REPRODUCTION
RESEARCH

Biological differences between *in vitro* produced bovine embryos and parthenotes

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Abstract

Parthenotes may represent an alternate ethical source of stem cells, once biological differences between parthenotes and embryos can be understood. In this study, we analyzed development, trophectoderm (TE) differentiation, apoptosis/necrosis, and ploidy in parthenotes and *in vitro* produced bovine embryos. Subsequently, using real-time PCR, we analyzed the expression of genes expected to underlie the observed differences at the blastocyst stage. *In vitro* matured oocytes were either fertilized or activated with ionomycin +6-DMAP and cultured in simple medium. Parthenotes showed enhanced blastocyst development and diploidy and reduced TE cell counts. Apoptotic and necrotic indexes did not vary, but parthenotes evidenced a higher relative proportion of apoptotic cells between inner cell mass and TE. The pluripotence-related *POU5F1* and the methylation *DNMT3A* genes were downregulated in parthenotes. Among pregnancy recognition genes, *TP-1* was upregulated in parthenotes, while *PGRMC1* and *PLAC8* did not change. Expression of *p66^{shc}* and *BAX/BCL2* ratio were higher, and *p53* lower, in parthenotes. Among metabolism genes, *SLC2A1* was downregulated, while *AKR1B1*, *PTGS2*, *H6PD*, and *TXN* were upregulated in parthenotes, and *SLC2A5* did not differ. Among genes involved in compaction/blastulation, *GJA1* was downregulated in parthenotes, but no differences were detected within *ATP1A1* and *CDH1*. Within parthenotes, the expression levels of *SLC2A1*, *TP-1*, and *H6PD*, and possibly *AKR1B1*, resemble patterns described in female embryos. The pro-apoptotic profile is more pronounced in parthenotes than in embryos, which may differ in their way to channel apoptotic stimuli, through *p66^{shc}* and *p53* respectively, and in their mechanisms to control pluripotency and *de novo* methylation.

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Introduction

Parthenogenetic activation of oocytes allows study of the roles of paternal and maternal genomes in early mammalian development. Parthenogenesis is a process by which the oocyte develops without the male gamete. This form of reproduction is not spontaneous in mammals, although it is an extended reproductive procedure in fish, amphibians, and snake, among other organisms. Parthenotes may represent an alternative ethical source of stem cells, once biological differences between parthenotes and embryos can be understood (Brevini & Gandolfi 2008). Parthenogenetic activation is a major step in nuclear transfer, which may facilitate or improve the genomic reprogramming of a reconstructed embryo and increase its chance of reproductive success (Kim *et al*. 1996). Matured mammalian oocytes remain arrested in metaphase II stage, a blockage in development that has been suggested to occur as an evolutionary safety mechanism to prevent parthenogenesis inside the female (Cibelli *et al*. 2006). The oocyte is released from its blocked stage when the fertilizing spermatozoon triggers multiple and rhythmic oscillations of intracellular free calcium (Yang *et al*. 1994). A variety of chemicals can mimic the intracellular calcium patterns and activate the oocyte without sperm (Cibelli *et al*. 2006) to yield parthenogenetic embryos. The combination of activators with either protein synthesis inhibitors or kinase inhibitors seems to be more efficient than single activation procedures (Jellerette *et al*. 2004), which has been recently confirmed in the bovine species (Wang *et al*. 2008).

Once transferred to surrogate mothers, parthenotes are unable to develop to term, although they can go through gastrulation and early stages of organogenesis. Essential alterations in parthenotes affect not only the trophoblast (something precociously evident at the blastocyst stage), but also parts of the embryo proper, such as muscle, liver, and pancreas (Fundele *et al*. 1989, 1990). In the cow, the

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Table 1 Cumulative embryo development of *in vitro* matured bovine oocytes fertilized or parthenogenetically activated with ionomycin +6-DMAP.

			Percentage of Day 3 embryos			Percentage of Day 8 blastocysts		
Group	***N***	**R**	Cleaved	5–8 Cells	8–16 Cells	Total	Expanded	Hatched
Parthenotes	899	17	81.4±1.6	38.1±2.3[x]	7.3±1.7[x]	42.4±3.0[x]	29.5±2.7[x]	3.0±1.1
Embryos	1089	17	85.1±1.5	68.2±2.3[y]	24.2±1.7[y]	31.2±2.9[y]	20.4±2.7[y]	3.1±1.0

Data are LSM±S.E.M. as proportions of cultured oocytes. Different superscripts express significant differences (x, y: $P<0.01$). *N*, oocytes in culture; R, replicates.

maximum development in uterus reached by a parthenogenetic embryo has been reported to be of 48 days (Fukui *et al.* 1992). The arrest in development of parthenotes is thought to be due to alterations in their genomic imprinting, which in turn seems to be an evolutive result of conflicts between the parental genomes during growth (Brevini & Gandolfi 2008). Maternal and paternal genes are reciprocally imprinted, such that both genomes are needed for correct growth, being complementary but not equivalent (Smith 2001). Therefore, monoparental duplication of gene expression would lead to embryonic lethality. A recent review identified more than 50 imprinted genes expressed in humans (Brevini & Gandolfi 2008); these and other non-imprinted genes could be therefore altered at early embryonic stages. The endpoint normally used in parthenogenetic experiments *in vitro* is the development up to the blastocyst stage (Cibelli *et al.* 2006). Studies on differential cell counts between IVF embryos and parthenotes are scarce (de la Fuente & King 1998, Van de Velde *et al.* 1999, Neuber *et al.* 2002), and the information on apoptosis is usually limited to the DNA damage phenotype (Neuber *et al.* 2002, Hao *et al.* 2004, Wang *et al.* 2008) without a focus to distinguish histological traits of necrotic and apoptotic nuclei and without a detailed gene expression study as a way to explore underlying mechanisms. An expression analysis covering genes representative of essential events in development could help to understand alterations in genomic imprinting, cell differentiation, and apoptosis showed by parthenotes.

