Zygote **25** (August), pp. 462–471. © Cambridge University Press 2017
doi:10.1017/S0967199417000284 First Published Online 3 July 2017

MBD1 and MeCP2 expression in embryos and placentas from transgenic cloned goats

Ruoxin Jia[2,3], *Guomin Zhang*[2], *Yixuan Fan*[2], *Zhengrong Zhou*[2], *Yongjie Wan*[2], *Yanli Zhang*[1], *Ziyu Wang*[2] *and Feng Wang*[2]

Jiangsu Engineering Technology Research Center of Meat Sheep and Goat Industry, College of Animal Science and Technology, Nanjing Agricultural University, Nanjing, Jiangsu, People's Republic of China; and Department of Animal Husbandry and Veterinary Science, Zhejiang Academy of Agricultural Sciences, Hangzhou, Zhejiang, People's Republic of China

Date submitted: 23.12.2016. Date revised: 25.04.2017. Date accepted: 08.05.2017

Summary

DNA methylation is an important form of epigenetic regulation in mammalian development. Methyl-CpG-binding domain protein 1 (MBD1) and methyl-CpG-binding domain protein 2 (MeCP2) are two members of the MBD subfamily of proteins that bind methylated CpG to maintain the silencing effect of DNA methylation. Given their important roles in linking DNA methylation with gene silencing, this study characterized the coordinated mRNA expression and protein localization of MBD1 and MeCP2 in embryos and placentas and aimed to analysis the effects of MBD1 and MeCP2 on transgenic cloned goats. Our result showed that *MBD1* expression of transgenic cloned embryo increased significantly at the 2–4-cell and 8–16-cell stages ($P < 0.05$), then decreased at the morula and blastocyst stages ($P < 0.05$); *MeCP2* expression in transgenic cloned embryo was significant decreased at the 2–4-cell stage and increased at the 8–16-cell stage ($P < 0.05$). Placenta morphology analysis showed that the cotyledon number of deceased transgenic cloned group (DTCG) was significantly lower than that the normal goats (NG) and in the live transgenic cloned goats (LTCG) ($P < 0.05$). MBD1 and MeCP2 were clearly detectable in the placental trophoblastic binucleate cells by immunohistochemical staining. Moreover, *MBD1* and *MeCP2* expression in DTCG was significant higher than in the NG and the LTCG ($P < 0.05$). In summary, aberrant expression of methylation CpG binding proteins MBD1 and MeCP2 was detected in embryonic and placental development, which reflected abnormal transcription regulation and DNA methylation involved in MBD1 and MeCP2. These findings have implications in understanding the low efficiency of transgenic cloning.

Keywords: Embryo, Goat, MBD1, MeCP2, Placenta

Introduction

Somatic cell nuclear transfer (SCNT) is a promising technique to produce mammalian transgenic clones. Embryos and fetuses have been successfully generated using SCNT in a range of species (Wells *et al*., 1997, 1999; Wakayama *et al*., 1998; Wilmut *et al*., 1997; Polejaeva *et al*., 2000; Chesné *et al*., 2002). In livestock, however, SCNT cloning is inefficiently developed to full-term (Cibelli *et al*., 1998; Kato *et al*., 1998; De Sousa *et al*., 2001; Watanabe & Nagai, 2009, 2011; Meng *et al*., 2014; Jia *et al*., 2016). Most cloned embryos die during post-implantation development, and those that survive to term are frequently defective (Wrenzycki *et al*., 2001; Cibelli *et al*., 2002). Abnormal placentation

[1]All correspondence to: Yanli Zhang. Jiangsu Livestock Embryo Engineering Laboratory, College of Animal Science and Technology, Nanjing Agricultural University, Weigang 1, Nanjing, Jiangsu 210095, People's Republic of China. Tel: +86 25 84395381; Fax: +86 25 84395314. E-mail: zhangyanli@njau.edu.cn
[2]Jiangsu Livestock Embryo Engineering Laboratory; Jiangsu Engineering Technology Research Center of Meat Sheep and Goat Industry, College of Animal Science and Technology, Nanjing Agricultural University, Nanjing, Jiangsu 210095, People's Republic of China.
[3]Department of Animal Husbandry and Veterinary Science, Zhejiang Academy of Agricultural Sciences, Hangzhou, Zhejiang 310021, People's Republic of China.

https://doi.org/10.1017/S0967199417000284 Published online by Cambridge University Press

has been described in several cloned species (Suemizu *et al*., 2003; Farin *et al*., 2006). Moreover, recent molecular evidence supports the hypothesis that the placental lineage is particularly vulnerable to problems arising from somatic nucleus reprogramming after nuclear transfer (Yang *et al*., 2007).

Successful nuclear transfer depends on nuclear reprogramming of an adult somatic nucleus to an embryonic state. Normal development involves many epigenetic modifications. DNA methylation, a well studied epigenetic modification, has been regarded as a key contributor to mammalian development, gene regulation and genome stability (Klose & Bird, 2006). Methyl-CpG-binding domain protein 1 (MBD1) is highly related to DNA methylation. Its MBD domain recognizes and binds to methylated CpGs. This binding allows it to trigger methylation of H3K9 and results in transcriptional repression. The CXXC3 domain of MBD1 makes it a unique member of the MBD family due to its affinity to non-methylated DNA. MBD1 acts as an epigenetic regulator via different mechanisms, such as the formation of the MCAF1/MBD1/SETDB1 complex or the MBD1-HDAC3 complex (Li *et al*., 2015). Methyl-CpG-binding protein 2 (MeCP2), the first MBD to be characterized, localizes to densely methylated DNA. This protein can bind to as little as one methylated CpG dinucleotide and represses transcription through a transcriptional repression domain (TRD). MeCP2 can associate with histone deacetylases (HDACs) and histone methyltransferases (HMTs), providing a link between DNA methylation and histone deacetylation and methylation, which act as a transcriptional repressor by binding to target gene promoters and silencing their transcription (Nan *et al*., 1996; Fuks *et al*., 2003).

As methylation levels always changes along with embryogenesis, MBDs with its interacting partners, including proteins and non-coding RNAs, participate in normal or pathological processes and function in different regulatory systems. Because of the important role of MBDs in epigenetic regulation, we hypothesized that MBD1 and MeCP2 may have a crucial function in developmental events, including embryo cleavage and placenta development. This study aimed to characterize the coordinated mRNA expression and protein localizations of MBD1 and MeCP2 in embryos and placentas from transgenic cloned goats in an effort to determine how MBD1 and MeCP2 functions. These findings could provide clues to understanding the low efficiency of transgenic cloning.

Materials and Methods

This study was performed in strict accordance with recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Nanjing Agricultural University. All surgery was performed under sodium pentobarbital anesthesia, and every effort was made to minimize suffering.

Embryos

Embryos in our study consisted of two groups: N (normal) group and T (transgenic clone) group. The N group was *in vivo* embryos produced by the internal fertilization. The T group was *in vitro* embryos produced by SCNT.

