Monoallelic expression of nine imprinted genes in the sheep embryo occurs after the blastocyst stage

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Abstract

The preimplantation embryos of a range of mammals can be susceptible to disruptions in genomic imprinting mechanisms, resulting in loss of imprinting. Such disruptions can have developmental consequences involving foetal and placental growth such as Beckwith–Wiedemann syndrome in humans and large offspring syndrome in sheep. Our objective was to investigate the dynamics of establishing monoallelic expression of individual sheep imprinted genes post-fertilisation. Semi-quantitative RT-PCR was used to amplify cDNA from the sheep blastocyst, day 21 foetus and day 21 chorioallantois, to compare expression levels between biparental and parthenogenetic embryos in order to indicate allelic expression status. In common with other mammals, IGF2, PEG1 and PEG3 were paternally expressed in the day 21 conceptus, while H19, IGF2R, GRB10 and p57KIP were maternally expressed. Interestingly, GNAS was maternally expressed in the foetus, but paternally expressed in the chorioallantois at day 21. Overall, the imprinting of ovine GRB10 and IGF2R was comparable with mouse but not with human. Contrary to the trophoblast-restricted maternal expression in both mouse and human, SASH2 (sheep homologue of Mash2/HASH2) was expressed in the ovine foetus and was biallelically expressed in the chorioallantois. Differential methylation of the H19 CTCF III upstream region and IGF2R DMR2 in the chorioallantois revealed predominantly paternal and maternal methylation respectively, indicating conservation of these imprinting regulatory regions. In blastocysts, IGF2R, GRB10 and SASH2 were expressed biallelically, while the other genes were not detected. Thus, for the majority of ovine imprinted genes examined, monoallelic expression does not occur until after the blastocyst stage.


Introduction

In the germline, epigenetic marks are added in a parent-specific manner to a small subset of genes that permit tissue and developmental stage-specific monoallelic expression of affected genes post-fertilisation, a phenomenon known as genomic imprinting (reviewed by Preece & Moore 2000). The germ line marks that permit monoallelic gene expression are still not fully understood, and appear to be added to specific imprinted loci at variable stages of gametogenesis and post-fertilisation development (reviewed by Allegrucci et al. 2005). There is also evidence that the initial germ line ‘imprints’ are reinforced or completed after fertilisation (Srivastava et al. 2003). The dynamics of imprint development is of interest due to the increasing evidence that both in vivo and in vitro influences on the preimplantation embryo can alter genomic imprinting with a range of developmental consequences (reviewed by Swales & Spears 2005). Intriguingly, some imprinted genes seem to be more susceptible to preimplantation perturbation by embryo culture environments or somatic cell nuclear transfer (SCNT) than others for reasons as yet unknown (Young et al. 2003, Mann et al. 2004). For example, both SCNT and embryo culture often disrupt the insulin-like growth factor 2 receptor (IGF2R) gene in the sheep (Young et al. 2001, 2003), but this gene is rarely affected in either humans or mice (Gicquel et al. 2004).

To date, the developmental stage-specific allelic expression of imprinted genes has received limited attention, especially during the preimplantation stages, where the paucity of material hinders molecular analysis. Although the expression of several imprinted genes has been examined in the preimplantation embryos from the mouse, human and cow, whether
this is biallelic or monoallelic has been uncovered in only relatively few cases. A gene-specific temporal imprinting pattern has been suggested by the observation of both monoallelically and biallelically expressed imprinted genes in human, mouse and bovine blastocysts (Lighten et al. 1997, Huntriss et al. 1998, Monk & Salpekar 2001, Mann et al. 2003, Ruddock et al. 2004). Furthermore, within these studies, imprinted genes have also been identified with no detectable expression in the preimplantation embryos, although the use of small numbers of embryos, amplified cDNA and supernumerary embryos from human fertility patients may all confound interpretation. As a model species for unravelling potential developmental mechanisms, sheep have an advantage over rodents of being monotocous with a pattern of foetal development more akin to that of humans. Thus, we investigated the allelic expression of nine imprinted genes in sheep blastocysts and day 21 foetuses and placentae.