In the present work, we analyze differences between the parthenotes and IVF embryos in blastocyst development, allocation of cells to the trophectoderm (TE) and the inner cell mass (ICM), and apoptosis/necrosis. Subsequently, we quantify the levels of mRNA transcription within a panel of genes related to functionality of the epiblast, TE, and pregnancy recognition, metabolism, apoptosis and growth arrest, and morula compaction and blastocyst formation.

Results

Development, differential cell counts, and apoptosis/necrosis analysis

In vitro development is shown in Table 1. Parthenotes and embryos exhibited a markedly different developmental pattern. Embryos showed significantly increased development rates at cleavage stages and at the embryonic genome major activation stage (i.e. 8- to 16-cell stage), while parthenotes showed enhanced blastocyst and expansion rates. No differences were noted at hatching. Parthenotes showed a significant reduction in TE cell counts (Table 2), which in turn accounted for a reduction in total cells. However, ICM cells and the proportions of ICM/total cells did not differ between parthenotes and embryos. Apoptotic and necrotic indexes (NI) did not vary (Table 3), although parthenotes evidenced a higher proportion of apoptotic cells in their ICM over that in TE cells.

Gene transcription analysis

Four pools of 15 embryos per treatment were analyzed in duplicated PCRs.

Figure 1 shows the genes involved in metabolism and compaction/blastocyst formation. Among metabolism related genes, *H6PD*, *AKR1B1*, *PTGS2* (*COX2*), and *TXN* genes were upregulated in parthenotes, while *SLC2A1* was upregulated in embryos, and *SLC2A5* did not show differences. Within compaction and blastocyst formation genes, *GJA1* (connexin-43; *CX43*) was upregulated in embryos, while *CDH1* (E-cadherin) and *ATP1A1* did not differ between embryos and

Table 2 Numbers of cells contained in the trophectoderm (TE), inner cell mass (ICM), and percentage of inner cell mass/total cells from bovine-expanded blastocysts derived from oocytes activated with 6-DMAP-ionomycin or fertilized.

		ICM		TE			
Treatment	***N***	No. of cells	Range	No. of cells	Range	**Total**	**Percentage of ICM/total**
Parthenotes	24	31.4±3.5	0–60	94.1±9.9[x]	52–178	123.9±11.4[x]	24.7±2.2
Embryos	20	32.5±3.4	0–57	126.3±9.5[y]	60–202	161.3±10.7[y]	22.2±2.1

Data are LSM±S.E.M. (four replicates). *N*, number of embryos counted. *x*, *y* ($P<0.01$).

Table 3 Apoptotic and necrotic index in the inner cell mass (ICM), trophectoderm (TE), and total cells, dead cell index (DCI: total TUNEL positive cells), plus relative proportions of apoptotic cells between the ICM and TE (ICM/TE, as a percentage) of day 8 parthenogenetic and fertilized bovine blastoycsts.

Group	*N*	Apoptotic index			Apoptosis	Necrotic index			DCI
		ICM	TE	Total	ICM/TE	ICM	TE	Total	
Parthenotes	23	3.1±0.8	1.3±0.4	2.1±0.3	1.40±0.2[a]	3.5±1.0	3.1±0.8	3.2±0.6	5.3±0.8
Embryos	20	2.3±0.8	2.3±0.4	2.3±0.4	0.86±0.2[b]	2.3±0.9	2.7±0.7	2.4±0.6	4.7±0.8

Data are LSM±S.E.M. from four replicates (a,b: $P=0.03$).

parthenotes. Figure 2 shows the genes involved in pluripotency/*de novo* methylation, apoptosis, and pregnancy recognition. The pluripotency-related gene *POU5F1* (*Oct3/4*) and the *de novo* methylation *DNMT3A* gene are downregulated in parthenotes. Among the growth arrest and apoptotic genes, the stress sensor *SHC1SHC* (*p66*^{shc}) is upregulated in parthenotes; this is contrary to *TP53* (*p53*) that shows downregulation in these blastocysts. The pro-apoptotic gene *BAX* did not change, while *BCL2L1* (*BCL2*) expression decreases in parthenotes. The resulting *BAX/BCL2L1* ratio was higher in parthenotes, suggesting a pro-apoptotic status. *TP-1* doubled its expression level in parthenotes, while *PGRMC1* and *PLAC8* did not differ.

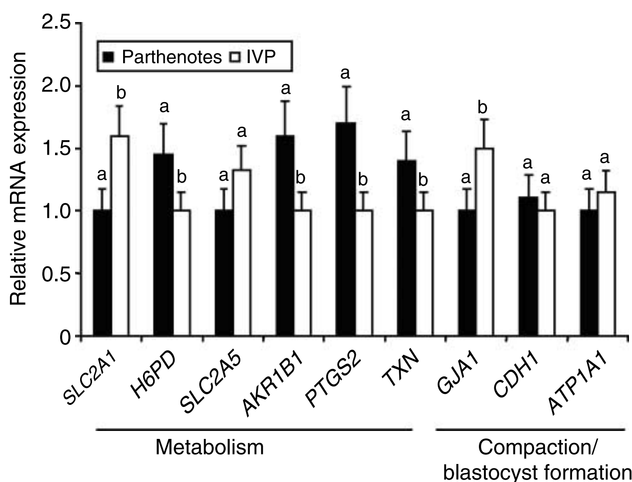

Figure 1 Relative mRNA transcription in bovine *in vitro* fertilized and parthenogenetic blastocysts of genes involved in metabolism and compaction/blastocyst formation (superscripts express significant differences within a gene; $P<0.05$).

