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Genomic imprinting in ruminants: allele-specific gene expression in parthenogenetic sheep

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Abstract. Studies in the mouse have established that both parental genomes are essential for normal embryonic development. Parthenogenetic mouse embryos (which have two maternal genomes and no paternal genome), for example, are growth-retarded and die at early postimplantation stages. The distinct maternal and paternal contributions are mediated by genomic imprinting, an epigenetic mechanism by which the expression of certain genes is dependent on whether they are inherited from mother or father. Although comparative studies have established that many imprinted mouse (and rat) genes are allele-specifically expressed in humans as well (and vice versa), so far imprinting studies have not been performed in other mammalian species. When considering evolutionary theories of genomic imprinting, it would be important to know how widely it is conserved among placental mammals. We have investigated its conservation in a bovid ruminant, the domestic sheep, by comparing parthenogenetic and normal control embryos. Our study establishes that, like in the mouse, parthenogenetic development in sheep is associated with growth-retardation and does not proceed beyond early fetal stages. These developmental abnormalities are most likely caused by imprinted genes. We demonstrate that, indeed, like in mice and humans, the growth-related *PEG1/MEST* and Insulin-like Growth Factor 2 (*IGF2*) genes are expressed from the paternal chromosome in sheep. These observations suggest that genomic imprinting is conserved in a third, evolutionarily rather diverged group of placental mammals, the ruminants. **Key words:** Genomic imprinting—Epigenetic—Evolution—Sheep—*IGF2*—*PEG1/MEST*

Introduction

In humans and mice, the maternal and paternal genomes are functionally different and are therefore both required for normal embryonic development. The distinct contributions from male and female gametes are mediated by genomic imprinting, an epigenetic mechanism that gives rise to differential expression of the maternally and paternally inherited alleles of certain genes (Hall 1990; John and Surani 1996). Dissection of the parental contributions, by analysis of mice that are uniparentally disomic for the entire genome or for individual chromosomes, has established that maternally and paternally imprinted genes play different and rather opposite developmental roles (Surani et al. 1984; McGrath and Solter 1984; Cattanach and Kirk 1985; Hall 1990). An extreme consequence of imprinting is that parthenogenetic mouse embryos (which have two maternal and no paternal genome) are small and die at early postimplantation stages because of the lack of paternally expressed genes (Surani et al. 1984; McGrath and Solter 1984). Indeed, it has been demonstrated for the *Igf2*, *Peg1/Mest*, *Peg3*, and *Snrpn* imprinted genes, which are paternally expressed, that they are fully repressed in parthenogenetic mouse embryos (Sasaki et al. 1992; Walsh et al. 1994; Barr et al. 1995; Kaneko-Ishino et al. 1995; Szabó and Mann 1996).

In the mouse, 27 parental allele-specifically expressed genes have been identified to date (Beechey and Cattanach 1998) and for two of these it has been shown that they are imprinted in rats as well (Pedone et al. 1994; Overall et al. 1997). Although comparative studies have established that most of the imprinted rodent genes are parental allele-specifically expressed in humans as well (John and Surani 1996), and vice versa, it is not known whether genomic imprinting is conserved among other mammalian groups. Many theories have been developed that consider the evolution of genomic imprinting (Hurst 1997), possibly the most inclusive of these says that imprinting evolved because of the conflicting interests of maternal and paternal genes in relation to transfer of nutrients from the mother to her offspring during pre- and postnatal development (the ‘‘conflict hypothesis’’: Haig and Graham 1991; Moore and Haig 1991). It would be important to know to which extent genomic imprinting is conserved among eutherian mammals in order to evaluate this and other evolutionary theories of imprinting. We set out to investigate its conservation in a bovid ruminant, the domestic sheep (*Ovis aries*). First, because ruminants are phylogenetically quite distinct from the rodent and primate lineages from which (based on comparative studies on mitochondrial proteins) they diverged about 110 and 100 million years ago, respectively (Penny and Hasegawa 1997, and references therein; Janke et al. 1997). Secondly, in contrast to rodents and primates, which have a placenta that invades all uterine layers and acquires nutrients directly from maternal blood vessels, ruminants have a nonaggressive form of placentation, with a chorion that does not invade the uterine layers (Steven 1975). Comparatively, implantation is also delayed in ruminants. In sheep, for example, the total gestation length is about 150 days, with gastrulation taking place at day 9.5 and implantation (indicated by placentome organization) occurring between days 23 and 25 of gestation (Steven 1975). The pronounced differences in placentation between ruminants and other groups of placental mammals should allow evaluation of other evolutionary theories which say that imprinting prevents ovarian trophoblast disease and restrains aggressive placentas from harming the pregnant mother (Hall 1990; Varmuza and Mann 1994).