A range of experimental models and molecular strategies have been employed to identify imprinted genes and to determine whether their imprinting is conserved among mammals. However, the lack of published sheep genome sequence information and also the lack of identified polymorphisms to facilitate allele-specific expression analysis has prompted several studies to utilise experimentally generated, monoparental embryos (Feil et al. 1998, Young et al. 2003, Ruddock et al. 2004), where quantitative gene expression differences are expected to reflect the relevant copy number of a maternal or paternal allele. Monoparental embryos of various species can develop to the blastocyst stage, including sheep (Loi et al. 1998), mouse (Kono et al. 2002), cow (Meo et al. 2004) and pig (Yi & Park 2005). Transfer of gynogenetic/parthenogenetic blastocysts to a receptive uterus also allows apparently normal early foetal development (Hagemann et al. 1998, Loi et al. 1998, Kono et al. 2002), although embryos die before mid-gestation. While parthenogenetic embryos show apparently normal development till day 21 in the sheep (Hagemann et al. 1998, Loi et al. 1998), they die before ~25 d.p.c (Feil et al. 1998).

In the present study, we generated sheep parthenogenotes to test whether there were time-dependent changes in the allelic expression of a range of putative imprinted genes in the sheep between the preimplantation and peri-implantation stages, and also to test whether allele-specific expression varied between the foetus and the placenta (chorioallantois) at gestational day 21. We employed a previously validated RT-PCR technique (Young et al. 2001) to quantify whether expression level varied significantly between the biparental control conceptus and the parthenogenote with two female genomes.

All the genes selected for analysis had potential roles in regulating foetal/placental size and/or development, and have been shown to be imprinted in at least some mouse or human tissues. Three of the imprinted genes analysed map to human chromosome 11p15.5 region and are associated with Beckwith–Wiedemann syndrome (IGF2, H19 and p57Kip2; reviewed by Enklaar et al. 2006). Disruptions of imprinting in the paternally expressed foetal mitogen, IGF2 or its adjacent non-coding regulator, H19 (maternally expressed), affect foetal size. p57Kip2 is a maternally expressed cell-cycle inhibitor, with disruption increasing the risk of exomphalos upon loss of imprinting. IGF2R and GRB10, both maternally expressed, have independent roles in regulating the IGF pathway, although deletion of the maternal allele in mice in both cases results in overgrowth of the embryo and placenta (Wutz et al. 2001, Charalambous et al. 2003). GNAS, a maternally expressed gene, couples multiple receptors to adenylyl cyclase, with roles in regulating birth weight, obesity and hormone resistance (Weinstein et al. 2006). Mash2/HASH2 is a maternally expressed transcription factor that when deleted in mice results in lethal trophoblast defects at 10 d.p.c (Guillemot et al. 1994). PEG1 encodes a α/β-hydrolase that when deleted paternally in mice generated growth-retarded pups with smaller placentae (Lefebvre et al. 1998). Alternative transcripts of PEG1 (isoforms 1 and 2) both contain exons 2–12, though the 5′ ends are alternatively spliced (Nakabayashi et al. 2002). Finally, we examined PEG3 encoding a differentiation-promoting, Kruppel-type zinc finger protein, paternal deletion of which results in embryonic, placental and neonatal growth retardation (Li et al. 1999).

Results

Sequencing of sheep RT-PCR products

Initially, RT-PCR products obtained for each putative sheep imprinted gene using day 21 chorioallantois cDNA were cloned and sequenced. BLAST searches were performed (http://www.ncbi.nlm.nih.gov/BLAST) to compare sheep sequences with human, cow and mouse (Table 1). Sheep homologues of all genes tested were identified, showing between 49 and 98% homology with the other mammals in the sequences investigated. Hereafter, the sheep MASH2 homologue is referred to as sheep achaete-scute homologue (SASH2). In general, homology was the highest with the cow and the lowest with mouse. IGF2R showed the least conservation between the sheep/cow and human/mouse.

