprinted genes can be biallelically expressed at the blastocyst stage, which may mask imprinting mechanisms, they may show quantitative effects of imprinting in terms of differences in gene expression between monoparental and biparental embryos (11, 32). Most of the X-linked genes up-regulated in female embryos exhibited a fold-change lower than 2, suggesting that X-Chr inactivation occurred partially. Unfortunately, very little is known about X-chromosome inactivation during preimplantation development in other species than mouse (33). In adult tissues, X-chromosome inactivation is a random and stable process, although some paternally expressed

X-linked genes, such as *XIST* and *RHOX5* (12), have been reported (<http://igc.otago.ac.nz/home.html>). Furthermore, females with Turner syndrome (XO) differ in their cognitive and behavioral phenotypes, according to the parental origin of their single X chromosome in human and mice (34), which may be caused by imprinting. Imprinting mechanisms have been proposed to evolve from a mechanism to defend the genome against transposable elements and, compared to autosomal chromosomes, the X chromosome has generated a disproportionately high number of functional retroposed genes in mammalian species (35).

We do not know if these differences in gene expression are similar in other species. However, in one study on mouse embryos, using a microarray analysis with 20,371 transcripts and three pools of male and female blastocysts, fewer than 600 sex-biased genes were detected (12). The large degree of sex bias in gene expression that we detected in bovine blastocysts can partly be attributed to the large power we had to detect highly significant sex effects on gene expression, even when the absolute effect was small because of the large sample size (nine pools each of male and female blastocysts). In our study, when only one bull and three pools each of male and female bovine blastocysts were analyzed, a small number of sex-biased genes was detected because of the small sample size and the low power to detect small effects as significant.

The high number of genes exhibiting sexual dimorphism shows an extensive transcriptional regulation lead by the sex Chrs and provides a basis for the study of transcriptional sexual dimorphism without hormonal interaction, which occurs both for *in vivo*- and *in vitro*-derived embryos. Furthermore, they suggest that data from male and female embryos should be analyzed separately in gene expression analyses focused on individual blastocysts and embryos produced by nuclear transfer from a male or female cell line, on embryo-maternal gene communication, and on stem cell lines. In addition, we have identified five X-Chr linked genes whose expression level in female embryos differs from male and parthenogenetic embryos, suggesting that they are predominantly expressed by the paternally inherited X-Chr and opening the possibility of a synergistic effect of imprinting and double-X dosage on X-linked genes transcriptional sex-related differences. Future analysis of gene expression in *in vivo* embryos produced under different environmental conditions may help to understand the nature and consequences that these early sex differences may have on sex ratio control in mammals. By analyzing these early sex differences it may be possible to exert greater control on sex ratio manipulation in domestic animals and to better understand other aspects of early embryo development, early sex determination mechanisms, X inactivation, and epigenetic and genetic processes related to early development that may have a short-term effect on implantation and a long-term effect on offspring.

Materials and Methods

Sperm Collection, Sorting, and Verification of the Sorting Procedure. Semen was collected and sorted flow cytometrically for sex from each of three bulls as previously described (36). Approximately 90% of the embryos produced with sorted sperm were of the predicted sex (17).

Embryo Production. *In vitro* fertilization procedures were performed as previously described (36). Details in [SI Materials and Methods](#). *In vivo*-derived blastocysts were obtained from superovulated cows on day 7 after artificial insemination. Details in [SI Materials and Methods](#).

RNA Isolation and Target Preparation. Total RNA was isolated and purified from 18 pools of 60 day 7 blastocysts each (three bulls, two sexes, three replicates) using the RNeasy Micro kit (Qiagen). Embryos were pooled randomly to avoid batch-to-batch variation. Labeled cRNA was synthesized with a linear RNA amplification method (Message Amp Premier Kit, Ambion) following the manufacturer's instructions and using 50 ng of total RNA as template for reverse transcription.

Array Hybridization and Scanning. Labeled cRNA was fragmented in fragmentation buffer [5× buffer: 200 mM Tris-acetate (pH 8.1)/500 mM KOAC/150 mM MgOAc] and hybridized to the microarrays ($n = 18$ arrays, as described above) in 200 μ L of hybridization solution containing 15 μ g labeled target in 1× Mes buffer (0.1 M Mes/1.0 M NaCl/20 mM EDTA 0.01% Tween20) and 0.1 mg/mL herring sperm DNA, 10% DMSO, 0.5 mg/mL BSA, 50 pM control oligonucleotide B2 and 1× eukaryotic hybridization controls (bioB, bioC, bioD, cre). Both control oligonucleotide B2 and eukaryotic hybridization controls were purchased from Affymetrix. The hybridization mix was applied to the GeneChip Bovine Genome Array (Affymetrix), which contains 24,072 bovine gene probe sets, representing more than 23,000 transcripts, including assemblies from 19,000 UniGene clusters. Arrays were placed on a rotisserie and rotated at $0.4 \times g$ for 16 h at 45 °C. Following hybridization, the arrays were washed and stained with a streptavidin-phycoerythrin conjugate (Molecular Probes). The arrays were scanned using a confocal scanner (GC3000_Affymetrix). The image data were analyzed by GeneChip Operating Software (GCOS 1.4, Affymetrix).

Array Bioinformatic and Statistical Analysis. For the bioinformatic analysis dChip and Affy/AffyPLM (Bioconductor) software were used to detect outlier samples and Partek Genomics Suite 6.4 (Partek software, Partek Inc.) to perform gene expression analysis. RMA processing was used for normalizing the data as well as a global median normalization. The change in expression of each gene was calculated by determining the fold-change (ratio) of the mean intensity of each group. Statistical analysis was based on a regression model and ANOVA to look for significant genes between conditions and hierarchical un/supervised analysis performed using Average linkage and Euclidean distance for classifying the samples. Bonferroni or FDR corrections were applied to reduce the total number of false-positives. Chr distribution was performed based on the Entrez link provided in annotation file Bovine.na29.annot.csv available on the Affymetrix Web site among the 9,322 expressed transcripts.

Gene ontology (FatiGO: <http://babelomics.bioinfo.cipf.es>) was used for categorizing embryo expressed sequence tags with respect to gene function, including molecular function, biological process, and cellular component (37). Raw data from microarray experiments was submitted to the Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo>). The platform ID is GPL2112. The accession ID is GSE17921.

Independent Verification of Array Data Using Real-Time RT-PCR. Poly(A) RNA was extracted after sexing from individual blastocysts produced in vitro with unsorted semen or by parthenogenetic activation or in vivo with unsorted semen following the manufacturer's instructions using the Dynabeads mRNA Direct Extraction KIT (DynaL Biotech) with minor modifications (17). (Details are available in *SI Materials and Methods*). The quantification of all mRNA transcripts was carried out by real-time qRT-PCR following a previously described protocol (1). Five pools of cDNA per experimental group, each obtained from 10 in vitro-produced or 5 in vivo-derived embryos, were used with two repetitions for all genes of interest. Details of protocol for quantification of mRNA transcripts are available in *SI Materials and Methods*. The primers listed in Table S6 were used to amplify specific fragments referring to the selected transcripts. PCR fragments were sequenced to verify the resulting PCR product.

YZRSR2 Cloning and Sequencing. Reverse transcription was performed in blastocyst RNA and full-length cDNA of YZRSR2 was cloned with 5'- and 3'-RACE using SMART Technology (Clontech Laboratories, Inc.).

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