In vivo *embryo production*

To obtain sufficient *in vivo* embryos, oestrous synchronization was performed using an intravaginal device (EAZI-BREED™ CIDR®, Animal Health Supplies, Ascot Vale, VIC, Australia) for 12 days each group ($n = 5$). Superovulation treatments were initiated at 48 h (Day 10) before the end of progesterone exposure. All Saanen dairy goats were superovulated with a total dose of 180 IU follicle-stimulating hormone (FSH; Pituitary Follitropin For Injection, Ningbo Sansheng Pharmaceutical Co., Ltd, China), which was divided into six decreasing doses i.m. given twice daily. Two injections of PGF2α analogue and 50 μg of D-cloprostenol (Cloprostenol Sodium for Injection, Ningbo Sansheng Pharmaceutical Co., Ltd, China) were given concurrent with fifth and sixth FSH treatments. After CIDR removal, the does were checked for oestrus with an aproned adult male twice daily and were randomly mated to one of three fertile bucks every 12 h as long as they displayed standing oestrus. At 24–36 h after the last mating, the embryos were collected.

In vitro *embryo production*

The manufacturing process of *in vitro* embryos using SCNT is shown in Fig. 1. Briefly, goat fibroblast cells from ears of 3-month-old goats were used to produce donor somatic cells harboring the *hLF* gene for SCNT. The culture and passage of donor cells, oocyte collection and enucleation, nuclear transferring, oocyte activation, embryo culture, and embryo transfer were carried out in order (Wan *et al*., 2012).

Embryo collection

Reconstructed and internal fertilization embryos were washed three times, transferred into 150-μl droplets of M16 medium (Sigma, USA) under mineral oil, and cultured for 6 days at 38.5°C with 5% CO_2 in air. Cleavage and development to the blastocyst stage were observed from day 2 to day 7. One-cell embryos, 2–4-cell embryos, 8–16-cell embryos,

https://doi.org/10.1017/S0967199417000284 Published online by Cambridge University Press

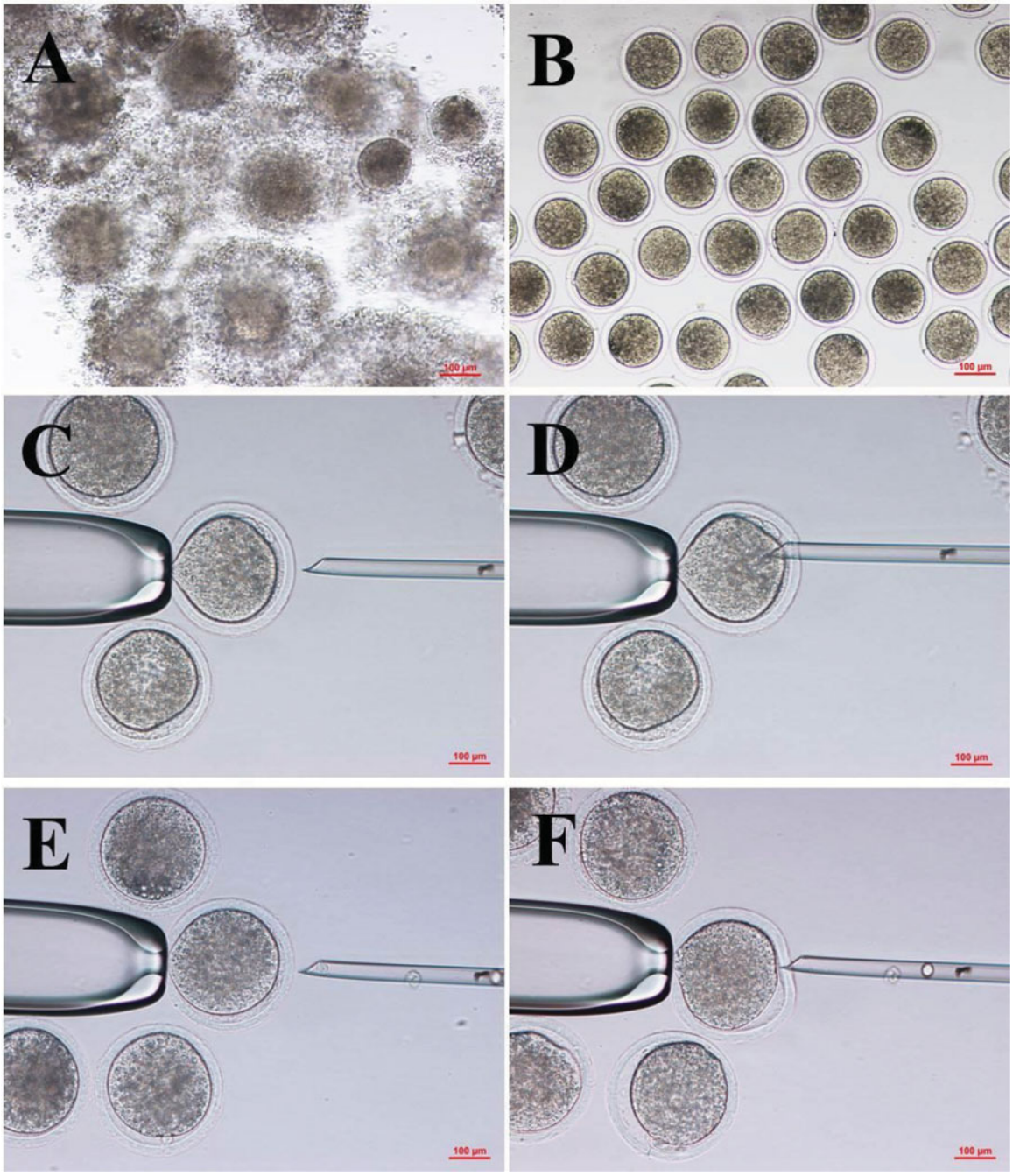


Figure 1 Manufacturing process of *in vitro* embryo using somatic cell nuclear transfer. (*A*) *In vitro* maturation oocytes. (*B*) Mature oocytes without cumulus oophorus. (*C*, *D*) enucleation; (*E*, *F*) nuclear transfer.

morulas, and blastocysts were also collected (Fig. 2). Twenty embryos from each stage were randomly selected and stored in Sample Protector for RNA (TaKaRa, China) until RNA isolation.

