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Putative imprinted gene expression in uniparental bovine embryo models

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Abstract. Altered patterns of gene expression and the imprinted status of genes have a profound effect on cell physiology and can markedly alter embryonic and fetal development. Failure to maintain correct imprinting patterns can lead to abnormal growth and behavioural problems, or to early pregnancy loss. Recently, it has been reported that the *Igf2R* and *Grb10* genes are biallelically expressed in sheep blastocysts, but monoallelically expressed at Day 21 of development. The present study investigated the imprinting status of 17 genes in *in vivo*, parthenogenetic and androgenetic bovine blastocysts in order to determine the prevalence of this unique phenomenon. Specifically, the putatively imprinted genes *Ata3*, *Impact*, *L3Mbtl*, *Magel2*, *Mkrn3*, *Peg3*, *Snrpn*, *Ube3a* and *Zac1* were investigated for the first time in bovine *in vitro* fertilised embryos. *Ata3* was the only gene not detected. The results of the present study revealed that all genes, except *Xist*, failed to display monoallelic expression patterns in bovine embryos and support recent results reported for ovine embryos. Collectively, the data suggest that monoallelic expression may not be required for most imprinted genes during preimplantation development, especially in ruminants. The research also suggests that monoallelic expression of genes may develop in a gene- and time-dependent manner.

Additional keywords: androgenetic, genomic imprinting, parthenogenetic, preimplantation.

Introduction

Imprinting is a process by which parents leave their mark on the DNA of future offspring. This mark results in the selective repression of mRNA expression from one parental allele (Surani 1994; Delaval and Feil 2004). Imprinting was first described following the discovery that both genomes are required for completion of murine embryogenesis (McGrath and Solter 1984). Surani *et al*. (1984) suggested that imprinting of specific genes is established during early gametogenesis. Although it has been widely believed that, following establishment of imprinting in the gametes, imprints are maintained throughout development (Young and Fairburn 2000; Ferguson-Smith *et al*. 2003; Murphy and Jirtle 2003; Delaval and Feil 2004), recent research in the ovine model has shown that monoallelic expression may not be developed until after Day 21 of development in this species (Thurston *et al.* 2008).

In the human, imprinting defects result in a variety of disorders involving growth, brain development and behavioural defects (Walter and Paulsen 2003). In livestock industries, the phenomenon of imprinting has implications for assisted reproductive technologies (ART), as well as for the inheritance of quantitative trait loci (QTL) and marker-assisted selection (MAS) programs. Imprinting has been implicated in the large offspring syndrome (LOS) seen in both *in vitro* fertilised (IVF) and cloned offspring, as well as in placental irregularities observed during cloned pregnancies (Hiendleder *et al*. 2004; Wrzeska and Rejduch 2004).

In the present study, nine genes were targeted for characterisation during bovine preimplantation embryo development, including genes unknown or known to be imprinted in cattle, as well as genes with unknown and known embryonic expression patterns. Amino acid transport system A3 (Ata3) mediates the transport of α-[^{14}C]-(methylamino)isobutyric acid and [^{3}H]-alanine (Sugawara *et al*. 2000). It is imprinted in the mouse (unknown in human), with paternal expression on embryonic Day 15.5 and in all adult tissues examined, except the liver and viscera (Mizuno *et al*. 2002). The gene *Imprinted and ancient* (*Impact*) is paternally expressed from chromosome 18

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in the mouse (Hagiwara *et al*. 1997). *Impact* is also paternally expressed in the rabbit and rat, but is expressed biallelically in the human, macaque and pig (Okamura *et al.* 2005). In the human, *IMPACT* is highly expressed in the brain and is a candidate for bipolar affective disorder (Kosaki *et al*. 2001). Although the mouse and human genes share significant regions of homology, human *IMPACT* is biallelically expressed, possibly due to differences within the first intron. In the mouse, this intron contains a cytosine guanine dinucleotide (CpG) island with characteristic tandem repeats; however, this island is missing from the first intron of the human gene (Okamura *et al*. 2000). Analysis of the bovine sequence shows the presence of two CpG islands within the first intron, suggesting that this gene will be monoallelically expressed in the bovine, similar to the mouse (D'Cruz, unpubl. obs.).