Semi-quantitative RT-PCR in control and parthenogenetic blastocysts

The expression of IGF2R, GRB10 and SASH2 was detected in cDNA from a pool of five sheep in vitro-produced blastocysts (Fig. 1). The expression of IGF2, H19, GNAS, p57Kip2, PEG1 and PEG3 was not detected in the same blastocyst cDNA, although the expression of each of these genes was detected in 80 d.p.c sheep cotyledon ‘positive control’ cDNA.

No significant differences in IGF2R, GRB10 and SASH2 expression were observed between five replicate
Table 1 Sheep imprinted gene sequences: species homology.

<table>
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<tr>
<th>Gene</th>
<th>Species designed</th>
<th>Exon</th>
<th>Sheep</th>
<th>Human</th>
<th>Mouse</th>
<th>Cow</th>
<th>Expected</th>
<th>Actual</th>
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<td>% Homology (accession number)</td>
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<td></td>
<td></td>
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<td>IGF2</td>
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<td>100% (NM_001009311)</td>
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<td>5</td>
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<td>83% (NR_001926)</td>
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<td>99% GNAS (NM_010309)</td>
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<td>91% Gnas (NM_010309)</td>
<td>29% Nesp55 (AJ010163)</td>
<td>88% (Iso 1)</td>
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<td>Human</td>
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<td>82% (NM_006210)</td>
<td>79% (NM_008817)</td>
<td>86% (NM_001002887)</td>
<td>250</td>
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*Iso, isoform.*
determine whether this epigenetic mechanism was involved in the imprinting of these loci in the sheep placenta. Sequencing of ten clones for H19 CTCF III DNA revealed significant hypomethylation in parthenogenotes (mean percentage methylation ± s.e.m. for controls: 25% ± 1.4 and for parthenogenotes: 5% ± 0.6) for CpG dinucleotides 2–5, 7–8, 10, 13–17 (P < 0.001) and 9 and 18 (P < 0.05; Fig. 5A). Within individual control chorioallantois samples, clones with both highly methylated and highly unmethylated CpGs are obvious, as expected for normal imprinting. Out of 50 parthenogenetic clones, none exhibited methylation at the CTCF-binding site compared with 14 out of 50 in the controls. Seventeen CpG dinucleotides of the IGF2R DMR 2 were also subjected to bisulphite sequencing. Significant differential DNA methylation was observed between the control and parthenogenetic chorioallantois groups for CpG dinucleotides 1–4, 6–13, 17–18 (P < 0.001) and 5 (P < 0.05; Fig. 5B). Overall, the parthenogenetic group was hypermethylated (mean percentage methylation ± s.e.m.: 86% ± 2.5) relative to the controls (25% ± 5.7).

**Discussion**

For the first time in any mammal, this study examined in sheep the ontogeny of development of monoallelic expression in nine genes previously identified as imprinted in other species. Sheep parthenogenetic and biparental embryos were used to examine parent-of-origin expression in blastocysts and then in the day 21 foetus and chorioallantois. In addition, we examined the relationship of DNA methylation at two DMRs (H19 CTCF III and IGF2R DMR2) with allelic expression of the relevant genes.

**Validation of the sheep parthenogenetic model for imprinting analysis**

Although parthenogenetic embryos have been used extensively to examine genomic imprinting, there is a possibility that the presence of two female genomes disrupts normal imprinting. This has been suggested as a barrier to the use of monoparental embryos for the identification of new imprinted genes (Ruf et al. 2006). However, the imprinting status determined in the present study for ovine H19, IGF2 and IGF2R concur with previous reports for various stages of sheep gestation where, in the case of IGF2, results inferred...
from parthenogenetic studies have been confirmed by allele-specific PCR utilising relevant identified polymorphisms (McLaren & Montgomery 1999). Overall, the results shown are entirely consistent with previous sheep studies of fertilised embryos (Young et al. 2001, 2003), as well as imprinting of these genes in the mouse (Thorvaldsen et al. 1998, Vu et al. 2004) and human (Takai et al. 2001). Thus, it seems unlikely that the sheep parthenogenotes exhibit generally abnormal imprinting, although disruptions in individual genes cannot be ruled out. At least for the genes studied, the monoallelic expression observed does not concur with the proposal of Loi et al. (1998) that the lack of morphological differences between control and parthenogenetic sheep embryos at day 21 may be due to the development of monoallelic expression of imprinted genes later than this developmental stage.