We recently demonstrated that parthenogenetic sheep conceptuses can be produced efficiently by chemical activation of metaphase II oocytes with a combination of ionomycin and 6-dimethylaminopurine. This preliminary study (Loi et al. 1998) showed that, when cultured in vivo to the blastocysts stage and transferred into recipient ewes, thus-derived parthenogenetic embryos can develop to day 21 of gestation, but apparently not to later fetal stages of development. For our analysis of imprinting we derived a larger number of day 21 parthenogenetic fetuses and compared these with appropriate biparental control fetuses. Devel-

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Table 1. Development of parthenogenetic and biparental control fetuses.

	Blastocysts/ 2-cell embryos (%)	d21 fetuses/ blastocysts (%)	d28 fetuses/ blastocysts (%)
PG	26/42 (68)[a]	6/10 (60)[b]	0/8 (0)[b]
N	11/12 (91)[a]	7/11 (64)[b]	—[c]

In vivo development of 2-cell stage parthenogenetic (PG) and normal control (N) embryos into blastocysts, and viable intra-uterine development of implanted blastocysts to days 21 and 28 of gestation, with the percentage success rates in parenthesis.
[a] Only cavitated blastocysts were scored seven days after transfer of two-cell embryos into the oviduct
[b] Only live fetuses (with a beating heart) were scored.
[c] Liveborn animals (7/7 implanted blastocysts) without apparent abnormalities were obtained with birthweights (1.7–2.6 kg) and pregnancy lengths (148–151 days) which were within the normal range of the Sarda breed.

opmental comparison established that parthenogenetic sheep fetuses not only die shortly after implantation, but are also growth-retarded, which suggests that imprinting is conserved in this ruminant species. Conservation of imprinting in sheep was confirmed by analysis of candidate genes in the parthenogenetic versus control fetuses. This demonstrated for two growth-related genes which are imprinted in mice and humans, that they are parental-allele specifically expressed in sheep as well.

Materials and methods

Ovine fetuses. Embryological procedures were chosen such that activated oocytes and fertilized eggs could develop entirely in vivo in order to minimize external influences on embryogenesis. All animal procedures and surgical interventions were in accordance with the PPR 27/1/1992 (Animal Protection Regulations of Italy) in conformity with EC regulation 86/609. Parthenogenetic fetuses were derived as described in detail before (Loi et al. 1998). Briefly, metaphase II oocytes obtained from superovulated adult Sarda breed ewes were activated with a combination of ionomycin and 6-dimethylaminopurine. After they reached the two-cell stage, parthenogenetic embryos were cultured to the blastocyst stage in the oviducts of recipient ewes. After recovery and morphological evaluation, blastocysts with >100 cells and a clear inner cell mass were transferred to the uteri of foster ewes (2 blastocysts per animal). Parthenogenetic fetuses were obtained from recipients on day 21 or 28 of pregnancy, and were analyzed for their external morphology and size. For the control fetuses (also of the Sarda breed), naturally fertilized two-cell stage embryos were cultured in vivo and transferred into recipient ewes, as for the parthenogenetic fetuses.