L(3)malignant brain tumour (L3MBTL) is a member of the Polycomb group of proteins involved in transcriptional regulation. Mutations of *L3MBTL* in *Drosophila* cause asynchronous mitotic divisions and disruptions to nuclear migration in the early embryo (Yohn *et al*. 2003). *L3MBTL* has been suggested to play a role in histone deacetylase-independent transcriptional repression and shows paternal-only expression in human haematopoietic cells (Li *et al*. 2004), but biallelic expression in the mouse (Li *et al.* 2005). There have been no reports of its expression in mammalian embryos. *MAGE-like 2* (*Magel2*) has been mapped to the Prader–Willi syndrome (PWS) region and is imprinted in the mouse, cow and human, with paternal expression only in the adult brain of both the mouse and human (Boccaccio *et al*. 1999; Khatib *et al.* 2007). *Makorin, ring finger protein, 3* (*Mkrn3/Znf127*) is paternally expressed in both the mouse and human and is predicted to function as a ribonucleoprotein (Jong *et al*. 1999*a*). Paternal expression was found in the mouse brain, heart and kidney, as well as in normal and androgenetic fibroblasts, but not in parthenogenetic embryo-derived fibroblasts (Jong *et al*. 1999*b*). *Mkrn3* was also found to be expressed in growing murine oocytes, but not in fully grown oocytes (Obata and Kono 2002). *Paternally expressed gene 3* (*Peg3*), a zinc-finger gene, is also imprinted in the cow, with high expression levels in the brain, testis and ovary (Kim *et al.* 2004). It was originally found through a screen of novel myogenic regulatory factors, but was later found to have a key role in p53-mediated apoptosis (Relaix *et al.* 2000). *Small nuclear ribonucleoprotein polypeptide N* (*Snrpn*) is well characterised in the mouse and human, with paternal expression in both species (Glenn *et al*. 1993; Szabo and Mann 1995). In the human and mouse preimplantation embryo, monoallelic (paternal) expression is detected from the four-cell stage onwards (Szabo and Mann 1995; Huntriss *et al*. 1998).

Ubiquitin protein ligase E3A (*Ube3a*) is maternally expressed in the mouse and human brain, but biallelically expressed elsewhere (Rougeulle *et al*. 1997). *Ube3a* is expressed in human oocytes and preimplantation embryos (Monk and Salpekar 2001; Salpekar *et al*. 2001). Finally, Zac1/Plagl1, a zinc finger protein, is paternally expressed in fetal and adult mouse tissues, except for the liver, where biallelic expression is observed (Piras *et al*. 2000). Knockout of *Zac1* results in intrauterine growth restriction, altered bone formation and neonatal lethality in mice (Varrault *et al.* 2006).

The aims of the present study were to characterise eight putatively imprinted genes during early bovine *in vitro* embryo development and to then investigate 16 genes [those from the present study and those reported by Ruddock *et al.* (2004)] at the blastocyst stage of development in *in vivo*, parthenogenetic (PA) and androgenetic (AN) embryos in order to determine genomic imprinting status.

Materials and methods

All chemicals were purchased from Sigma Chemical (St Louis, MO, USA) unless stated otherwise in the text.

Animal ethics

Experiments were approved by and performed under the guidelines of the Monash University Animal Ethics Committee for animal experimentation. Monash University adheres to the Victorian Prevention of Cruelty to Animals Act and Regulation 1986 (the law), and NHMRC (2004).

Bovine in vitro *maturation and IVF*

Bovine oocytes were collected, matured, fertilised and cultured as described previously (Ruddock *et al*. 2004). Briefly, bovine immature oocytes surrounded by cumulus cells were aspirated from abattoir-derived ovaries and matured in TCM199 medium supplemented with 10 $\mu g\,mL^{-1}$ follicle-stimulating hormone (FSH; Ovagen; ICPBio, Auckland, New Zealand), 0.1 $IU\,mL^{-1}$ luteinising hormone (LH; Chorulon; Intervet, Bendigo East, Vic., Australia), 1 $\mu g\,mL^{-1}$ β-oestradiol, 1 $ng\,mL^{-1}$ insulin-like growth factor (IGF)-I, 100 μM cysteamine and 10% (v/v) fetal calf serum (FCS; Lot No. IL0403; JRH Biosciences, Brooklyn, Vic., Australia). Following 20–22 h maturation at 39°C in 5% CO_2 in air, cumulus–oocyte complexes (COCs) were either denuded of cumulus cells for oocyte mRNA extraction or fertilised in modified Fert-Talp medium (Daniels *et al*. 2001) and cultured in modified synthetic oviduct fluid (SOF) medium (Gardner *et al*. 1994) until the desired stage of development. To collect morula- and blastocyst-stage embryos, the culture medium was supplemented on Day 5 with 5% (v/v) fibroblast growth factor (FGF) 4-conditioned medium from mouse embryonal carcinoma cells (Hall *et al*. 2005*a*), 25 $ng\,mL^{-1}$ heparin and 10% (v/v) charcoal-treated FCS.

Parthenogenetic embryo production

Oocytes were activated with 5 μM calcium ionophore A-23187 for 4 min in tissue culture medium 199 (TCM-199) supplemented with 25 mM HEPES (TCM-H) and 20% FCS before being transferred to SOF medium containing 2.5 $\mu g\,mL^{-1}$ cytochalasin D and 10 $\mu g\,mL^{-1}$ cycloheximide for a further 5 h incubation in 5% CO_2 in air and a further 3 h incubation in 5% CO_2 in air. Following 5 h incubation, oocytes were cultured *in vitro* according to the IVF procedure described above.