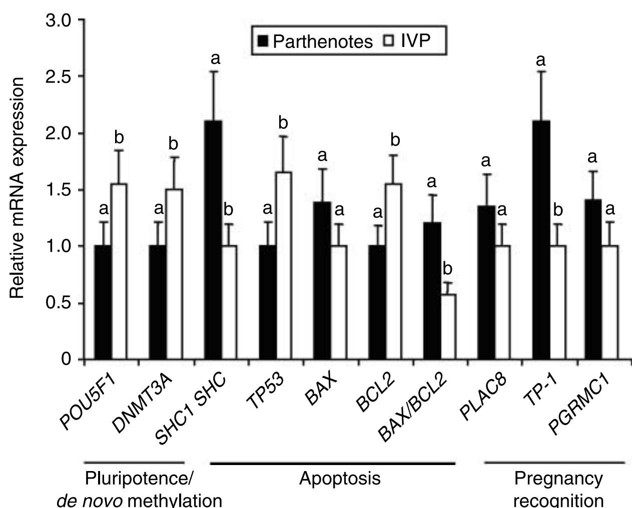

Figure 2 Relative mRNA transcription in bovine *in vitro* fertilized and parthenogenetic blastocysts of genes involved in pluripotency/*de novo* methylation, apoptosis, and pregnancy recognition (superscripts express significant differences within a gene; $P<0.05$).

Ploidy analysis

Parthenotes showed higher diploid and lower haploid rates than IVF embryos (see Table 4). In fact, no parthenote was found to be haploid, while no IVF embryo was judged as polyploid. Mixoploid rates did not differ.

Discussion

The present study shows that parthenotes and IVF bovine embryos have different patterns of development. Both groups have similar first cleavage rate, but parthenotes developed at rates lower than IVF embryos during subsequent earlier stages, as seen on day 3. However, parthenotes proceeded faster once the compacted morula stage was reached (day 6). In agreement with our results, parthenotes were reported to show a first cleavage rate comparable with IVF embryos (Van de Velde *et al*. 1999). Using time-lapse cinematography, Neuber *et al*. (2002) showed that the first cleavage occurred earlier in parthenotes than in IVF embryos, although, in agreement with our observations, second and third cleavages showed lower rates in parthenotes than in IVF embryos. DNA synthesis starts earlier in parthenotes than in IVF embryos (De la Fuente & King 1998). Therefore, the differences reported herein could be due to the use of a specific bull for IVF, as bulls affect

Table 4 Ploidy in bovine parthenogenetic and IVF bovine embryos.

Group	*N*	Diploid	Polyploid	Mixoploid	Haploid
Parthenote	29	79.3±8.5[a]	6.9±3.3	13.4±7.6	0.0[a]
Embryos	30	56.6±8.4[b]	0.0	30.0±7.5	13.8±4[b]

N, blastocysts counted. Chromosomal status expressed as LSM±S.E.M. Superscripts (a, b) express significant differences ($P<0.05$).

cleavage rates, developmental kinetics, blastocyst yields, and blastocyst quality (reviewed in Vandaele *et al*. (2006)). The paternal genome is actively demethylated in IVF embryos and it transcribes before the maternal complement does (Gutierrez-Adan *et al*. 1997, Reik *et al*. 2003). The slower development in parthenotes at earlier stages may relate to the absence of an active paternal genome. The results of this study indicated that parthenotes contained fewer cells in the TE than IVF embryos, which led to a reduction in total cells. This abnormality has been well described by other authors who studied total cells in bovine parthenotes (Van de Velde *et al*. 1999, Kubisch *et al*. 2003, Wang *et al*. 2008) or, TE and ICM differentially (de la Fuente & King 1998, Neuber *et al*. 2002). However, cells in the ICM were reported to be reduced (de la Fuente & King 1998) or, consistent with our results, unaltered (Neuber *et al*. 2002). Reductions in cell numbers seem to be a rule in parthenotes of all species (Surani *et al*. 1984, Newman-Smith & Werb 1995, Hardy & Handyside 1996, Hao *et al*. 2004). The reduction in TE cells could account for the shortage in extra-embryonic tissue and almost no trophoblast found in parthenotes arrested development (Kaufman *et al*. 1977).

The relative frequency of apoptosis (i.e. apoptotic ICM/TE cells) was higher in parthenotes than in IVF embryos. However, we did not observe the changes in apoptosis and necrosis indexes between parthenotes and IVF embryos, By contrast, other studies found higher apoptotic indexes (AI) in parthenotes than in IVF embryos (Neuber *et al*. 2002, Hao *et al*. 2004, Wang *et al*. 2008). Frequency of diploid blastocysts was reported to be lower in parthenotes than in IVF embryos (Wang *et al*. 2008). Interestingly, haploidy increases the incidence of apoptosis in preimplantation embryos, while parthenogenetic activation and parthenogenesis themselves would not lead to apoptosis (Liu *et al*. 2002*a*, 2002*b*, Jeong *et al*. 2005). Haploidy is also responsible for diminished blastocyst rates over normal embryos and diploid parthenotes (Henery & Kaufman 1992, Jeong *et al*. 2005). Therefore, the enhanced diploidy we found in parthenotes is consistent with their AI (that was not increased) and improved development rates.