**Imprinting in the sheep blastocyst**

**Genes undetectable in the blastocyst**

No expression of IGF2, H19, p57Kip2, GNAS, PEG1 or PEG3 could be detected in the sheep blastocyst, despite careful optimisation of RT-PCR conditions. This is consistent with the expression of H19 and IGF2 observed previously in sheep foetus and placenta at 17 d.p.c., but not in elongated 11 d.p.c. blastocysts (Lee et al. 2002). Whereas sheep blastocysts failed to express GNAS and PEG1 isoform 2, Ruddock et al. (2004) detected biallelic expression in bovine blastocysts utilising the same primer sequences. In addition to true species differences, the fact that bovine embryos were cultured in the presence of foetal calf serum in that study raises the possibility of culture-induced effects, as reported for IGF2R in the sheep (Young et al. 2001) and H19 and Grb10 in the mouse (Khosla et al. 2001). To overcome this issue, the IVF embryos utilised in the present study were cultured in serum-free medium, which we have shown previously to yield comparable embryo and placental development with entire development in vivo (De Sousa et al. 2001, Young et al. 2001).

**Genes expressed in the blastocyst**

Only three out of nine imprinted genes were found to be expressed in the sheep blastocyst (IGF2R, GRB10 and SASH2), and at this stage all exhibited biallelic expression.

The SASH2 homologue is also expressed biallelically in the mouse (Tanaka et al. 1999, Mann et al. 2003) and cow (Arnold et al. 2006). Notably, although Mash2 is biallelically expressed in all cells of the murine preimplantation embryo, it is not essential for blastocyst development or implantation (Tanaka et al. 1999) reiterating that the expression of an imprinted gene is not a sufficient condition for its biological function. The expression of imprinted genes is typically regulated by specific epigenetic modifications, such as DNA methylation and histone modifications, which are actively maintained throughout cell division and development.
(or non-imprinted) gene is not sufficient to imply an essential function. GRB10 is also expressed in human (Shen et al. 2005) and bovine (Ruddock et al. 2004) preimplantation embryos, although the allelic status is undetermined. Igf2r is biallelically expressed in mouse blastocysts (Szabo & Mann 1995, Mann et al. 2003) and expressed in human blastocysts (allelic status unknown: Lighten et al. 1997).

In the limited number of allele-specific studies on human preimplantation embryos, only IGF2 (Lighten et al. 1997) and SNRPN (Monk & Salpekar 2001) have been found to be monoallelically expressed. PEG1 and H19 were both expressed in only a minority of blastocysts tested and KVLQT1 was not expressed (Salpekar et al. 2001). Monoallelic expression has been demonstrated for Igf2 (Rappolee et al. 1992), H19 (Doherty et al. 2000), Peg1 (Lefebvre et al. 1998) and Peg3 (Mann et al. 2004), although expression of Gnas (Peters et al. 1999) is biallelic. Similarly, of the eight imprinted genes studied in bovine blastocysts, only NNAT was monoallelically expressed, although later developmental stages were not examined (Ruddock et al. 2004). Collectively, these data suggest that monoallelic expression of the imprinted genes studied thus far is often not key to blastocyst development, but rather is a developmental mechanism that becomes progressively more important as the foetus and placenta form.