Androgenetic embryo production

Androgenotes were produced as described previously (Lagutina *et al*. 2004). Briefly, after *in vitro* maturation (IVM), oocytes were denuded of cumulus cells by vortexing in the presence

of hyaluronidase and oocytes with an extruded polar body were stained with Hoechst 33342. Enucleation was performed by aspiration of the polar body and associated metaphase II plate in a minimal volume of cytoplasm. Completeness of enucleation was confirmed by the identification of metaphase chromosomes within the enucleation pipette under ultaviolet light. All manipulations were performed in SOF medium supplemented with 20 mM HEPES, 6 mg mL^{-1} bovine serum albumin (BSA) and essential and non-essential amino acids (SOF-HEPES) in the presence of cytochalasin B (5 μg mL^{-1}). IVF was then performed with 0.5–1 × 10^6 sperm mL^{-1} in fertilisation SOF medium supplemented with essential and non-essential amino acids (SOFaa) (Gardner *et al*. 1994) without glucose and supplemented with 1 μg mL^{-1} heparin, 20 μM D-penicillamine, 100 μM hypotaurine and 1 μM epinephrine in 5% CO_2 and 5% O_2 in humidified air at 38.5°C. At 17 h of IVF, embryos were washed to remove sperm and centrifuged at room temperature for 3 min at 15 000*g* in 45% Percoll in SOF-HEPES in order to visualise pronuclei by differential interference contrast optics (DIC). The pronucleus with a small volume of cytoplasm was enucleated with a 25–30-μm diameter pipette and transferred into the perivitelline space of another single pronuclear zygote. All manipulations were performed in SOF-HEPES in the presence of cytochalasin B (5 μg mL^{-1}). The cytoplast–karyoplast constructions were fused in 0.3 M mannitol solution, containing 50 μM $CaCl_2$ and 100 μM $MgCl_2$, by a single direct current (DC) pulse of 1.2 kV cm^{-1} applied for 30 μs at 20–22 h after IVF. Embryos were cultured in SOFaa medium. During embryo culture, half of the medium was renewed on Day 3 with fresh SOFaa and on Day 6 with TCM199 containing 16 mg mL^{-1} BSA (where Day 0 was the day of IVF).

In vivo *blastocyst collection*

Superovulation and non-surgical embryo collection (Day 7) of mixed-breed, multiparous, postpartum cycling beef cows (BioXcell, Holbrook, NSW, Australia) was performed as described previously (Hall *et al.* 2005*b*).

Isolation of mRNA and synthesis and amplification of cDNA

Extraction of mRNA from single oocytes or embryos for cDNA production and amplification was as described previously (Ruddock *et al*. 2004). Briefly, single oocytes or embryos were lysed in 3–5 μL lysis buffer (0.8% (w/v) Igepal, 5 mM dithiothreitol (DTT) and 1 IU μL^{-1} RNAsin) followed by mRNA extraction with Dynabeads (Dynal, Carlton South, Vic., Australia). The synthesis and amplification of cDNA were performed using SMART cDNA synthesis kits (Becton Dickinson, North Ryde, NSW, Australia).

Gene-specific polymerase chain reaction

Amplified cDNA was diluted 1 : 10 and then tested for several control genes before gene-specific polymerase chain reaction (PCR) of the genes of interest (at least three replicate PCRs were run for each gene in each embryo; 1 μL diluted amplified cDNA was used per reaction). The PCR primers for control genes and the genes of interest are listed in Table 1. The PCRs were run for 30–35 cycles and consisted of denaturation (95°C, 45 s), annealing (54–68°C, 45 s) and extension (72°C, 1 min) steps, with annealing temperatures for each gene listed in Table 1. All PCR products were confirmed by direct sequencing.

Results

Putative imprinted gene expression in IVM oocytes and IVF embryos at various stages of development

Eight putatively imprinted genes were investigated in amplified cDNA from individual oocytes, two-, four-, eight- and 16–32-cell embryos, morula, blastocysts and hatched blastocysts ($n = 3$ per stage). Investigation of the expression of the control genes *PolyA polymerase*, *Oct4*, *Gdf9* and *Ifn-τ* has been performed and reported previously for these amplified oocyte and embryo cDNA stocks (Ruddock *et al*. 2004). The following genes, which have been shown previously to be imprinted in either the mouse, human or bovine, were investigated in the present study: *Ata3*, *Impact*, *L3Mbtl*, *Magel2*, *Mkrn3*, *Peg3*, *Snrpn*, *Ube3a* and *Zac1*. Expression profiles are shown in Fig. 1. *Ata3* was not detected at any stage of preimplantation development. *Impact* and *Ube3a* appeared to be expressed in almost all embryos tested. *L3Mbtl* transcripts were detected in only two hatched blastocysts. *Magel2* was expressed in one oocyte and one eight-cell embryo, and then in most samples from the 16–32-cell stage onwards. *Mkrn3* transcripts were found in almost all embryos from the 16–32-cell stage onwards. *Peg3* was detected in one four-cell embryo. *Snrpn* transcripts were detected in most of the two- to eight-cell embryos, but were not found beyond this stage of development. *Zac1* expression was detected in almost all oocytes and early embryos, as well as in two of three morulae, but not in any blastocysts.