Analysis of gene expression. Total RNA was isolated from day 21 fetuses and from the corresponding extra-embryonic membranes according to Chomczynski and Sacchi (1987). After electrophoresis on 1% formaldehyde gels, RNA (5 μg/sample) was transferred to Hybond N^+ membrane (Amersham). Hybridizations with radioactively labeled cDNA probes were performed as described by Church and Gilbert (1984). For quantification of expression, band intensities were measured with a Biorad Geldoc 1000 system and compared with band intensities corresponding to control (*GAPDH*) hybridizations.

Results and Discussion

Parthenogenetic and naturally fertilized ovine embryos were compared for their developmental potential in vivo (Table 1). Two-cell parthenogenetic embryos, obtained by chemical activation of mature oocytes, developed with high efficiency (26 out of 42) to cavitated blastocysts, which appeared morphologically identical to normal control blastocysts (data not shown). After transfer into synchronized recipient ewes, parthenogenetic (6 out of 10) and control blastocysts (7 out of 11) were found to also undergo embryonic development to day 21 of gestation at similar frequencies. At day 21, parthenogenetic fetuses appeared morphologically normal and were viable, as witnessed by a beating heart (Fig. 1). In our previous study, the few parthenogenetic day 21 fetuses produced were used for histological preparations and this did not

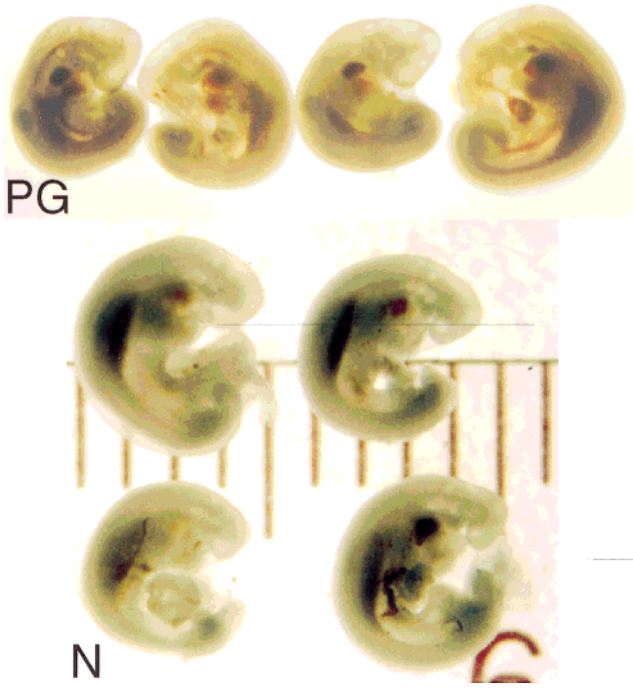

Fig. 1. Parthenogenetic sheep development is associated with growth-retardation. Parthenogenetic sheep fetuses (PG) were derived by chemical activation of oocytes and in vivo culture to day 21 of gestation. For the normal control day 21 fetuses (N), naturally fertilized two cell stage embryos were cultured in vivo, as for the parthenogenetic fetuses. Statistical comparison (chi square analysis) showed that parthenogenetic fetuses ($N = 6$) were significantly smaller than control ($N = 7$) fetuses (4.5 ± 0.5 mm versus 5.2 ± 0.3 mm; $P < 0.01$). All fetuses derived in this study were of the Sarda breed.

reveal any developmental abnormalities (Loi et al. 1998). Comparison of the crown-rump lengths of all day 21 fetuses derived in this study showed that the parthenogenetic fetuses were significantly smaller than the control fetuses (Fig. 1). At day 28 of gestation, only moribund parthenogenetic fetuses were obtained (8 out of 8) with partly resorbed extra-embryonic membranes (not shown). Although its causes remain to be determined, we have previously shown that this fetal lethality likely occurs at days 25–26 of gestation (Loi et al. 1998). This is the stage at which chorio-alantoic placenta formation takes place (Steven 1975), and it should therefore be interesting to determine whether (like in mice) embryonic death in parthenogenetic sheep is related to aberrant development of extra-embryonic membranes. In conclusion, our developmental observations demonstrate that, like in mice (Surani et al. 1984; McGrath and Solter 1984), parthenogenetic development in sheep is associated with growth-retardation and does not proceed beyond early fetal stages. These similarities suggested that imprinting is conserved in sheep, a question that we addressed by comparing the expression of candidate imprinted genes in parthenogenetic and fertilized control fetuses.