**Imprinted gene expression in day 21 sheep foetus and chorioallantois**

**IGF2, H19 and p57Kip2**

The three human imprinted genes clustered on chromosome 11p15.5, IGF2 and H19, p57Kip2, were not expressed in the sheep blastocysts. However, at day 21, all exhibited the parental expression pattern expected from mouse and human post-implantation tissues (Morison et al. 2005). This suggests that imprinted genes in this Beckwith–Wiedemann associated chromosomal region are coordinately activated during early development. Furthermore, the sheep chorioallantois at day 21 exhibited the expected paternal DNA methylation for the H19 CTCF III DMR (Young et al. 2003), which correlates with the observed maternal expression of the gene.

**GRB10 and IGF2R**

A switch from both GRB10 and IGF2R biallelic expression in the sheep blastocyst to maternal expression in the day 21 foetus and chorioallantois suggests more similarity to the mouse than human (Szabo & Mann 1995, Blagitko et al. 2000, Killian et al. 2001, Monk et al. 2006). Although a DMR homologue was confirmed in the sheep, whether the observed lack of differential methylation within individual control
animals for the IGF2R DMR2 is due to the examination of an insufficient number of clones or true individual variation remains to be fully established. A previous explanation for the differential imprinting of IGF2R between humans and mice alluded to the differences in monotocous/litter-bearing reproductive strategies (Monk et al. 2006). However, the imprinting of IGF2R in the monotocous sheep does not lend support to this hypothesis. Differences in epigenetic regulatory mechanisms (Vu et al. 2004) between species remain a possibility.

**PEG1 and PEG3**

PEG1 isoform 1 was detected in the day 21 chorioallantois (but not foetus), with clear paternal expression. Isoform 2 was paternally expressed in both the foetus and the chorioallantois. Human isoform 1 shows paternal expression in foetal tissues and placenta (Nakabayashi et al. 2002), whereas isoform 2 exhibits biallelic expression in some foetal tissues but is paternally expressed in chorionic villi, kidney and placenta (Nakabayashi et al. 2002). Thus, although paternal

**SASH2**

In the day 21 sheep conceptus, neither the expression pattern nor the allelic expression status of SASH2 homologue correlates with reports in mouse or human. Mash2/HASH2/MASH2 in the mouse, human and bovine preimplantation embryos becomes tropho-blast-restricted and maternally expressed post-implantation (Tanaka et al. 1999, Miyamoto et al. 2002, Arnold et al. 2006). No evidence for monoallelic expression of SASH2 was seen in the sheep chorioallantois by day 21, although imprinting at later stages or in the trophoblast cannot be ruled out. Indeed, the cow exhibits biallelic expression in the elongated trophoblast at 17 d.p.c, but MASH2 maternal expression is detected in day 40 cotyledonary tissue (Arnold et al. 2006). Mash2/HASH2 has not been detected in the foetal tissues of any of the above species but was clearly expressed in the day 21 sheep foetus, despite careful removal of all trophoblast tissue. Mash2 expression has been reported, however, in adult rat sciatic nerve and Schwann cells (Kury et al. 2002). Notably, that SASH2 expression was only 18% greater in parthenogenetic versus biparental sheep foetuses raises the possibility of stage-specific expression in some tissue/cell types within the developing foetus that requires more comprehensive interspecies analysis to clarify.

**GNAS**

The limited number of human and mouse studies post-implantation indicate Gnas/GNAS tissue-specific maternal expression (Peters et al. 1999; reviewed by Weinstein et al. 2004). Intriguingly, the current study indicates that the sheep exhibits contrasting parental expression of GNAS between the foetus (maternal) and the chorioallantois (paternal). However, the primers utilised from Ruddock et al. (2004) detect the entire range of potential transcripts from the complex Gnas/GNAS locus. Thus, it is possible that maternal expression observed in the sheep foetus was from the sheep homologue of a maternally expressed transcript such as NESP55, with paternal expression detected in the placenta possibly attributable to XLas (reviewed by Weinstein et al. 2004).