Expression in in vivo*, PA and AN blastocysts*

In vivo, PA and AN blastocysts were analysed for expression of the genes of interest in order to determine imprinting status. Five individual blastocysts were analysed from each group. All blastocysts showed expression of *Actin*, *Oct4* and *Ifn-τ*, and no blastocysts contained transcripts for *Gdf9*, an oocyte-specific gene (Fig. 2). Each blastocyst was then tested for 16 known or putatively imprinted genes: *Ata3*, *Dlk1*, *Gnas*, *Grb10*, *Impact*, *L3Mbtl*, *Magel2*, *Mest isoform 1*, *Mkrn3*, *Ndn*, *Nnat*, *Peg3*, *Sgce*, *Snrpn*, *Ube3a*, *Xist* and *Zac1* (Fig. 3). *Gnas*, *Impact*, *Ndn* and *Ube3a* were expressed in all blastocysts tested. *Ata3*, *Dlk1*, *Mest isoform 1*, *Nnat*, *Peg3* and *Snrpn* transcripts were not detected in any blastocysts. The other genes showed varying expression patterns within and between groups. *Grb10* was detected in all but two AN blastocysts. *L3Mbtl* was only detected in one AN blastocyst. *Magel2* transcripts were detected in two *in vivo* blastocysts and one AN blastocyst. *Mkrn3* was expressed in all *in vivo* blastocysts, four of five PA blastocysts and one of five AN blastocysts. *Sgce* was expressed in all PA blastocysts. *Xist* was expressed in one of five *in vivo* blastocysts and all five PA blastocysts. *Zac1* was expressed in two of five AN blastocysts.

Discussion

Imprinted genes play critical roles in fetal and placental growth and brain development in human (Walter and Paulsen 2003)

Table 1. Reverse transcription–polymerase chain reaction primers

Gene	Primer (5′ → 3′)	GenBank accession no.	Length (bp)	Annealing temperature (°C)
Poly(A) polym.	Forward: GTTGCAGGGTAACCGATGAA Reverse: TGTTGTGGGTATGCTGGTGT	X63436	361	56
Oct4	Forward: GGTTCTCTTTGGAAAGGTGTTC Reverse: ACACTCGGACCACGTCTTTC	AF022987	314	56
GDF9	Forward: GCTGCTTTGCCTGGCTCTGT Reverse: TGTCACATCAATCTCAATCC	NM_174681	595	56
IFN-τ	Forward: GCCCTGGTGCTGGTCAGCTA Reverse: CATCTTAGTCAGCGAGAGTC	AF238611	584	56
Ata3	Forward: ATCYTGGGCTTGTCCTATGC Reverse: GAGGGCAGGGWATTTGGAAT	AY948548	474	64
Dlk1	Forward: GTGACCAGTGCGTGACCTTT Reverse: GCAGGTCTTGTCCATGAAGC	AY360448	454	54
Gnas	Forward: GAAGGACAAGCAGGTCTACC Reverse: GACCATGTTGTAGCTGCTG	AY376066	675	60
Grb10	Forward: GAAGATGGGACAAGCAAAGT Reverse: CTGGCACCAAGTAACCATCTG	AY376067	290	58
Impact	Forward: TGGCGAGGAGTGGTGTGTCA Reverse: GGCATAGATGTTGTGGGTGG	AY948549	594	68
L3Mbtl	Forward: CGAGAACGAATCTGAGCCA Reverse: CGGAATACCCATCAAAGTGC	AY948550	351	66
Magel2	Forward: CTGATGGTGGTTCTGAGCCT Reverse: CAGGACAATCATCTTGCTGG	AY948551	257	60
Mest	Forward: CGCCGAGATCGTCTCCGCAG Reverse: CTCCACGATGCTGGCCTGCTC	AY376068	377	58
Mkrn3	Forward: TGAAGCCGAGAKWGACAATG Reverse: CCTGCGGATACACCTAAKACA	AY948552	498	56
Ndn	Forward: GTGAARGATGTCATCGGCAG Reverse: GTCCTCWGAGACACTGYTGC	AY360449	590	60
Nnat	Forward: CCTCGGCWGAACTGCTCATC Reverse: GCGKTGCCTRTGCCCAGAT	AY360450	517	60
Peg3	Forward: CTTCGCGGTCATTTCTGAGT Reverse: TTGTCCTTGCCGTACATCTTC	AY427787	282	60
Sgce	Forward: CCCGTTACCCTATCAAGCAG Reverse: GGCAGCACATGATATAAGCG	AY376070	557	56
Snrpn	Forward: TGGGAAGGAGCAGCAAGGTG Reverse: TGGTCAACTGATGGTGGCGG	AY948553	532	62
Ube3a	Forward: GGAGTTGATGAGGGAGGTGTT Reverse: TCTGTAGTTTCTTCTAGTGCTTGGA	AY948554	635	58
Xist	Forward: AGCATTGCTTAGCATGGCTC Reverse: TGGCTGTGACCGATTCTACC	AF104906	365	60
Zac1	Forward: GGGAAGAAGTACAACACCATGC Reverse: CTGTGTGGACCACCAGGT	AY995187	249	63