Placentas

Placentas in our study consisted of three groups: normal goats (NG), live transgenic cloned goats (LTCG) and deceased transgenic cloned goats (DTCG). NG included four newborn normal goats from conventional reproduction, which were used as the control. LTCG included four live cloned goats that were lived healthy up to now. DTCG included four dead cloned goats that died within 3-day after birth. All placental samples were weighed out, the size and number of placenta cotyledon were measured, and frozen in liquid nitrogen and stored at −80°C until further use. Other portions of the placentas were fixed for 24 h in 4% formaldehyde, and stored in 75%

https://doi.org/10.1017/S0967199417000284 Published online by Cambridge University Press

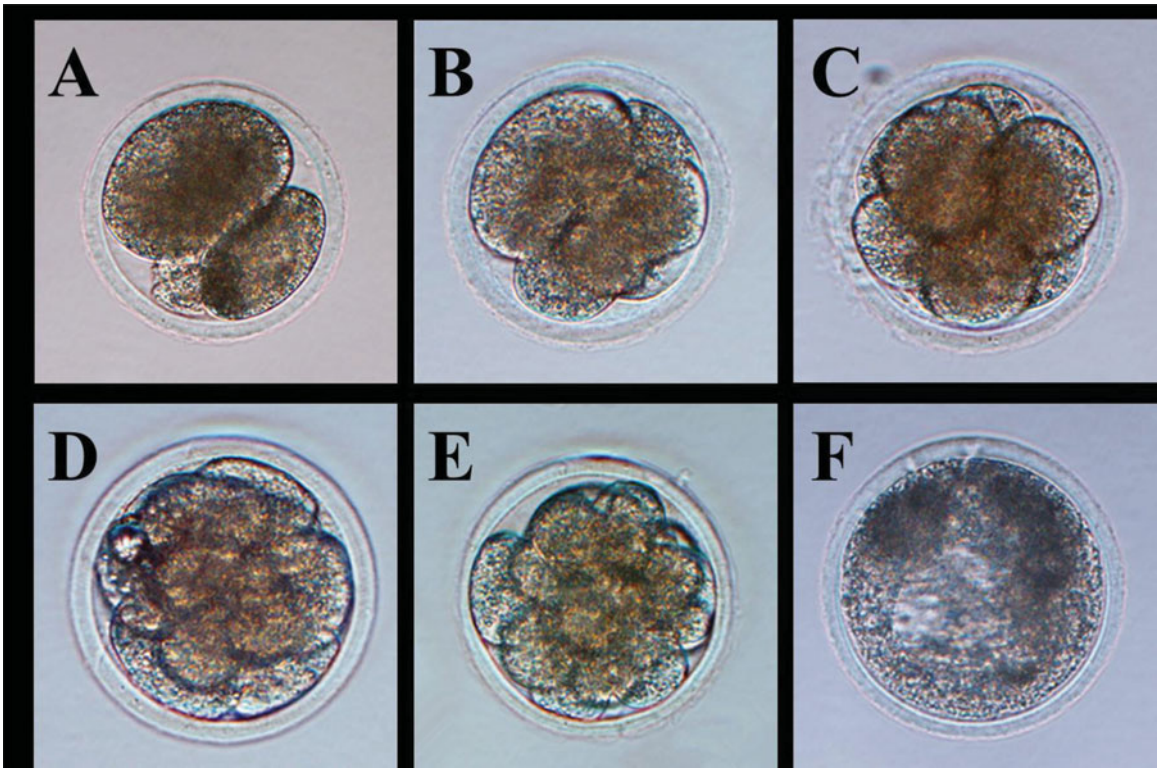


Figure 2 Transgenic cloned embryos at different developmental stages. (*A*) 2-cell stage; (*B*) 4-cell stage; (*C*) 8-cell stage; (*D*) 16-cell stage; (*E*) 32-cell stage; (*F*) blastocyst stage.

alcohol. Fixed tissues were analysed for structural organization.

Gene expression analyses

Total RNA was isolated from embryos and placentas using RNeasy Micro kits (Qiagen, Germany), and placental tissue RNA was extracted using RNAprep Pure kits (Tiangen, China) according to the manufacturers' instructions. Real-time PCR was performed on an ABI 7300 Real-Time PCR System (Applied BioSystems, USA) and detected using SYBR Green Master Mix in a reaction volume of 20 µl. The primers used in real-time PCR are shown in Table 1. Comparative quantification of MBD1 and MeCP2 mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001).

Protein location analyses

After fixation, placental tissues were embedded in paraffin, and 6-µm sections were cut and mounted on slides. Sections were processed for immunohistochemical staining as described previously (Shi *et al.*, 1991). A primary rabbit anti-MBD1 antibody (1:500, Abcam, USA) and secondary antibody (1:1000, Beyotime, China) were used in the immunohistochemical analyses. Specific protein immunoreactivity was visualized by incubation with DAB (3,3′-diaminobenzidine, Beyotime, China). The primary antibody was replaced with normal rabbit serum as a negative control.

Statistical analyses

Statistical analyses were performed using SPSS 17.0 (IBM, USA). Experimental data are shown as the mean ± standard error of the mean (SEM). Differences in the mean were evaluated using one-way analysis of variance (ANOVA). Statistically significant differences were defined as $P < 0.05$.

Results

Relative expression of MBD1 and MeCP2 mRNA in transgenic cloned embryos

During the early embryonic developmental stage, *MBD1* and *MeCP2* expression showed significant differences between transgenic cloned embryos and fertilized eggs (Fig. 3). Specifically, at the 2–4-cell embryo stages, compared with fertilization embryos, *MBD1* expression was significantly higher in transgenic cloned embryos ($P < 0.05$, Fig. 3*A*), but *MeCP2* was significantly lower in transgenic cloned embryos ($P < 0.05$, Fig. 3*B*). At 8–16-cell embryo stages, the *MBD1* and *MeCP2* mRNA levels in transgenic cloned

https://doi.org/10.1017/S0967199417000284 Published online by Cambridge University Press

Table 1 Primers for quantitative real-time polymerase chain reaction

Genes	Primer sequence (5′→3′)	Product size (bp)	Sequence accession number	Annealing temperature (°C)
MBD1	F-GGACGCTCAGACACCTACTACCAG; R-CCAGGTATCGGGTCAGCTCAA	76	NM_001046481.1	60
MeCP2	F-CCGCTGGGAAGTATGATGTG; R-GGGAGATTTGGGCTTCTTAGG	191	XM_588477	55
GAPDH	F-CGACTTCAACAGCGACACTCAC; R-CCCTGTTGCTGTAGCCCAATTC	118	NM_001034034	58

Abbreviations: bp, base pair; F, forward; R, reverse.