**Figure 5** Clonal DNA bisulphite sequencing of 6–10 clones from individual control and parthenogenote (Parth) chorioallantois samples at (A) H19 CTCF III DMR and (B) IGF2R DMR 2. Mean % methylation ± s.e.m. is shown. The grey box highlights the CTCF-binding site. C1–C5 and P1–P5 denote individual control and parthenogenetic animals respectively.
expression of sheep *isoform 2* is similar to other species, the lack of expression detected for *isoform 1* in the foetus represents a clear difference. Since deletion of the murine *Peg1* paternal allele results in abnormal maternal behaviour in regard to placentophagia and poor nest building (*Lefebvre et al. 1998*), one possibility is that the species differences in this gene reflect variable types of maternal behaviour post-partum. However, sheep express *PEG3* paternally in both the day 21 foetus and the chorioallantois, and a similar pattern of expression is seen in virtually all tissues from the mouse, human, as well as in bovine adult brain and testis (*Kim et al. 2004*), although disrupted imprinting in this gene also affects murine maternal behaviour (*Li et al. 1999, Murphy et al. 2001*).

**Perspective**

While the general phenomenon of genomic imprinting appears to be conserved in the sheep, the expression of several imprinted genes in blastocysts varies between species. The observations that many imprinted genes are either not expressed or expressed from both alleles at the blastocyst stage suggest that there is not a major requirement for genomic imprinting in preimplantation development. The lack of blastocyst monoallelic expression may be particularly prevalent in ruminants due to delayed implantation of the embryo after formation of an elongated blastocyst (foetal cotyledons and maternal caruncles do not form full connections until approximately day 23 in the sheep; *Boshier 1969, Guillomot et al. 1981*). However, variable placentation and the analysis of non-comparable stages between mammals can also confound interspecies comparisons, urging more systematic studies of the ontogeny of imprinting. A greater understanding of the time course of progressive monoallelic expression and the epigenetic changes that occur during preimplantation development will be important for ensuring the safety of assisted reproductive technologies, determining normal reprogramming of imprints in SCNT and monitoring the stability of imprinted genes in embryonic stem cells.

**Materials and Methods**

All experimental procedures were carried out in accordance with the UK Home Office Regulations and the National Research Council publication Guide for Care and Use of Laboratory Animals (copyright 1996, National Academy of Science).

**Production of biparental and parthenogenetic embryos**

Control biparental blastocysts were derived from sheep (*Ovis aries*) oocytes (from local abattoirs) that had been *in vitro* matured and *in vitro* fertilised with Suffolk breed semen, as described by *De Sousa et al. (2001)*. Culture to the blastocyst stage was in embryo culture medium consisting of synthetic oviduct fluid containing 1× essential and non-essential amino acids and 4 mg/ml BSA. Blastocysts were washed with sterile PBS and then dispersed into 1–2 μl PBS-containing sterile 0.5 ml Eppendorf tubes, prior to snap freezing and storage at −80°C.

Control day 21 foetal and chorioallantois tissues were recovered after post-mortem from naturally mated Scottish Blackface ewes (3–4 years) inseminated by a single Suffolk ram. After killing by i.v. administration of 25 ml 20% (w/v) pentobarbitone sodium (Euthatal, Rhône Mérieux Ltd, Harlow, UK), all foetuses and placental tissues were individually snap frozen in 1.5 ml sterile Eppendorf tubes and then stored at −80°C.

Sheep parthenogenetic embryo production was as described by *Loi et al. (1998)*. Briefly, metaphase II oocytes were chemically activated with ionomycin, followed by 6-dimethylaminopurine incubation, to prevent polar body exclusion. *In vitro* culture to the blastocyst stage was as described above for biparental embryos. For day 21 tissue, single parthenogenetic embryos were transferred to the uteri of foster ewes, as described by *De Sousa et al. (2001)*. Obviously fused chorioallantois was carefully separated from peripheral trophoblast tissue and yolk sac prior to freezing.

**Primer design and RT-PCR**

Imprinted gene primers were designed using published sheep sequences (where available) or from alignments of the relevant human, mouse and bovine sequences using regions of high homology wherever possible (*Table 2*).