and bovine (Wrzeska and Rejduch 2004). Previous preliminary results on imprinted gene expression in the bovine (Ruddock *et al*. 2004) and ovine (Thurston *et al.* 2008) suggested that some genes known to be imprinted in other species were either not imprinted or that imprinting occurred at different stages of development. To help elucidate which of these equally plausible events was occurring, the imprinting status of an additional eight genes was investigated in bovine embryos. All but one gene displayed mRNA expression during this crucial period of development. The expression patterns were similar to those seen previously in relation to the maternal to embryonic transition (MET), which occurs around the 16–32-cell stage in bovine embryos (Memili and First 2000; Hatanaka *et al*. 2001). Specifically, some genes were on before MET, some were on following MET and others displayed transcripts continuously during all cleavage stages of development. One gene (*Ata3*) showed no expression at all.

Putative imprinted gene expression

Ata3 transcripts were undetectable in all oocytes and IVF embryos tested, suggesting that the transporter is not used during bovine early embryo development *in vitro*. This was not surprising because *Ata3* is thought to encode a primarily liver-specific amino acid transporter (Sugawara *et al*. 2000; Hatanaka *et al*.

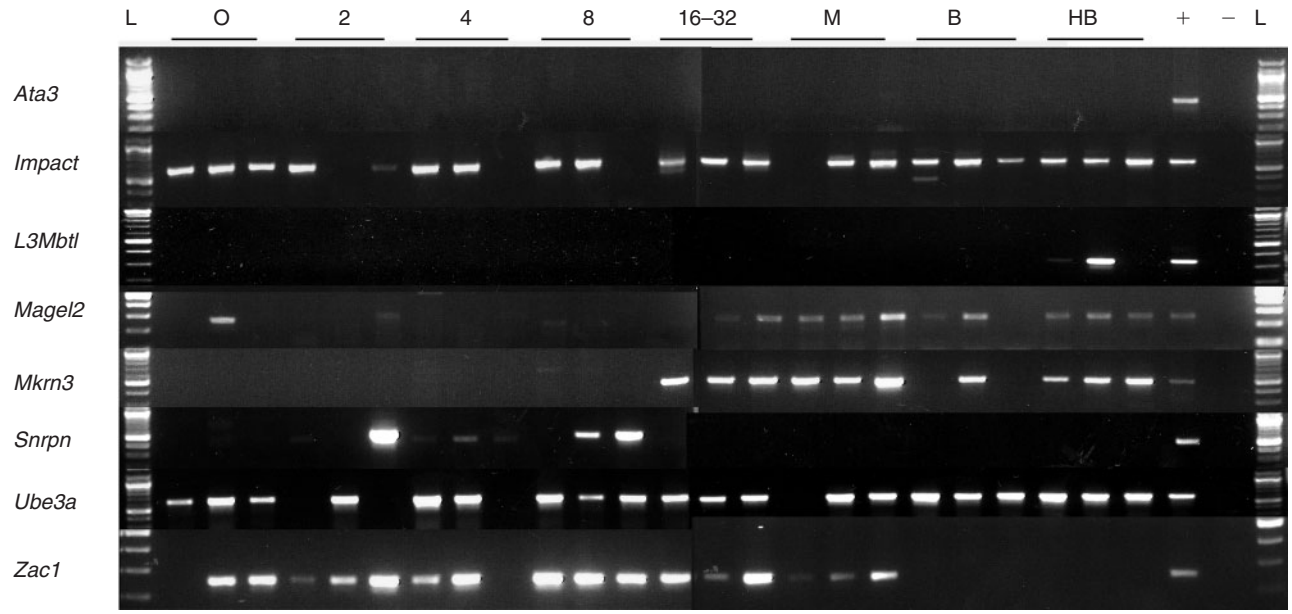

Fig. 1. mRNA expression of the *Ata3*, *Impact*, *L3Mbtl*, *Magel2*, *Mkrn3*, *Peg3*, *Snrpn*, *Ube3a* and *Zac1* genes in *in vitro*-matured oocytes (O), two-cell (2), four-cell (4), eight-cell (8) and 16–32-cell (16–32) embryos, morulae (M), Day 7 blastocysts (B), Day 8 hatched blastocysts (HB), positive tissue (+) and water (−). The positive control was testis, except for *Ata3* (liver), *Magel2* (bovine trophectoderm cell line) and *Zac1* (male adult fibroblasts). L, 100-bp DNA ladder.