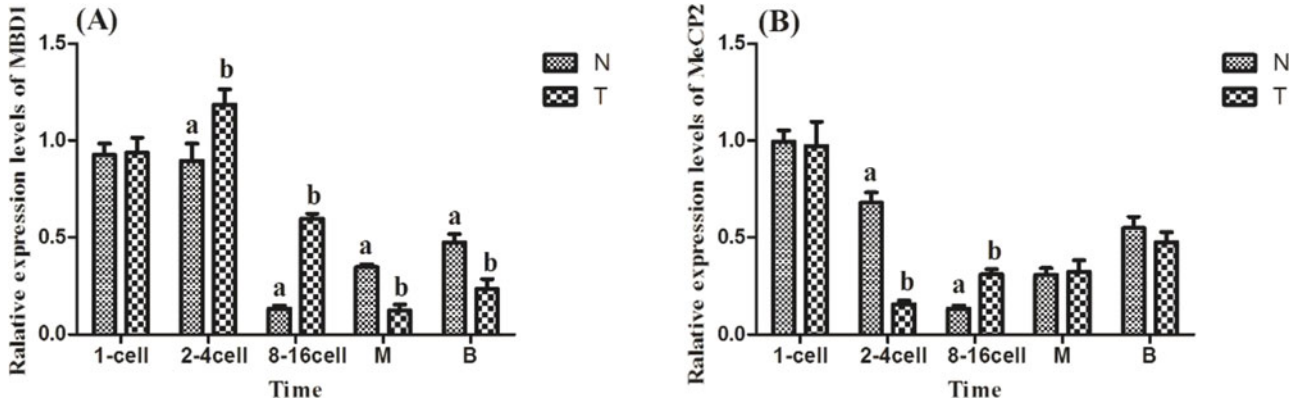


Figure 3 *MBD1* and *MeCP2* transcript levels in zygotes and transgenic cloned embryos of goats during early development. (*A*) transcript levels of *MBD1*; (*B*) transcript levels of *MeCP2*. N: fertilized eggs; T: transgenic cloned embryos. 1-cell: single cell embryo; 2–4 cell: 2–4-cell blastomere; 8–16 cell: 8–16-cell blastomere; M: morula; B: blastocyst. Bars with different superscripts are statistically different ($P < 0.05$).

embryos were higher compared with fertilization embryos ($P < 0.05$, Fig. 3). At the morula and blastocyst stages, *MBD1* expression was extremely lower in transgenic cloned embryos than fertilization embryos ($P < 0.01$, Fig. 3*A*).

Placenta morphology analyse of transgenic cloned goats

The fetal weight of the normal goats (NG) and LTCG were lower than the DTCG, which was the opposite of the placentome number results (Table 2). The average placentome number of NG and LTCG was higher than that of DTCG. Moreover, the significantly variance displayed on the placentome at diameter 2 cm, 2–4 cm, 6–8 cm and >8 cm (Table 3).

Placenta fetalis were sectioned and stained with hematoxylin and eosin. The histologic characteristics of placenta fetalis are shown in Fig. 4. In the trophoblastic epithelium, cylindrical cells arranged in palisades and numerous binucleate cells can be visualized. In addition, fetal mesenchyme, a connective lax tissue with elongated cores that is vascularized with blood vessels of diverse caliber, is located close to the epithelium.

Protein localizations of MBD1 and MeCP2 in placenta fetalis of transgenic cloned goats

MBD1 and MeCP2 immunoreactivities were detected in binucleate cells of the trophoblastic epithelium (Fig. 5). In control sections, virtually no immunoreactivity was observed when normal rabbit serum was used in place of the primary antibody.

Relative expression of MBD1 and MeCP2 mRNA in placenta fetalis

MBD1 expression levels of DTCG on placental cotyledons at diameter 2 cm, 2–4 cm, 4–6 cm and 6–8 cm were significantly higher than that of normal and LTCG ($P < 0.05$, Fig. 6). Analogously, in all placental cotyledons with different diameters, the total level of *MeCP2* expression in DTCG was significantly higher than normal and LTCG ($P < 0.05$, Fig. 7).

Discussion

In SCNT, active demethylation of donor somatic cell DNA shortly after nuclear transplantation is thought to be important for reprogramming subsequent

https://doi.org/10.1017/S0967199417000284 Published online by Cambridge University Press

Table 2 Fetal weight, placental weight, placentomes number of different groups

Group	Fetal weight (kg)	Placental weight (kg)	No. of placentomes
NG ($n = 4$)	2.26 ± 0.25^{a}	0.65 ± 0.05	80.50 ± 4.56^{a}
LTCG ($n = 4$)	3.65 ± 0.36^{a}	0.50 ± 0.11	65.00 ± 7.82^{a}
DTCG ($n = 4$)	1.71 ± 0.06^{b}	0.78 ± 0.16	31.00 ± 4.18^{b}

DTCG, dead transgenic cloned goats, LTCG, live transgenic cloned goats; NG, normal goats.
[a,b]Means with a, b within each column within each factor represent significant difference ($P < 0.05$).

Table 3 Number of placentomes in different diameters of different groups

	Number of placentomes in different diameters (dia., cm)				
Group	$d \leq 2$	$2 < d \leq 4$	$4 < d \leq 6$	$6 < d \leq 8$	> 8
NG ($n = 4$)	19.25 ± 1.03^{a}	35.50 ± 4.21^{a}	12.50 ± 1.04	11.75 ± 1.03^{a}	1.50 ± 0.29^{a}
LTCG ($n = 4$)	17.00 ± 1.47^{a}	30.00 ± 4.34^{a}	11.25 ± 1.65	6.00 ± 1.29^{b}	1.25 ± 0.48^{a}
DTCG ($n = 4$)	7.00 ± 1.22^{b}	11.50 ± 1.85^{b}	9.50 ± 1.04	3.00 ± 0.91^{b}	0^{b}

DTCG, dead transgenic cloned goats; LTCG, live transgenic cloned goats; NG, normal goats.
[a,b]Means with a, b within each column within each factor represent significant difference ($P < 0.05$).

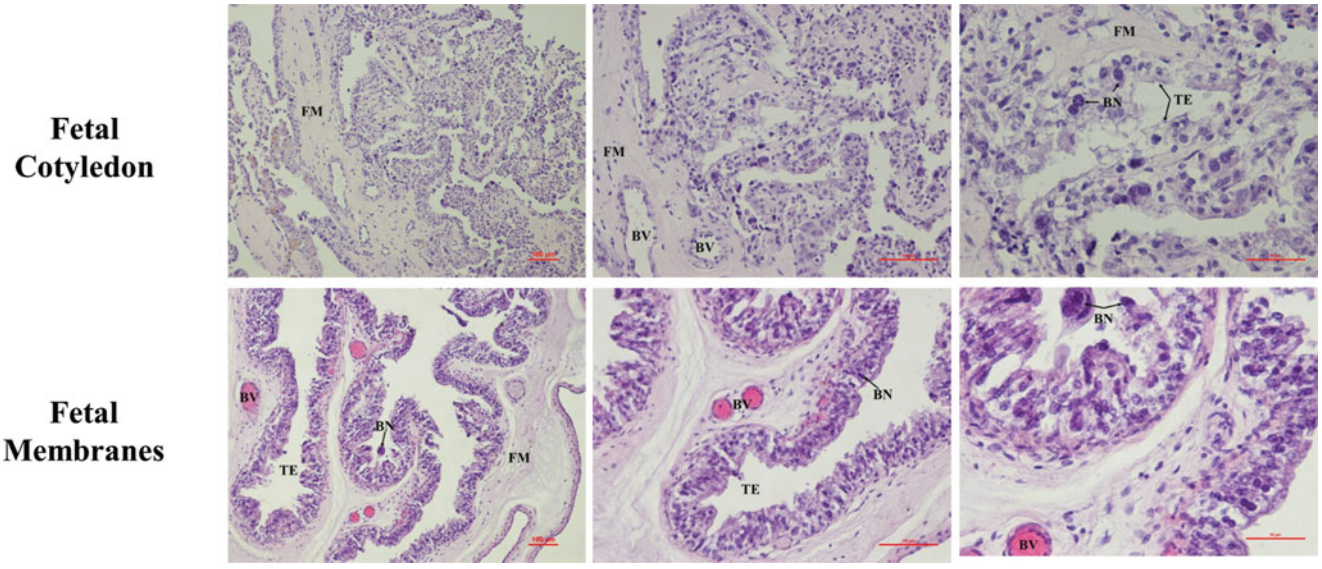


Figure 4 Photographs of placenta stained with haematoxylin–eosin. BN: binucleate cells; BVs: blood vessels; FM: fetal mesenchyme; TE: trophoblastic epithelium.