Sheep tissue was homogenised by adding 1 ml TRI reagent (Sigma) per 100 mg tissue to a tube of Lysing Matrix D (Q Biogene, Nottingham, UK). Once the tissue had been thoroughly homogenised in a Ribolyser (speed 6 for 45 s; Thermo Electron Corporation, Hampshire, UK), samples were pipetted into 1.5 ml Eppendorf tubes, and RNA was extracted according to the TRI reagent protocol.

RNA extraction from embryos employed the Qiagen QIA Shredder and RNeasy Mini kits, according to the manufacturer’s instructions, with the following alterations to optimally retrieve RNA from single blastocysts. After RNA elution from the RNeasy column in 50 μl RNase/DNase-free water (Sigma), 2 μl Pellet Paint co-precipitant (Novagen, Nottingham, UK) was added per sample, followed by 5 μl 3 M sodium acetate (Novagen) and pipetting to mix. Ice-cold 100% ethanol (100 μl) was then added and vortexed prior to centrifugation at 16000 g (14 000 r.p.m.) for 5 min. After removal of the supernatant and a further 70% ethanol (500 μl) wash with brief vortexing and centrifugation as above, the supernatant was removed and tubes were placed on ice to allow the remaining ethanol to evaporate. Each RNA pellet was then resuspended in 8 μl RNase/DNase-free water for immediate cDNA synthesis using the First Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech) utilising the random hexamer pd(N)₆ primer (0.2 μg/μl), according to the manufacturer’s protocol. Final cDNA volume was 50 μl for day 21 tissue samples and 15 μl for blastocyst cDNA. Reverse transcriptase negative (RT−) controls contained all components, except the
Table 2 Sequences and primers used to amplify putative sheep imprinted genes.

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<th>Imprinted gene</th>
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</table>

IGF2, H19 and IGF2R primers were designed to published sheep sequences. GNAS, GRB10 and PEG1 were designed from published cow sequences (Ruddock et al. 2004), with PEG3, MASH2 and P5<sup>APP</sup> designed from human sequences. Primer sequences, expected amplicon sizes, optimal magnesium concentrations and optimal annealing temperatures are indicated.

F, forward; R, reverse; ISO, isoform.
RT-containing bulk first strand mix. RNA-negative (RNA−) controls contained all cDNA synthesis components but no sample RNA. All cDNA was stored at −20 °C.

In a final volume of 25 µl, RT-PCR comprised Taq polymerase (0.125 µl) and 10× buffer (2.5 µl, containing 1.5 mM Mg), both from Roche Diagnostics; 10× dNTP (2.5 µl, Invitrogen); 5 µM forward and reverse primers (2.5 µl, Sigma Genosys) andRNase/DNase-free water (Sigma). In some reactions, magnesium chloride was added to 2.5 mM final concentration (Roche Diagnostics), and/or Q Solution (5 µl, Qiagen; for PEG3) was added to assist with regions of secondary structure, adjusting water volume accordingly. Optimal annealing temperature and magnesium concentration were empirically determined for each primer set (Table 2). The PCR cycling conditions were 95 °C for 5 min followed by cycles of 95 °C for 1 min, × °C (optimum annealing temperature) for 30 s and 72 °C for 1 min, and a final incubation at 72 °C for 5 min.

**Semi-quantitative RT-PCR**

Semi-quantitative gene expression analysis was performed using the Quantum internal standard, 18S with 18S Competimers (classic 18S, 488 bp and classic II 18S, 324 bp; Ambion, Huntington, UK) in a multiplex reaction, according to the manufacturer’s protocol and as described by Young et al. (2001). In total, 2.5 µl 18S mix was added to each reaction, reducing the PCR mix water volume accordingly. The optimal 18S to Competimer ratios ranged from 1:2 to 1:16, with cycle number ranging from 31 to 40 cycles and optimised final cDNA concentrations that ensured linear amplification varied from between 0.002 and 0.04 µg per 25 µl reaction for the foetus and chorioallantois. All PCR were performed in duplicate and replicated at least twice. Image analysis of ethidium bromide-stained agarose gel was used to quantify gene expression levels that were then statistically analysed by the Mann–Whitney U test.