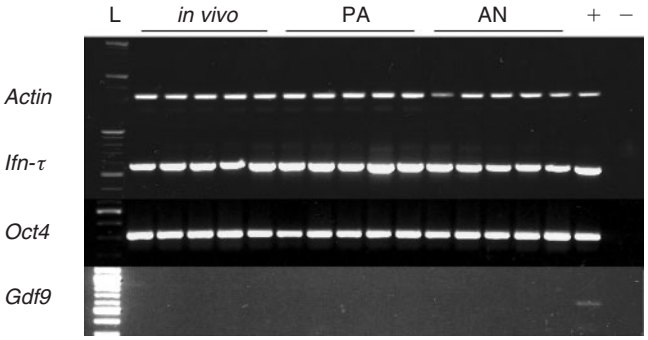

Fig. 2. mRNA expression of the control genes *Actin*, *Ifn-τ*, *Oct4* and *Gdf9* in Day 7 *in vivo*-flushed blastocysts (*in vivo*), Day 7 *in vitro*-produced parthenogenetic blastocysts (PA), Day 7 *in vitro*-produced androgenetic blastocysts (AN), positive tissue (+) and water (−). The positive control was the bovine trophectoderm cell line, except for *Gdf9* (oocyte). L, 100-bp DNA ladder.

2001). Blastocysts produced *in vivo* were also tested to determine whether the lack of mRNA expression was due to *in vitro* culture techniques. Results were highly consistent between the IVF and *in vivo* blastocysts for this gene, as well as for the remaining genes tested.

Three genes (*Impact*, *Magel2* and *Ube3a*) were expressed both before and after MET, suggesting a crucial and ubiquitous role in early embryo development. *Impact* is imprinted in the mouse, but not the human (Hagiwara *et al*. 1997; Okamura *et al*. 2000). While the bovine displayed CpG islands within the first intron, similar to those found in the mouse, the ubiquitous expression in the early bovine embryo suggests that the role of *Impact* may be more homologous to the human than the mouse during early embryo development. *Impact* belongs to the YCR59c/yigZ hypothetical protein family or Uncharacterised Protein Family 29 (UPF0029) and has unknown function (Doerks *et al*. 1998; Okamura *et al*. 2000).

Magel2 and Necdin (Ndn) are related proteins involved in PWS that have been shown recently to bind to Fez1 (a protein involved in axonal outgrowth and kinesin-mediated transport) at or near the centrosomes, thus preventing its proteasomal degradation (Lee *et al*. 2005). *Ndn* and *Magel2* show a similar pattern of expression following MET, with the exception of weak expression of *Magel2* in two samples before MET. This coordinated expression has been described previously in the mouse embryo (Lee *et al*. 2000).

The third gene to be ubiquitously expressed was *Ube3a*, a candidate gene for the human disorder Angelman syndrome (Rougeulle *et al*. 1997; Wrzeska and Rejduch 2004). The *Ube3a* gene is ubiquitously expressed in human oocytes and early embryos, although its role during early preimplantation development is not known (Monk and Salpekar 2001; Salpekar *et al*. 2001). The *Ube3a* gene encodes the E6-associated protein (E6AP), which acts as a ubiquitin ligase enzyme (Williams 2005).

The remaining five genes examined for the first time in bovine embryos showed varying patterns of expression. In the present study, *L3Mbtl* was expressed only in two *in vitro*-produced Day 8 hatched blastocysts. Mutations of *L3Mbtl* in *Drosophila* result in asynchronous mitotic divisions in the embryo (Yohn *et al*. 2003). This role of *L3Mbtl* and the suggested role of transcriptional repressor in human haematopoietic cells (Li *et al*. 2004), make *L3Mbtl* an interesting candidate for the regulation of germ