embryonic development. MBD1 and MeCP2 are members of the MBD protein subfamily that bind methylated CpG. The DNA methylation pattern is believed to be 'read' by the conserved MBD family of proteins (Wade, 2001; Jaenisch & Bird, 2003).

During embryonic preimplantation development, significant changes occur in DNA methylation amount and patterns (Santos & Dean, 2004). A further passive loss of methylation has been observed as DNA replicates between the 2-cell and morula stages, with somatic cell levels being re-established at or after the blastocyst stage (Mayer *et al*., 2000; Bourc'His *et al*., 2001; Dean *et al*., 2001). In this study, *MBD1* and *MeCP2* mRNA levels from fertilized eggs were further reduced with embryonic development to a minimum at the 8–16-cell stage, demonstrating the important role of *MBD1* and *MeCP2* in linking DNA demethylation with embryogenesis. These results are consistent with a number of previous studies on dynamic changes in the localization of five members of the MBD gene family during murine and bovine preimplantation embryo development (Ruddock-D'Cruz *et al*., 2008).

Discrepancies were detected between transgenic cloned embryos and fertilized eggs in *MBD1* and *MeCP2* mRNA levels during preimplantation development. In fertilization embryos, the phase of *MBD1* and *MeCP2* minimal expression was at 8–16-cell embryo stage. However, in transgenic cloned

https://doi.org/10.1017/S0967199417000284 Published online by Cambridge University Press

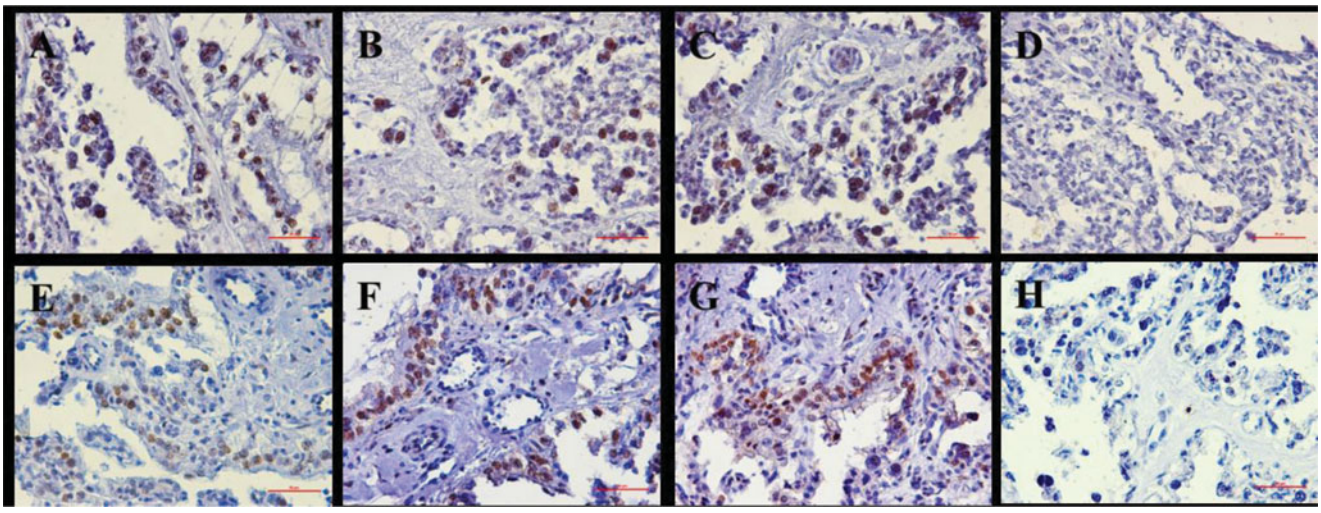


Figure 5 Protein localizations of MBD1 and MeCP2 in placentas. (*A*) MBD1-NG; (*B*) MBD1-LTCG; (*C*) MBD1-DTCG; (*D*) MBD1 control; (E) MeCP2-NG; (F) MeCP2-LTCG; (*G*) MeCP2-DTCG; (H) MeCP2 control. NG, Normal goats; LTCG, Live transgenic cloned goats; DTCG, Dead transgenic cloned goats; Control, the negative control. Positive signals appear brown in color, and counterstaining background appears blue in color.