**Bisulphite treatment**

DNA to be bisulphite treated (100 ng) was initially digested with EcoRI (1 µl 10 U/µl added per digest) with Buffer H (both Roche) overnight (~16 h) at 37 °C. The 25 µl EcoRI digest was then pipetted into screw-cap tubes, incubated at 100 °C for 5 min and then immediately placed on ice. Next, 2.5 µl fresh 3 M sodium hydroxide (Sigma) was added to each tube at 37 °C for 20 min. Subsequently, 3.8 g sodium bisulphite (Sigma) was mixed with 5 ml RNase/DNase-free water (Sigma) and 1.5 ml 2 M sodium hydroxide in a dark (foil-covered) tube. During this time, 110 mg hydroquinone (Sigma) was dissolved in 1 ml water by heating at 50 °C for 10 min and subsequently added to the bisulphite solution, inverting the tube to mix. In total, 270 µl bisulphite solution was then added to the digested and denatured DNA, pipetting twice to mix. Following overlay of 200 µl mineral oil (Sigma), each sample was incubated for 5 h at 55 °C in the dark.

After the bisulphite incubation, 2 ml sterile tubes were prepared containing 600 µl RNase/DNase-free water, 90 µl 3 M sodium acetate (pH 5.2, Sigma) and 2.5 µl Pellet Paint (Novagen) and the bisulphite-treated DNA solution (minus mineral oil). Nine hundred microlitres of isopropanol (Sigma) were added and mixed by inversion, followed by centrifugation at 13 000 g (13 000 r.p.m.) for 20 min. The pellet was washed with 800 µl 70% ethanol (Sigma), centrifuged for 5 min at 13 000 g and then the supernatant was removed with a pipette. The pellet was air-dried on ice for 10 min and resuspended in 50 µl of water (RNase/DNase-free), followed by the addition of 5 µl fresh 3 M sodium hydroxide and incubated at 37 °C for 15 min. DNA clean up was performed using the Qiagen PCR purification kit, according to the manufacturer’s instructions.

**PCR of bisulphite-treated DNA**

Table 3 shows the bisulphite primers (Sigma Genosys) designed using ‘MethPrimer’ (Li & Dahiya 2002) for H19 and IGF2R. In a final volume of 20 µl. the bisulphite PCR comprised AmpliTaq Gold (10 µl; Applied Biosystems, Cheshire, UK), 5 µM forward and reverse primers (2 µl, Sigma Genosys), RNase/DNase-free water (7 µl, Sigma) and bisulphite-converted DNA. Optimal annealing temperature was empirically determined for each primer set (Table 3). The PCR cycling conditions were 95 °C for 5 min followed by 40 cycles of 95 °C for 30 s, X°C (optimum annealing temperature) for 45 s and 72 °C for 1 min, and a final incubation at 72 °C for 5 min. All PCR products (10 µl) were examined on agarose gel prior to cloning. Primers were tested for PCR bias (data not shown), for which there was none.

**Cloning of PCR products**

Bisulphite PCR products were cloned using the TOPO XL cloning kit (Invitrogen), according to the manufacturer’s instructions, except the ligation reaction, which consisted of 1 µl PCR product, 0.2 µl TOPO XL PCR vector and 3.8 µl water (RNase/DNase-free,

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Table 3 Bisulphite primers for ovine H19 and IGF2R methylation analysis.

<table>
<thead>
<tr>
<th>Imprinted gene bisulphite primers*</th>
<th>Primer sequence</th>
<th>Annealing temp (°C)</th>
<th>Band size</th>
<th>Primer region (bp)</th>
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<td>ACC TCC TCA ACA CCT TAC TCA AA</td>
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</table>

*F, forward; R, reverse.

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Sigma). Plasmid DNA purification occurred using the Qiagen QIAprep Spin miniprep kit, according to the manufacturer's instructions. Sequencing of DNA was performed by the DNA sequencing laboratory (University of Nottingham). Statistical analysis was performed using the χ² test.

Acknowledgements
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