Parthenotes exhibited a pro-apoptotic gene expression profile as defined by their increased *BAX/BCL2* ratio. This condition was not consistent with an enhanced apoptotic phenotype in parthenotes, but it could relate to pattern differences because an increased relative frequency of apoptosis was observed in the ICM over the TE. *BAX* and *BCL2* are pro- and anti-apoptotic genes respectively, and the tendency of a cell to undergo apoptosis is continuously counterbalanced by pro- and anti-apoptotic gene expression (Duan *et al*. 2005). Interestingly, the mRNA for $p66^{shc}$ was more abundant in parthenotes, contrary to *p53*, which increased in IVF embryos. In IVF bovine embryos, the stress related protein $p66^{shc}$ is up-regulated in the early arrest, while *p53* does not seem to be involved in the same function (Favetta *et al*. 2004, 2007*a*, 2007*b*). Apoptosis is involved in the bovine early developmental arrest *in vivo* (Yang & Rajamahendran 2002), although developmental arrest independent of apoptosis has been also described to occur in the uterus (Ikeda *et al*. 2006). To the best of our knowledge, there are no reports on $p66^{shc}$ gene expression in parthenotes. However, although $p66^{shc}$ and *p53* might have common regulatory mechanisms under particular circumstances (Rodríguez *et al*. 2007), parthenotes and embryos could differ in their ability to channel apoptotic stimuli through $p66^{shc}$ and *p53* (Matsumoto *et al*. 1997, Yin *et al*. 2002).

The increase in *BAX/BCL2* ratio in parthenotes is consistent with a reduced expression of *POU5F1*. In the pig, parthenotes showed increased blastocyst yields and cell numbers in coincidence with decreased TUNEL-positive nuclei and *BAX* expression, while increasing *BCL2L1* and *POU5F1* expression (Choi *et al*. 2008). Knockdown of *POU5F1* mRNA causes a reduction in the ICM cells in bovine IVF embryos (Nganvongpanit *et al*. 2006*a*), which could be a sign of diminished pluripotency. However, the ICM of our parthenotes showed no reduced cell counts and produces outgrowths (data not shown). This suggests that a reduction in *POU5F1* gene expression could be not always limitative of the ICM viability as an outgrowth. In fact, embryos with different development potential, such as those produced by nuclear transfer, *in vivo*-derived and IVF embryos do not differ between their *POU5F1* expression (Long *et al*. 2007). The role of *POU5F1* in maintenance of pluripotency in bovine is controversial, as *POU5F1* transcripts have been found both in the ICM and the TE (Degrelle *et al*. 2005). *DNMT3A* sets up the methyl-CG landscape of the genome early in the development. The reduced *DNMT3A* levels found in our study suggest a lower methylation activity in parthenotes, which agrees with a low expression of *DNMT3A* reported in female blastocysts (Bermejo-Alvarez *et al*. 2008) and XX ES cell lines as it compares with XY or XO lines (Zvetkova *et al*. 2005). Sagirkaya *et al*. (2006) found that the *DNMT3A* expression associates with a lower rate of blastocyst development. Accordingly, in the present work, reduced *DNMT3A* expression was observed in parthenotes, which in turn showed high blastocyst development rates. From our and the above results, it is likely that the reduced *DNMT3A* levels are associated with the female genome.

The *PLAC8*, an invasion specific gene, is more abundant in blastocysts resulting in calf delivery compared with those resulting in resorption (El-Sayed *et al*. 2006), and in endometrium of pregnant as compared with non-pregnant cows (Galaviz-Hernandez *et al*. 2003, Klein *et al*. 2006). These findings suggest a potential role for PLAC8 in placental development and fetus–maternal interface. Our work shows that parthenotes are not defective in expressed *PLAC8*. Interferon-tau (*TP-1*) is produced by the trophoblast of ruminant

species before placentation is initiated. The rapid upregulation of *TP-1* expression at a time when the corpus luteum is wavering on the point of regression is essential if the pregnancy is to be maintained, while a less than robust production may contribute to embryonic loss (Roberts *et al*. 1990). Female bovine (Larson *et al*. 2001, Kimura *et al*. 2004), and deer embryos (Flint *et al*. 1997) express higher *TP-1* than males and could be responsible for differential blastocyst loss. Female embryos and parthenotes produce more *TP-1* than male embryos (Kubisch *et al*. 2003). The above findings are consistent with our increased *TP-1* mRNA abundance in parthenotes. *PGRMC1* is a membrane-bound progesterone receptor that mediates anti-apoptotic effects of progesterone in granulosa cells (Engmann *et al*. 2006, Peluso *et al*. 2006, 2008). Bovine embryos at early cleavage stages (Dode *et al*. 2006) and rat oocytes (Peluso *et al*. 2006) express *PGRMC1*. The role of *PGRMC1* in development seems to be related to pregnancy recognition. Equine conceptuses from day 7 to day 14 show increased *PGRMC1* (Rambags *et al*. 2008). The non-differential expression of *PGRMC1* and *PLAC8* between parthenotes and embryos, as well as the higher *TP-1* expression in parthenotes, suggest that the activities represented by these genes would not be a limitation in the viability of parthenotes.