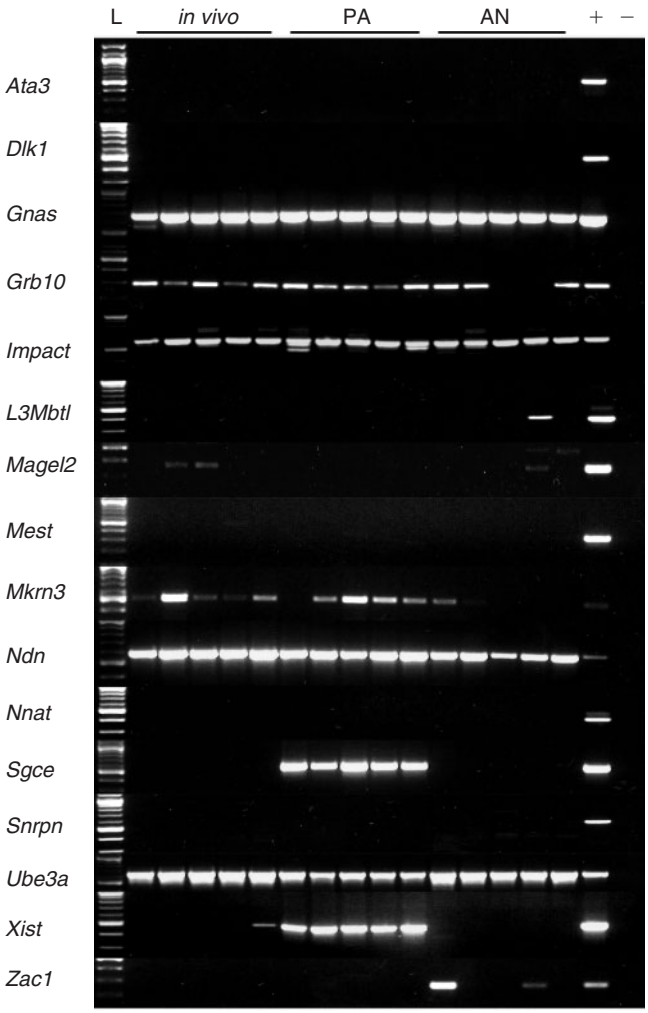

Fig. 3. mRNA expression of *Ata3*, *Dlk1*, *Gnas*, *Grb10*, *Impact*, *L3Mbtl*, *Magel2*, *Mest isoform1* (*Mest e1*), *Mkrn3*, *Ndn*, *Nnat*, *Peg3*, *Sgce*, *Snrpn*, *Ube3a*, *Xist* and *Zac1* in Day 7 *in vivo*-flushed blastocysts (*in vivo*), Day 7 *in vitro*-produced parthenogenetic blastocysts (PA), Day 7 *in vitro*-produced androgenetic blastocysts (AN), bovine positive tissue (+) and water (−). The positive control was the bovine trophectoderm cell line, except for *Ata3* (liver), *Impact* (testis), *L3Mbtl* (testis), *Mkrn3* (testis), *Nnat* (fetal brain), *Snrpn* (testis), *Ube3a* (testis), *Xist* (cow adult fibroblast) and *Zac1* (bull adult fibroblast). L, 100-bp DNA ladder.

layer differentiation and embryo implantation. The *Mkrn3* gene was expressed from MET onwards, again indicating a role in early development, possibly before that of *L3Mbtl* (only present at Day 8), in inner cell mass : trophectoderm differentiation or blastocoel formation. Mkrn3 is thought to function as a ribonucleoprotein (Jong *et al*. 1999*a*), suggesting a possible role in the control of RNA splicing, degradation and translation around the time of MET. *Peg3* was expressed in only one four-cell embryo, likely indicating precocious expression in the single embryo, but also the possibility of a role just before MET. *Peg3* does not appear to have been investigated previously in embryos at this stage of development, but was shown to be undetectable in sheep blastocysts (Thurston *et al.* 2008), consistent with the present data. Finally, *Snrpn* and *Zac1* were expressed up to MET, indicating a role in oocyte development, fertilisation or the first cleavage divisions before destruction of maternal stores of mRNA. Neither of the two genes was expressed in morulae or blastocysts. *Snrpn*, or *small nuclear ribonucleoprotein polypeptide N*, may be playing a role in mRNA stabilisation in the oocyte or may be involved in the translation of maternal mRNA after fertilisation. *Zac1*, or *Lot1*, is a zinc-finger nuclear transcription factor. The protein is often silenced in ovarian and breast cancer cells and has antiproliferative effects (Abdollahi *et al*. 2003). This transcription factor has been shown to play a critical role in the regulation of a network of imprinted genes crucial for embryonic growth (Varrault *et al.* 2006) and the results of the present study suggest a role in oocyte growth and the storage of mRNA for early embryo cleavage following fertilisation.

Genomic imprinting status

To decipher genomic imprinting status in the bovine embryo, parthenogenetically and androgenetically activated embryos were created. These embryos, first created in the 1980s in mice (Barton *et al*. 1984; McGrath and Solter 1984), elucidated the process of genomic imprinting. These embryos have been further used to demonstrate *Igf2* and *H19* imprinting in sheep (Hagemann *et al*. 1998) and to show that *GRB10* and *IGF2R* switch from biallelic to monoallelic expression patterns between the blastocyst stage and Day 21 of ovine development (Thurston *et al.* 2008). In the present study, mRNA expression was compared between *in vivo*-flushed Day 7 blastocysts and PA and AN embryos created *in vitro*. In cattle, the embryo survives on maternal stores of mRNA until MET at the 16–32-cell stage. Blastocysts were chosen for imprinting analysis because maternal stores of mRNA are carried over from the oocyte, making allelic discrimination of mRNA impossible until after degradation of maternal transcripts and the initiation of new transcription.