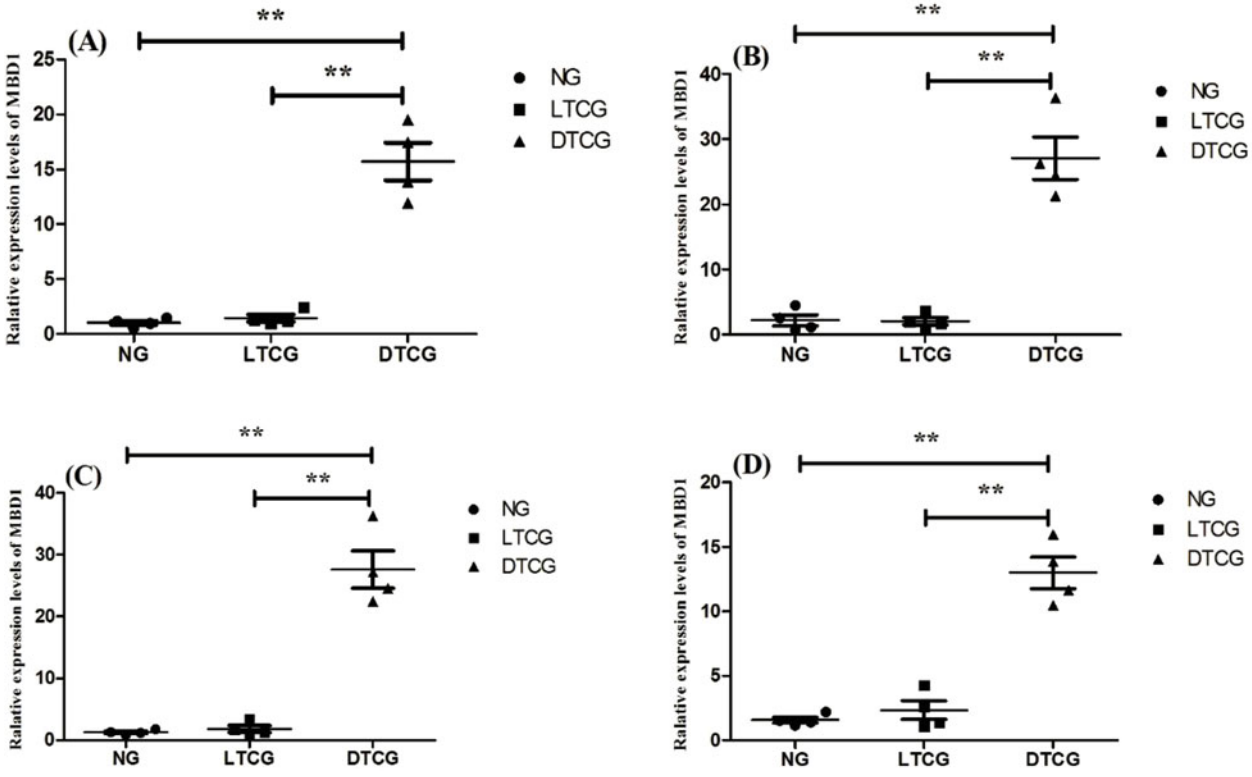


Figure 6 *MBD1* transcript levels in placental cotyledon with different diameters. (*A*) dia. ≤ 2 cm; (*B*) 2 cm < dia. ≤ 4 cm; (C) 4 cm < dia. ≤ 6 cm; (*D*) 6 cm < dia. ≤ 8 cm; **Represent extremely significant deviation ($P < 0.01$). NG, Normal goats; LTCG, Live transgenic cloned goats; DTCG, Dead transgenic cloned goats.

embryos, the timing of *MBD1* minimal expression was postponed until the morula stage, and the time for *MeCP2* minimal expression was advanced to the 2–4-cell embryo stage. During embryonic preimplantation development, at the time of the maternal to zygotic transition (MZT), DNA methylation is minimal, maternal transcripts are degraded and the newly formed embryo must begin transcription (Dean *et al.*, 2001; Oswald *et al.*, 2000; Mayer *et al.*, 2000). MBD proteins have been implicated in both global transcriptional

https://doi.org/10.1017/S0967199417000284 Published online by Cambridge University Press

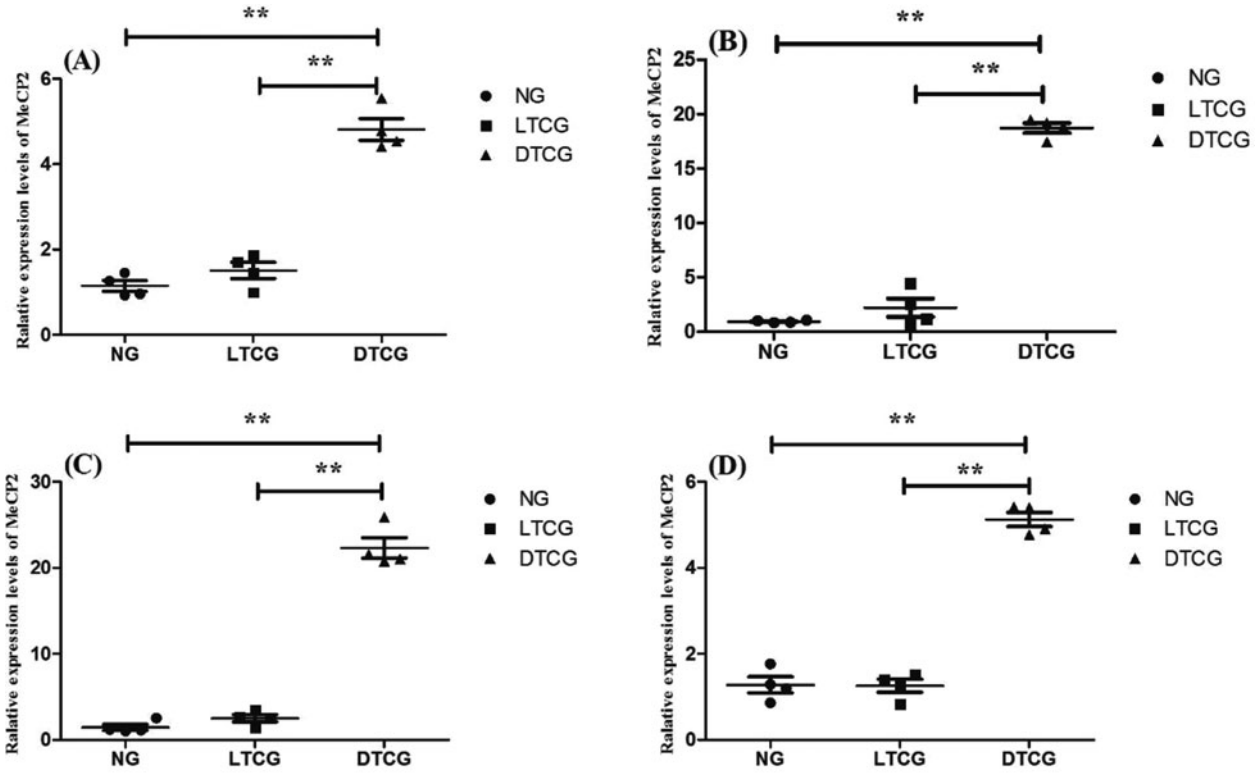


Figure 7 *MeCP2* transcript levels in placental cotyledon with different diameters. (*A*) dia. ≤ 2 cm; (*B*) 2 cm < dia. ≤ 4 cm; (*C*) 4 cm < dia. ≤ 6 cm; (*D*) 6 cm < dia. ≤ 8 cm. **Represent extremely significant deviation ($P < 0.01$). NG, Normal goats; LTCG, Live transgenic cloned goats; DTCG, Dead transgenic cloned goats.

regulation (Klose & Bird, 2006) and, more specifically, in repression of Oct4, a pluripotency marker expressed during early embryogenesis (Gu *et al.*, 2006). These results suggested that there was a disorder timing for the regulation of *MBD1*- and *MeCP2*-mediated gene transcription, which affects the demethylation of donor somatic cells and maternal gene activation during early embryogenesis.

Normal embryos undergo demethylation, followed by remethylation, to achieve genome-wide methylation reprogramming in early preimplantation development (Dean *et al.*, 2001). In particular, it might be significant if the inner cell mass (ICM) remains methylated because trophectoderm cells are the first differentiated cell type to form during development and trophoblast-specific gene expression is essential for embryonic nutrition and implantation (Beaujean *et al.*, 2004). We found that MBD1 levels were extremely low in transgenic cloned embryos at the blastocyst stage, suggesting that MBD1 affects global remethylation of the embryonic genome, which may cause cloned embryo implantation failure.