The enzyme of the aldose reductase gene (*AKR1B1*) exerts two activities, namely metabolizing progesterone that is important to implantation, and synthesizing PGF2α and, subsequently, terminating pregnancy. *AKR1B1* is also known as the cause for cell apoptosis in somatic cells that accumulate sorbitol (Galvez *et al*. 2003, Wirtu *et al*. 2004). Expression of *AKR1B1* has been detected in endometrium (Madore *et al*. 2003) and also in IVF bovine embryos (Dode *et al*. 2006, El-Sayed *et al*. 2006) and is associated with failure to establish pregnancy and resorption (El-Sayed *et al*. 2006). Perhaps, *AKR1B1* contributes to deciding the fate of the embryo through activation of apoptotic mechanisms. Its dependence on glucose could lead *AKR1B1* to address certain glucose-linked signs of sexual dimorphism, such as altered developmental kinetics and sex-based, stress resistance (Gutiérrez-Adán *et al*. 2006). We found an enhancement in the pentose phosphate pathway limiting rate *H6PD*, an X-linked gene, in parthenotes. Expression of *H6PD* is increased in female embryos (Wrenzycki *et al*. 2002, Lopes *et al*. 2007), being involved in metabolic differences between male and female embryos (Iwata *et al*. 2002, Kimura *et al*. 2005, Lopes *et al*. 2007). However, parthenotes have been reported not to overexpress the X-linked genes, *H6PD*, and *XIST* (Wrenzycki *et al*. 2002), although in the latter study, embryos were picked up on day 7 and the exact blastocyst stage at which they were analyzed was not described (Wrenzycki *et al*. 2002). In somatic cells, the overexpression of *H6PD* induces the expression of pro-oxidative enzymes (Leopold *et al*. 2003, Park *et al*. 2006), which is consistent with the overexpression of thioredoxin (*TXN*) in our parthenotes. The ubiquitous protein disulfide reductase TXN plays a role in the mouse implantation (Nonogaki *et al*. 1991), and responds to oxidative stress in bovine and mouse embryos (Nonogaki *et al*. 1991, Bing *et al*. 2003). The *TXN* gene is downregulated in embryos that fail to give pregnancies and those leading to resorption (El-Sayed *et al*. 2006), while the addition of the compound to a pig embryo culture improves blastocyst formation, cell numbers, and apoptosis (Ozawa *et al*. 2006). The up-regulation of *TXN* in parthenotes could be beneficial to the short-term survival of these blastocysts.

Prostaglandin G/H synthase-2 (*PTGS2*) is barely detected at a constitutive level, while it is markedly inducible in specialized cell types (Smith *et al*. 2000). Inhibition of PTGS2 increases pregnancy rates after embryo transfer and lowers PGF2α release (Scenna *et al*. 2005). Expression of *PTGS2* is more abundant in blastocysts resulting in calf delivery compared with those resulting in resorption (El-Sayed *et al*. 2006). In bovine, the *PTGS2* protein localizes to the TE, while the ICM does not express *PTGS2* (Charpigny *et al*. 1997), suggesting that this gene is necessary for the elongation and subsequent implantation. Moreover, higher expression of *PTGS2* throughout the implantation window indicates an important role for the PGs released by the embryo in mediating interactions with the uterus (Charpigny *et al*. 1997, Wang *et al*. 2002). As *PTGS2* expression is time regulated during ruminant embryo development, differential expression could be related to asynchronous development between IVF and parthenogenetic embryos. Expression of *SLC2A1* was reduced in parthenotes, while *SLC2A5* (a fructose transporter) did not change. Bovine *in vivo* derived embryos express *SLC2A1* and *SLC2A5* (Augustin *et al*. 2001, Balasubramanian *et al*. 2007). However, IVP embryos show reduced *SLC2A1* mRNA abundance as compared with *in vivo* counterparts (Lazzari *et al*. 2002, Balasubramanian *et al*. 2007, Rho *et al*. 2007). The expression of *SLC2A1* in IVP embryos has been found in the ICM and the TE (Wrenzycki *et al*. 2003). Embryonic respiration and expression of *SLC2A1* and *H6PD* correlate, and *SLC2A1* is down-regulated in female blastocysts (Lopes *et al*. 2007). Therefore, the down-regulation of *SLC2A1* in parthenotes may reflect an absence of pro-oxidative conditions, such as described to occur in female blastocysts.

Compaction in cattle is less pronounced in the IVP embryos as related to *in vivo* embryos (Prather & First 1993). The intercellular structures are altered in the IVP embryos (Boni *et al*. 1999), and the expression of genes responsible for compaction and cell-to-cell adhesion, such as *GJA1* (*CX43*) and E-cadherin (*CDH1*), is diminished (Wrenzycki *et al*. 1996, 2001, Rizos *et al*. 2002, 2003). A low expression of *GJA1* has been associated to low quality of IVP blastocysts (Lonergan *et al*. 2003*a*, 2003*b*, Rizos *et al*. 2003), low

developmental competence as oocytes (Nemcova *et al*. 2006), and reduced survival to cryopreservation (Rizos *et al*. 2002). *CDH1* intervenes in cell-to-cell adhesion as it associates to compaction (Pratt *et al*. 1982, Fleming *et al*. 1984), while its transcripts are found at comparable levels in the ICM and TE of bovine blastocysts (Shehu *et al*. 1996, Barcroft *et al*. 1998, Wrenzycki *et al*. 2003). *CDH1* plays an important role in the blastocyst formation and expansion, and knocking down *CDH1* mRNA in bovine embryos reduces the blastocyst rate (Nganvongpanit *et al*. 2006*b*). In our study, levels of *CDH1* did not differ between blastocysts and parthenotes, so the *CDH1* contribution to compaction and blastulation is not altered in parthenotes. However, *GJA1* levels are diminished in parthenotes, although coexpressed connexins may compensate for one another (Houghton *et al*. 2002).

The composition and accumulation of fluid in the blastocoele is regulated by Na/K-ATPase, an essential activity for the differentiation of the TE. In the mouse, the expression of multiple isoforms of α and β subunits by the blastocyst maintains cavitation, but targeted disruption in the Na/k-ATPase α1-subunit suppresses expansion and the embryo dies (Barcroft *et al*. 2004). Embryonic Na/K-ATPase activity increases when a nascent blastocoele cavity forms and decreases to baseline levels at full expansion (Houghton *et al*. 2003). We found lower *ATP1A1* expression in fully expanded than in hatched blastocysts (Rodríguez *et al*. 2006). According to their development rates and *ATP1A1* expression levels, our parthenotes did not seem to differ essentially from embryos during blastulation.