We studied the expression of candidate imprinted genes in five parthenogenetic and five naturally fertilized control fetuses at day 21 of gestation. For each fetus, the extra-embryonic membranes were studied as well (Fig. 2). We first analyzed the expression of the Insulin-like Growth Factor 2 gene (*IGF2*), which maps to sheep chromosome 21q21-qter (Ansari et al. 1994) and encodes a major fetal growth factor. In mice and humans, *IGF2* is expressed from the paternal chromosome exclusively (DeChiara et al. 1991; Ohlsson et al. 1993). In parthenogenetic mouse embryos, as a consequence, *Igf2* is not expressed (Walsh et al. 1994). We detected high levels of *IGF2* expression in the normal sheep fetuses

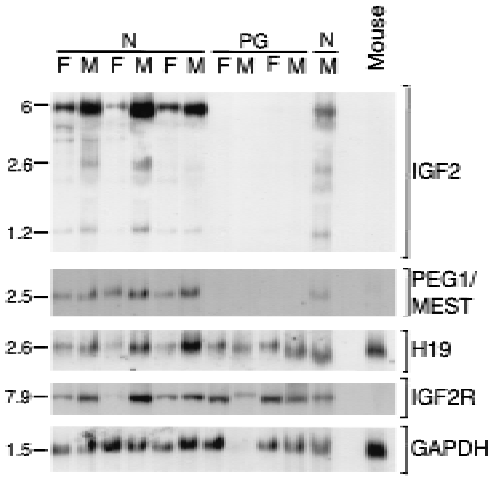

Fig. 2. The *IGF2* and *PEG1/MEST* genes are imprinted in the sheep. By northern analysis we studied 5 parthenogenetic and 5 normal fetuses at day 21 of gestation. Shown are total RNAs from two of the parthenogenetic (PG) and 3 of the normal control (N) fetuses (F) and their extra-embryonic membranes (M). To the far right, total RNA from an embryonic day 15 mouse fetus. For *IGF2* analysis, an ovine cDNA probe (Ohlsen et al. 1994) containing exons 1,3 and 8–10, was used (hybridization at 65°C; film exposure for 18 h). Transcript sizes, according to Ohlsen et al. 1994, are indicated in kb. *PEG1/MEST* hybridization was performed with a 963 bp probe from the highly conserved coding sequence, covering nucleotides 222-1185 of the human cDNA (GEN/EMBL: D78611; Nishita et al. 1996) (hybridization at 60°C; exposure for 5 days). For *H19* hybridization, a PCR-amplified 545 bp fragment containing nucleotides 1612-2156 of the human *H19* cDNA (GEN/EMBL: M32053; Zhang and Tycko 1992) was used (hybridization at 60°C; exposure for 60 h). For *IGF2R*, a full-length cDNA (7.8 kb) of the bovine *IGF2R* gene (12) was used as probe (hybridization at 60°C; exposure for 5 days). *GAPDH* expression was analyzed with a 250 bp probe from the 5′-most part of the murine cDNA (hybridisation at 60°C; exposure for 20 h). The ratio of the *IGF2R* and *GAPDH* band intensities was determined for each RNA sample. In the fetuses these ratios were 0.4, 0.1, 0.6, 0.7 and 1.6, in lanes 1, 3, 5, 7, and 9, respectively. In the extraembryonic membrane samples the ratios were 3.8, 2.5, 0.7, >2, 1.4, and 0.7 in lanes 2, 4, 6, 8, 10 and 11, respectively.

and their extra-embryonic membranes. In contrast, no *IGF2* expression was observed in the parthenogenetic fetuses and membranes (Fig. 2) and this expression pattern strongly suggests that *IGF2* is imprinted and paternally expressed in sheep as well. In analogy with studies in the mouse, where genetic ablation of the *Igf2* gene leads to reduced fetal growth (DeChiara et al. 1991), it seems likely that the absence of *IGF2* expression in parthenogenetic sheep fetuses is involved in their growth retardation.