After embryonic implantation, the placenta is designed to allow efficient exchange between the mother and fetus to optimize growth and development (Penninga & Longo, 1998). Furthermore, a correlation between placental size and litter weight has been determined, suggesting that smaller placentas are more efficient (Osgerby *et al.*, 2003; Konyalı *et al.*, 2007). In addition, the number of placentomes decreases under extreme conditions, such as malnutrition (Konyalı *et al.*, 2007).

Notable differences in fetal weight, placental weight, and placentome number and size were detected between live transgenic cloned (LTCG) and deceased transgenic cloned (DTCG) goats. Specifically, the LTCG fetal weight and placentome number values were significantly higher compared with the DTCG. However, the LTCG placental weight was lower than that in the DTCG. These results indicated that placental efficiency was reduced in DTCG, proving that placental dysfunction in transgenic cloned goats may lead to their death.

Similar to other ruminants, the goat has a characteristic placental epithelium with two morphologically and functionally distinct cell types: there are mononucleate and binucleate trophoblast cells in the trophectoderm (Igwebuike, 2009), which were observed in the HE staining analyses of placentas from transgenic goats. Binucleate cells produce hormones, such as placental lactogen and progesterone, and through a fusion process with an uterine epithelial cell

https://doi.org/10.1017/S0967199417000284 Published online by Cambridge University Press

or the fetomaternal syncytium, release their contents into maternal connective tissue (Wooding *et al*., 1996; Igwebuike, 2009). Binucleate cells are also involved in villi development (Wooding *et al*., 1996; Klisch *et al*., 2010). Moreover, binucleate cells account for 15–20% of cells in the ovine trophectoderm for most of the gestation period. However, close to term, their numbers decrease concomitantly with increased fetal cortisol levels (Ward *et al*., 2002). These studies revealed that binucleate cells are a unique feature in ruminants that have essential roles in placental development and function. Interestingly, MBD1 and MeCP2 proteins were strongly expressed in binucleate trophoblast cells, suggesting that MBD1 and MeCP2 may modulate the function of binucleate cells. Studies have shown that DNA methylation regulates placental lactogen I (*PRL-I*) gene expression, and MeCP2 overexpression could inhibit *PRL-I* gene expression (Cho *et al*., 2001). In addition, lack of MBD1 in mice could cause placental hypoplasia and eventually lead to miscarriage (Li *et al*., 1992; Tate *et al*., 1996). In this study, we found that *MBD1* and *MeCP2* expression levels in DTCG were higher compared with LTCG. These data suggest that *MBD1* and *MeCP2* expression or overexpression may be connected with the placental dysfunction and the death of transgenic cloned goats. However, the detailed mechanism needs to be further elucidated. A future study is needed in order to provide evidence of the direct role of *MBD1* and *MeCP2* during embryonic and placental development, possibly using modification of *MBD1* and *MeCP2* expression via overexpression or knockdown methods in donor cells.

In conclusion, MBD1 and MeCP2 likely have important roles in linking DNA demethylation and remethylation during embryogenesis. Aberrant MBD1 and MeCP2 expression was present in transgenic cloned embryos, which may cause embryo implantation to fail. Additionally, MBD1 and MeCP2 protein expression in binucleate cells highlights its possible role in placental development. MBD1 and MeCP2 overexpression was detected in the placentas from DTCG. These findings are presumably due to aberrant epigenetic nuclear reprogramming during SCNT, which may cause developmental insufficiencies and ultimately death in cloned transgenic goats.

Acknowledgements

The authors greatly appreciate the work of Jingang Wang for embryo transfer and goats raising, as well as thanking Lizhong Wang and Shan Lan for their technical assistance. This study was supported financially by the National Nature Science Foundation of China (No. 31672422).

References

Beaujean, N., Hartshorne, G., Cavilla, J., Taylor, J., Gardner, J., Wilmut, I., Meehan, R. & Young, L. (2004). Non-conservation of mammalian preimplantation methylation dynamics. *Curr. Biol.* **14**, R266–7.

Bourc'His, D., Le Bourhis, D., Patin, D., Niveleau, A., Comizzoli, P., Renard, J.-P. & Viegas-Pequignot, E. (2001). Delayed and incomplete reprogramming of chromosome methylation patterns in bovine cloned embryos. *Curr. Biol.* **11**, 1542–6.

Chesné, P., Adenot, P.G., Viglietta, C., Baratte, M., Boulanger, L. & Renard, J.-P. (2002). Cloned rabbits produced by nuclear transfer from adult somatic cells. *Nat. Biotechnol.* **20**, 366–9.

Cho, J.-H., Kimura, H., Minami, T., Ohgane, J., Hattori, N., Tanaka, S. & Shiota, K. (2001). DNA methylation regulates placental lactogen I gene expression. *Endocrinology* **142**, 3389–96.

Cibelli, J.B., Campbell, K.H., Seidel, G.E., West, M.D. & Lanza, R.P. (2002). The health profile of cloned animals. *Nat. Biotechnol.* **20**, 13–4.

Cibelli, J.B., Stice, S.L., Golueke, P.J., Kane, J.J., Jerry, J., Blackwell, C., de Leon, F.A.P. & Robl, J.M. (1998). Cloned transgenic calves produced from nonquiescent fetal fibroblasts. *Science* **280**, 1256–8.

De Sousa, P.A., King, T., Harkness, L., Young, L.E., Walker, S.K. & Wilmut, I. (2001). Evaluation of gestational deficiencies in cloned sheep fetuses and placentae. *Biol. Reprod.* **65**, 23–30.

Dean, W., Santos, F., Stojkovic, M., Zakhartchenko, V., Walter, J., Wolf, E. & Reik, W. (2001). Conservation of methylation reprogramming in mammalian development: aberrant reprogramming in cloned embryos. *Proc. Natl. Acad. Sci.* **98**, 13734–8.

Farin, P.W., Piedrahita, J.A. & Farin, C.E. (2006). Errors in development of fetuses and placentas from *in vitro*-produced bovine embryos. *Theriogenology* **65**, 178–91.

Fuks, F., Hurd, P.J., Wolf, D., Nan, X., Bird, A.P. & Kouzarides, T. (2003). The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. *J. Biol. Chem.* **278**, 4035–40.

Gu, P., Le Menuet, D., Chung, A.C.-K. & Cooney, A.J. (2006). Differential recruitment of methylated CpG binding domains by the orphan receptor GCNF initiates the repression and silencing of Oct4 expression. *Mol. Cell. Biol.* **26**, 9471–83.

Igwebuike, U. (2009). A review of uterine structural modifications that influence conceptus implantation and development in sheep and goats. *Anim. Reprod. Sci.* **112**, 1–7.

Jaenisch, R. & Bird, A. (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat. Genet.* **33**, 245–54.

Jia, R., Zhou, Z., Zhang, G., Wang, L., Fan, Y., Wan, Y., Zhang, Y., Wang, Z. & Wang, F. (2016). Analysis of imprinted messenger RNA expression in deceased transgenic cloned goats. *Genet. Mol. Res.* **15**, doi: 10.4238/gmr.15017455.