The results of this study show that 6 out of 18 genes analyzed (i.e. *SLC2A5*, *CDH1*, *ATP1A1*, *BAX*, *PLAC8*, *PGRMC1*) were equally expressed between embryos and parthenotes. Four genes (i.e. *DNMT3A*, *TP-1*, *H6PD*, and *SLC2A1*) varied in parthenotes such as reported to occur within female embryos. In addition, possible sexually dimorphic expression of *AKR1B1* merits further research, because of its association with metabolic traits differentially regulated between male and female embryos. Pregnancy recognition, as judged by the gene panel studied, would not differ from IVF embryos, as the only significant change observed was an increase in *TP-1*, typical from the female parthenogenetic genotype. Maybe genes involved in pregnancy recognition do not exert such a function in the blastocyst. A correct genomic imprinting could be unnecessary if a functional placenta is still not formed. Therefore, parthenogenetic blastocysts would serve as a source of stem cells, but not for implantation. Gene transcription indicates that the pro-apoptotic profile of parthenotes is more pronounced than that of embryos, which does not strictly correlate to an apoptotic phenotype. Parthenotes and embryos could differ in stress signaling (*p53* and $p66^{shc}$) that triggers growth arrest and eventually apoptosis. Gene expression did not shed light on compaction and blastocyst formation, as the lower expression in the parthenogenetic *GJA1* did not correspond to morphological or functional differences between both blastocyst types. Metabolism related genes accounted for most changes in gene expression: *H6PD*, *SLC2A1* and, as hypothesized, *AKR1B1*, showed changes consistent with embryos bearing the female genotype. A lower expression of *OCT3/4* in parthenotes may indicate that the pluripotency in such blastocysts could be compromized, although this decrease is probably not to occur in a short term. Ultimately, *de novo* methylation was lower in parthenotes, as it occurs in female embryos. A proportion of the genes analyzed may explain important traits of the parthenogenetic phenotype, especially concerning cell death. Other genes are in coincidence with the genotype exhibited by female embryos. Genes that differ between parthenotes and IVF embryos are potential candidates to be imprinted, which opens doors to further research.

Materials and Methods

All reagents were purchased from Sigma, except otherwise indicated.

In vitro *maturation*

Cumulus–oocyte complexes (COCs) from slaughterhouse ovaries were aspirated from 3 to 8 mm visible follicles and processed throughout as described in a previous work (Rodríguez *et al*. 2007). Briefly, COCs were washed three times in holding medium (HM) consisting of TCM199 (Invitrogen), 25 mM Hepes, and BSA 0.4 g/l, supplemented with 2 IU/ml heparin. For *in vitro* maturation (IVM), selected COCs were cultured for 24 h in bicarbonate-buffered TCM199, FSHp (1 μg/ml), LH (5 μg/ml), 17β-estradiol (1 μg/ml), and 10% fetal calf serum. Maturation was performed by culturing ~ 50 COCs in 500 μl of medium at 38.7 °C in 5% CO_2 in air with high humidity for 22–24 h.

In vitro *fertilization and parthenogenetic activation*

Matured oocytes were submitted to either IVF or parthenogenetic activation (PA).

For IVF, a frozen/thawed semen straw corresponding to a single bull was layered down 1 ml pre-equilibrated Sperm-TALP. After 1 h of incubation, ~ 700 μl of the upper layer of supernatant containing the motile sperm was removed. The sperm were centrifuged, washed, and cell sperm concentration was determined. The COCs were washed twice in HM and placed in four-well cultured dishes containing pre-equilibrated fertilization medium (Fert-TALP) with heparin (10 μg/ml, Calbiochem, La Jolla, CA, USA). Spermatozoa were then added at a concentration of 2×106 cells/ml in 500 μl medium per well containing 100 COCs for maximum. IVF was accomplished by incubating oocytes and sperm cells together for 18–20 h at 38.7 °C in 5% CO_2 with high humidity. Cumulus cells were detached from fertilized oocytes after IVF using a vortex.

For PA, the cumulus cells were removed by incubation in 0.1% hyaluronidase for 2 min and by vortexing in M199–BSA for another 2 min. Matured oocytes were activated with 5 mM ionomycin for 4 min followed by 2 mM 6-DMAP for 4 h.

Both presumptive zygotes and parthenotes were washed three times in culture medium which consisted of synthetic oviduct fluid containing amino acids, citrate, and myo-inositol (mSOF), as described by Holm *et al*. (1999), modified with 6 g/l BSA and no serum. *In vitro* culture (IVC) was made in four-well dishes with 400 μl of medium layered under mineral oil at 38.7 °C in 5% CO_2, 5% O_2, and humidified air. Cleavage stages, morulae, and blastocyst development were recorded on days 3, 6, and 8 respectively (day 0=onset of IVF or PA).

Differential cell counts

Embryonic cells were counted in the ICM and the TE of expanded blastocysts. Embryos were fixed and stained as reported by Thouas *et al*. (2001). Blastocysts were incubated in 500 μl BSA-free TCM199 Hepes (Invitrogen) with 1% Triton X-100, and 100 μg/ml propidium iodide for 30 s. Samples were then fixed in 500 μl ethanol with 25 μg/ml bisbenzimide (Hoechst 33342) and stored overnight at 4 °C. These fixed and stained blastocysts were transferred directly to a glycerol droplet on a glass microscope slide. Cell counts were made using digital images obtained with an inverted microscope equipped with a u.v. excitation filter at 330–385 nm and a barrier filter at 420 nm. TE cells were identified by their red fluorescence; ICM cells appeared blue.