We next analyzed the expression of *PEG1/MEST* (Paternally Expressed Gene-1/MEsoderm Specific Transcript), an *alpha/beta* hydrolase-encoding gene, which is expressed at high levels in mesodermal tissues (Sado et al. 1993). *PEG1/MEST* is paternally expressed in mice and humans (Kaneko-Ishino et al. 1995; Nishita et al. 1996). As a consequence, parthenogenetic mouse embryos do not express this gene (Kaneko-Ishino et al. 1995). The mouse *Peg1/Mest* gene has recently been targeted by homologous recombination and this genetic study indicates that absence of PEG1/MEST leads to a 20% reduction in fetal growth (L. Lefebvre and M.A. Surani, personal communication). The human *PEG1/MEST* gene has been mapped to chromosome 7 (Nishita et al. 1996), maternal disomy of which is associated with fetal growth retardation (Kotzot et al. 1995). On hybridization of the sheep RNAs, *PEG1/MEST* expression was detected in the control fetuses and their extra-embryonic membranes, but no expression was observed in the parthenogenetic fetuses and membranes. This result most likely indicates that in sheep also, *PEG1/MEST* is imprinted and expressed from the paternal chromosome exclusively. Given its involvement in the regulation of fetal growth, it seems likely that the absence of *PEG1/MEST* expression is one of the factors implicated in the growth-retardation of parthenogenetic sheep fetuses.

We also analyzed *H19* and *IGF2-receptor* ((*IGF2R*), two growth-related imprinted genes that are maternally expressed in fetal and neonatal mouse tissues (Bartolomei et al. 1991; Barlow et al. 1991). *H19* encodes a non-translatable RNA, and the expression of this maternally expressed gene is co-regulated with that of the neighbouring, paternally expressed *IGF2* gene. Hybridizing with a human probe corresponding to a highly conserved portion of the gene, we detected a transcript of the same size as the mouse and human *H19* RNA (Bartolomei et al. 1991; Zhang and Tycko 1992), which suggests the existence of an ovine *H19* homologue. From the levels of expression, however, we were unable to deduce whether *H19* is maternally expressed in sheep, as no double dose was detected in the parthenogenetic RNA samples. For the analysis of *IGF2R* expression, a bovine cDNA probe was used (Lobel et al. 1988). Transcripts were detected in all the sheep RNA samples, and expression in the parthenogenetic fetuses appeared to be somewhat higher than in the control fetuses. Further research should elucidate whether this corresponds to imprinting of the ovine *IGF2R* gene, or is indirectly caused by the absence of IGF2.

In summary, we have shown that parthenogenetic sheep embryos are growth-retarded and do not develop beyond early fetal stages. Most likely, these abnormalities are caused by imprinted genes. Indeed, for the growth-related *IGF2* and *PEG1/MEST* genes we provide evidence for their paternal expression being conserved in sheep. Our data on imprinting in sheep would support ''the conflict hypothesis'', a theory that predicts that imprinted genes that enhance embryonic growth are expressed off the paternally derived genome (Haig and Graham, 1991; Moore and Haig 1991). Our demonstration of imprinting in a mammal with a non-invasive placenta, however, seems not to fit hypotheses that say that imprinting restrains aggressive placentas from harming the pregnant mother (Hall 1990; Varmuza and Mann 1994). From our data we predict that other genes that are imprinted in humans and mice are allele-specifically expressed in sheep as well and play key roles in growth and development. One aberrant phenotype in which imprinting might be involved is the muscular hypertrophy at the *callipyge* locus on sheep chromosome 18, a non-Mendelian mutation that becomes apparent only when inherited from father (Cockett et al. 1996).

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