Kato, Y., Tani, T., Sotomaru, Y., Kurokawa, K., Kato, J.-Y., Doguchi, H., Yasue, H. & Tsunoda, Y. (1998). Eight calves

https://doi.org/10.1017/S0967199417000284 Published online by Cambridge University Press

cloned from somatic cells of a single adult. *Science* **282**, 2095–8.

Klisch, K., Wooding, F. & Jones, C. (2010). The glycosylation pattern of secretory granules in binucleate trophoblast cells is highly conserved in ruminants. *Placenta* **31**, 11–7.

Klose, R.J. & Bird, A.P. (2006). Genomic DNA methylation: the mark and its mediators. *Trends Biochem. Sci.* **31**, 89–97.

Konyalı, A., Tölü, C., Daş, G. & Savaş, T. (2007). Factors affecting placental traits and relationships of placental traits with neonatal behaviour in goat. *Anim. Reprod. Sci.* **97**, 394–401.

Li, E., Bestor, T.H. & Jaenisch, R. (1992). Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* **69**, 915–26.

Li, L., Chen, B.F. & Chan, W.Y. (2015). An epigenetic regulator: methyl-CpG-binding domain protein 1 (MBD1). *Internatl. J. Mol. Sci.* **16**, 5125–40.

Mayer, W., Niveleau, A., Walter, J., Fundele, R. & Haaf, T. (2000). Embryogenesis: demethylation of the zygotic paternal genome. *Nature* **403**, 501–2.

Meng, L., Jia, R.-X., Sun, Y.-Y., Wang, Z.-Y., Wan, Y.-J., Zhang, Y.-L., Zhong, B.-S. & Wang, F. (2014). Growth regulation, imprinting, and epigenetic transcription-related gene expression differs in lung of deceased transgenic cloned and normal goats. *Theriogenology* **81**, 459–66.

Nan, X., Tate, P., Li, E. & Bird, A. (1996). DNA methylation specifies chromosomal localization of MeCP2. *Mol. Cell. Biol.* **16**, 414–21.

Osgerby, J., Gadd, T. & Wathes, D. (2003). The effects of maternal nutrition and body condition on placental and foetal growth in the ewe. *Placenta* **24**, 236–47.

Oswald, J., Engemann, S., Lane, N., Mayer, W., Olek, A., Fundele, R., Dean, W., Reik, W. & Walter, J. (2000). Active demethylation of the paternal genome in the mouse zygote. *Curr. Biol.* **10**, 475–8.

Penninga, L. & Longo, L. (1998). Ovine placentome morphology: effect of high altitude, long-term hypoxia. *Placenta* **19**, 187–93.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Res*. **29**, e45.

Polejaeva, I.A., Chen, S.-H., Vaught, T.D., Page, R.L., Mullins, J., Ball, S., Dai, Y., Boone, J., Walker, S. & Ayares, D.L. (2000). Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature* **407**, 86–90.

Ruddock-D'Cruz, N.T., Xue, J., Wilson, K.J., Heffernan, C., Prashadkumar, S., Cooney, M.A., Sanchez-Partida, L.G., French, A.J. & Holland, M.K. (2008). Dynamic changes in the localization of five members of the methyl binding domain (MBD) gene family during murine and bovine preimplantation embryo development. *Mol. Reprod. Dev.* **75**, 48–59.

Santos, F. & Dean, W. (2004). Epigenetic reprogramming during early development in mammals. *Reproduction* **127**, 643–51.

Shi, S.R., Key, M.E. & Kalra, K.L. (1991). Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. *J. Histochem. Cytochem.* **39**, 741–8.

Suemizu, H., Aiba, K., Yoshikawa, T., Sharov, A.A., Shimozawa, N., Tamaoki, N. & Ko, M.S. (2003). Expression profiling of placentomegaly associated with nuclear transplantation of mouse ES cells. *Dev. Biol.* **253**, 36–53.

Tate, P., Skarnes, W. & Bird, A. (1996). The methyl-CpG binding protein MeCP2 is essential for embryonic development in the mouse. *Nat. Genet.* **12**, 205–8.

Wade, P.A. (2001). Methyl CpG binding proteins: coupling chromatin architecture to gene regulation. *Oncogene* **20**, 3166–73.

Wakayama, T., Perry, A.C., Zuccotti, M., Johnson, K.R. & Yanagimachi, R. (1998). Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* **394**, 369–74.

Wan, Y.J., Zhang, Y.L., Zhou, Z.R., Jia, R.X., Li, M., Song, H., Wang, Z.Y., Wang, L.Z., Zhang, G.M., You, J.H. & Wang, F. (2012). Efficiency of donor cell preparation and recipient oocyte source for production of transgenic cloned dairy goats harboring human lactoferrin. *Theriogenology* **78**, 583–92.

Ward, J., Wooding, F. & Fowden, A. (2002). The effects of cortisol on the binucleate cell population in the ovine placenta during late gestation. *Placenta* **23**, 451–8.

Watanabe, S. & Nagai, T. (2009). Death losses due to stillbirth, neonatal death and diseases in cloned cattle derived from somatic cell nuclear transfer and their progeny: a result of nationwide survey in Japan. *Anim. Sci. J.* **80**, 233–8.

Watanabe, S. & Nagai, T. (2011). Survival of embryos and calves derived from somatic cell nuclear transfer in cattle: a nationwide survey in Japan. *Anim. Sci. J.* **82**, 360–5.

Wells, D.N., Misica, P.M., Day, T. & Tervit, H.R. (1997). Production of cloned lambs from an established embryonic cell line: a comparison between *in vivo*-and *in vitro*-matured cytoplasts. *Biol. Reprod.* **57**, 385–93.

Wells, D.N., Misica, P.M. & Tervit, H.R. (1999). Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. *Biol. Reprod.* **60**, 996–1005.

Wilmut, I., Schnieke, A., McWhir, J., Kind, A. & Campbell, K. (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature* **385**, 810–3. Reprinted in Nussbaum, M.C. & Sunstein, C.R. (1998). *Clones and Clones: Facts and Fantasies about Human Cloning*, pp. 21–8. New York & London: W.W. Norton.

Wooding, F., Morgan, G., Monaghan, S., Hamon, M. & Heap, R. (1996). Functional specialization in the ruminant placenta: evidence for two populations of fetal binucleate cells of different selective synthetic capacity. *Placenta* **17**, 75–86.

Wrenzycki, C., Wells, D., Herrmann, D., Miller, A., Oliver, J., Tervit, R. & Niemann, H. (2001). Nuclear transfer protocol affects messenger RNA expression patterns in cloned bovine blastocysts. *Biol. Reprod.* **65**, 309–17.

Yang, X., Smith, S.L., Tian, X.C., Lewin, H.A., Renard, J.P. & Wakayama, T. (2007). Nuclear reprogramming of cloned embryos and its implications for therapeutic cloning. *Nat. Genet.* **39**, 295–302.

https://doi.org/10.1017/S0967199417000284 Published online by Cambridge University Press