Apoptosis and necrosis

Expanded blastocysts were fixed in 4% (w/v) PBS-buffered paraformaldehyde and permeabilized with PBS containing 0.5% (v/v) Triton-X 100 and stored overnight in PBS containing 10 μg/ml BSA. After washing with PBS containing 0.1 μg/ml PVA, the blastocysts were incubated for 1 h at 37 °C in 30 μl droplets of TUNEL (TdT -mediated dUTP nick-end labeling) reaction mixture (*in situ* Cell Death Detection Kit, Roche) under paraffin oil in darkness. Negative controls contained no TdT. Positive control was obtained by treating samples with 100 U/ml DNase I for 1 h at 37 °C. After the TUNEL reaction, the nuclei were stained with 10 μg/ml bisbenzimide diluted in PBS–PVA for 10 min. The blastocysts were washed in PBS with 0.1 μg/ml PVA, mounted on a glass slide with droplets of glycerol containing 2.5% (w/v) 1,4-diazabicyclo(2.2.2) octane, flattened with a cover slip and examined under an Olympus IX50 fluorescence microscope. The wide band filters for blue and uv excitation were used for the detection of TUNEL and bisbenzimide staining respectively. Discrimination between nuclei showing apoptotic or necrotic morphology was based on a previous description (Gjørret *et al*. 2003), as depicted in Rodríguez *et al*. (2006). Briefly, TUNEL positive nuclei consisting of condensed chromatin aggregated in granular masses, pycnotic appearance or multiple fragments scattered within cellular spaces were considered as apoptotic. Nuclei that showed unclear or fluffy edges and swelling or fragmentation into numerous fluffy elements plus swelling were considered necrotic.

TE and ICM cells were enumerated separately. The allocation of cells to the TE or ICM was based on their position in the embryos and the relative size of their nuclei. Cells within the ICM appear as a mass within the embryo and have smaller nuclei than those seen in the TE cells. The ratios of apoptotic and necrotic cells to total cells were termed as the AI and NI respectively. The relative frequencies of apoptotic cells within the ICM and TE (RF) were calculated as: AI of the ICM/AI of the TE.

Cytogenetic analysis

The embryos developed to blastocyst stage were evaluated for their ploidy by a modification of the method of Alexander *et al*. (2006). In brief, embryos were synchronized at metaphase by transferring to IVC medium containing 0.1 μg/ml demecolcine (KaryoMAX Colcemid, Gibco BRL) for 16 h. Then the embryos were exposed to a hypotonic solution (0.8% sodium citrate) for 3 min and placed in cold methanol–glacial acetic (3:1) solution for 6 h. The fixed embryos were carefully dropped individually onto a pre-cleaned microscope slide. Few drops of cold methanol–glacial acetic (1:1) solution were dropped over each embryo to spread the preparation. After air drying, the slides were stained with 4% Giemsa solution for 10 min and ploidy was evaluated at ×1000 with oil-immersion optics. Embryos were classified as being haploid (n), diploid (2n), and polyploid (≥3n).

Gene expression

RNA extraction

Pools of 15 expanded, day 8 blastocysts were snap frozen in LN_2 and then stored at −80 °C. Isolation of mRNA was performed using the Dynabeads mRNA Direct KIT (DYNAL, Oslo, Norway), following the manufacturer's instructions. The samples were lysed in 180 μl lysis/binding buffer (DYNAL). Hybridization was performed with 100 μl Dynabeads oligo (dT)25 for 10 min. Poly A RNA–beads complexes were separated from the binding buffer and rinsed in buffer A and B (DYNAL), using a magnetic separator. Poly A RNA was finally eluted in 180 μl 10 mM Tris–HCL.

Reverse transcription

Reverse transcription was achieved using the First-Strand cDNA synthesis kit for RT-PCR, (AMV, Roche) with oligo (dT)25, following the manufacturer's instructions. The tubes were then incubated at 25 °C for 10 min to allow annealing. The RNA was subsequently reverse transcribed at 42 °C for 60 min, followed by 5 min incubation at 99 °C to denature the RT enzyme. The samples were then cooled at 4 °C.

Real-time PCR

Quantification was performed using a real-time PCR (qRT-PCR) method. Briefly, a Rotorgene 2000 Real Time CyclerTM (Corbett Research, Sydney, Australia) and SYBR Green (Molecular Probes, Eurogene, OR, USA) as a double-stranded

DNA-specific fluorescent dye were used to determine the mtDNA copy number. The PCR reaction mixture (25 ml) contained: 1× PCR buffer, 3 mM MgCl2, 2 U Taq Express (MWGAG Biotech, Ebersberg, Germany), 100 mM of each dNTP, and 0.2 mM of each primer. In addition, the double-stranded DNA dye, SYBR Green I, (1:3000 of 10 000 × stock solution) was included in each reaction. The PCR protocol included an initial step of 94 °C (2 min), followed by 40 cycles of 94 °C (15 s), 56–59 °C (30 s)), and 72 °C (10 s). Fluorescence data were acquired at 83 °C after the elongation step. The melting protocol consisted of a hold temperature at 40 °C for 60 s and then heating from 50 to 94 °C, holding at each temperature for 5 s while monitoring fluorescence. Product identity was confirmed by ethidium bromide-stained 2% agarose gel electrophoresis. Experiments were conducted to contrast relative levels of each transcript and histone H2AFZ (*H2A*) in every sample. PCR was performed adding 2 μl aliquot of each sample to the PCR mix containing the specific primers to amplify each gene. Primer sequences, the sizes of the amplified fragments of all transcripts, and the GenBank accession number are shown in Table 5. The comparative CT method was used to quantify the expression levels (Gutierrez-Adan *et al*. 2004). Quantification was normalized to the endogenous control, *H2A*. Fluorescence was acquired in each cycle to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background for each sample. Within this region of the amplification curve, a difference of one cycle is equivalent to doubling of the amplified PCR product. According to the comparative C_t method, the ΔC_t value was determined by subtracting the $H2AC_t$ value for each sample from each gene C_t value of

Table 5 Primer sequences, the sizes of the amplified fragments of transcripts, and the GenBank accession number.