Only *Xist* displayed complete imprinting, with expression in *in vivo* and PA embryos only. All androgenotes lacked *Xist* expression, which suggests all were male, although the generation of androgenotes in a previous study by Lagutina *et al*. (2004) showed that XX and XY androgenotes were expected in a 1 : 2 ratio. Embryo sex could not be confirmed because *SRY* transcripts were not detectable in any of our embryo samples (data not shown). This lack of expression has been reported previously in ovine embryos (Bernardi *et al.* 1996) and bovine expression of *SRY* has only been described in pooled embryos (Gutierrez-Adan *et al.* 1997). This sexual bias could be due to the small sample size or possibly a preference for the selection of male blastocysts, because they develop faster *in vitro* (Avery *et al.* 1989; Xu *et al.* 1992).

Six genes were not expressed at the blastocyst stage (*Ata3*, *Dlk1*, *Mest isoform 1*, *Nnat*, *Peg3* and *Snrpn*), rendering imprinting analysis impossible. The expression of *Nnat* in IVF blastocysts, in contrast with the lack of expression in *in vivo*-produced blastocysts, suggested precocious gene expression following *in vitro* culture. The remaining 10 genes were not fully imprinted at the blastocyst stage of development and showed

variable expression among and within groups. Four genes (*Gnas*, *Impact*, *Ndn* and *Ube3a*) were expressed in all blastocysts tested, indicating a lack of imprinting. Three genes (*Sgce*, *Zac1* and *L3Mbtl*) were not expressed in *in vivo* embryos, but showed some expression in PA or AN embryos, again indicating precocious expression as a result of *in vitro* culture. *Sgce* expression in PA embryos was puzzling, because the gene was found to be on early in development, but switched off following MET, and was off in parthenogenotes in our previous study (Ruddock *et al.* 2004). This difference may be due to subtle differences in the *in vitro* culture or to the variation in the activation protocols used. These differences are currently being investigated. The last three genes (*Grb10*, *Magel2* and *Mkrn3*) showed a semblance of imprinting, with one or more blastocysts in at least one group not expressing the gene, although lack of paternal *Mkrn3* expression was opposite to what was expected from mouse and human data.

There are several potential reasons for this lack of clear-cut imprinting. First, *in vitro* culture may impinge on the imprinting process of IVF blastocysts. This hypothesis is supported by recent evidence that murine *H19* imprinting is lost in embryos following culture in Whitten's medium and that this loss of imprinting persists in mid-gestation conceptuses (Doherty *et al*. 2000; Mann *et al*. 2004). However, the fact that approximately 60% of IVF blastocysts, when compared with *in vivo* embryo transfers, result in live births suggests that the effects of *in vitro* culture may not explain fully the results obtained (Peterson and Lee 2003). Second, it may be that bovine genes are not imprinted, not completely imprinted ('leaky' expression) or show tissue-specific temporal imprinting in adult tissues. *IGF2R* has been found previously to be imprinted in the mouse and cow (Killian *et al*. 2001), but not in the human (Vu *et al*. 2000; Yang *et al*. 2003). There are also numerous genes that show imprinting in only a select number of tissues, such as *PPP1R9A*, which is maternally expressed in fetal muscle, eye and placenta but is biallelically expressed in other tissues (Nakabayashi *et al*. 2004), *OBPH1*, which shows predominant maternal expression in the placenta but biallelic expression in fetal and newborn organs in the human and mouse (Higashimoto *et al*. 2002), and *KCNQ1*, whose expression is imprinted in several tissues but not in the heart (Lee *et al*. 1997). This is unlikely to explain the lack or impartial imprinting of the numbers of genes investigated in the present study. Third, the data may indicate a loss of imprinting maintenance during preimplantation development. It is currently believed that genomic imprints are only erased and re-established during gametogenesis and that these imprints are maintained during preimplantation development. The recent discovery that *Xist* becomes biallelically expressed in the inner cell mass of the mouse substantiates the claim that imprinting may not be maintained as once thought (Okamoto *et al*. 2004). A transgenic human β-globin locus has also been shown recently to acquire methylation imprinting during the post-fertilisation period (Tanimoto *et al.* 2005). It is possible that imprints are erased following fertilisation and re-established too rapidly to have been discovered in the murine model system or that genomic imprinting is differentially regulated between species. The recent report of late establishment of imprinting in the sheep supports this theory (Thurston *et al.* 2008). Finally, it has also been theorised that, in certain cases, both parental alleles are required to establish imprinted gene expression (Sotomaru *et al.* 2002; Ruf *et al.* 2006).

In order to elucidate which explanation, or combination of explanations, is correct, future studies will be aimed at a more in-depth analysis of the genomic structure of the genes of interest. This will involve investigation by bisulfite sequencing to determine differentially methylated regions for each gene and/or by discovery of single nucleotide polymorphisms for quantitative monoallelic discrimination of mRNA expression.

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