Gene symbol	MGI or HGNC official name	Primers sequence (5′–3′)	Fragment size	GenBank accession numbers
H2AFZ	Histone H2AFZ	5′-AGGACGACTAGCCATGGACGTGTG 5′-CCACCACCAGCAATTGTAGCCTTG	212	NM_016750
POU5F1	POU domain class 5 1 (Oct3/4)	5′-CGAGTATCGAGAACCGAGTG 5′-CAGGGTTCTCTCCCTAGCTC	440	NM_174580.1
DNMT3A	DNA (cytosine-5)-methyltransferase 3 alpha	5′-CTGGTGCTGAAGGACTTGGGC 5′-CAGAAGAAGGGGCGGTCATC	318	XM_001252215.1
SHC1 SHC (*p66^{shc}*)	SHC (Src homology 2 domain containing) transforming protein 1	5′-GTGAGGTCTGGGGAGAAGC 5′-GGTTCGGACAAAGGATCACC	334	NM_001075305
TP53	Tumor protein p53	CTCAGTCCTCTGCCATACTA GGATCCAGGATAAGGTGAGC	364	U74486
BAX	BCL2-associated X protein	5′-CTACTTTGCCAGCAAACTGG 5′-TCCCAAAGTAGGAGAGGA	158	NM_173894.1
BCL2L1	BCL2-like 1	5′-GGAGCTGGTGGTTGACTTTC 5′-CTAGGTGGTCATTCAGGTAAG	517	BC147863.1
PLAC8	Placenta-specific 8	5′-CGGTGTTCCAGAGGTTTTTCC 5′-AAGATGCCAGTCTGCCAGTCA	163	NM_016619
TP-1	Interferon-tau (IFN-t)	5′-GCCCTGGTGCTGGTCAGCTA 5′-CATCTTAGTCAGCGAGAGTC	564	AF238612
PGRMC1	Progesterone receptor membrane component 1	5′-TGTGTGTCACAAATCCAGAAAG 5′-AATCATGCAGTTAGGTCAATCG	265	AF254804
SLC2A1	Solute carrier family 2 (facilitated glucose transporter), member 1	5′-AGCGTCATCTTCATCCCAGC 5′-CCACAATGCTCAGGTAGGAC	540	M60448
H6PD	Glucose-6-phosphate dehydrogenase	5′-TTGCGGCCGCCGTCCTCTATGTG	220	XM_583628
SLC2A5	Solute carrier family 2 (facilitated glucose transporter), member 5	5′-AGTCATCTCCATCATCGTCCT 5′-GTACCCGCCACCATGTAGGCAG	531	AF308830
AKRIBI	Aldose reductase mRNA	5′-CGTGATCCCCAAGTCAGTGA 5′-AATCCCTGTGGGAGGCACA	152	M314631
PTGS2	Prostaglandin G/H synthase-2	5′-ATCTACCCGCCTCATGTTCCT 5′-GGATTAGCCTGCTTGTCTGGA	187	AF031698
TXN	Thioredoxin	5′-ATGGTGAAACAGATTGAGAGCA 5′-CGTTGGAATACTTTTCAGAGAGA GAA	154	AF104105
GJA1	Gap junction protein, alpha 1, 43kDa	5′- TGCCTTTCGTTGTAACACTCA 5′- AGAACACATGAGCCAGGTACA	142	AY382593.1
CDH1	Cadherin 1, type 1, *CDH1*	5′- GACACTGGAGGTATCAGCGCAC 5′- TGATCTGGACCAGCGACTTAGG	193	AY508164.1
ATP1A1	ATPase, Na+/K+ transporting, alpha 1 polypeptide	5′- ACCTGCTGGGCATCCGAGTGAC 5′- GGGAAGGCACAGAACCACCAG	333	BC123864.1|

the sample. Calculation of $\Delta\Delta C_t$ involved using the highest sample ΔC_t value (i.e. the sample with the lowest target expression) as an arbitrary constant to subtract from all other ΔC_t sample values. Fold changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta C_t}$.

Statistical analysis

The data were analyzed in two steps. First, the factors with significant influence were identified by categorical data modeling (CATMOD) using SAS v. 8.2 software (1999; SAS Institute Inc., Cary, NC, USA). CATMOD fits linear models to frequency response functions. Treatment and replicate were found to have significant influence on the dependent variables. Secondly, those factors identified as significant were used to produce a linear model using the general linear models procedure (GLM; SAS software). This procedure performs ANOVA for the unbalanced data. GLM was used to estimate the least-square means (LSM) for each fixed effect having a significant *F* value. Development and apoptosis/necrosis data were transformed to frequency percentages. Blastocyst cell counts were handled as absolute values. One way repeated-measures ANOVA (followed by multiple pair-wise comparisons using Student–Newman–Kleus method) was used for the analysis of differences in the mRNA expression assayed by quantitative RT-PCR. Gene expression was expressed as arbitrary units.

Declaration of interest

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

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