—Mini Review—

Animal Cloning: Reprogramming the Donor Genome

Seiya Takahashi[1]*

[1]*Department of Animal Breeding and Reproduction, National Institute of Livestock and Grassland Science, NARO, 2 Ikenodai, Tsukuba, Ibaraki 305-0901, Japan*

Introduction

Nuclear transfer (NT) involves transferring the nucleus from a diploid cell to an unfertilized egg from which the maternal nucleus has been removed. The NT technique involves several steps. The nucleus itself can be injected or the intact cell can be transferred into the oocyte. In the latter case, the oocyte and donor cell are normally fused and the reconstructed embryo activated by an electrical pulse. The reconstructed embryos are then cultured and those that appear to be developing normally are implanted into foster mothers. The NT technique was first used to clone sheep [1, 2] and cattle [3] by using cells taken directly from early embryos. In 1995, Campbell *et al.* [4] produced live lambs from embryo derived cells from a 'differentiated' cell line that had been cultured for several weeks. In 1996, Wilmut *et al.* [5] created Dolly, the first animal cloned from a cell taken from an adult animal. Since then, although somatic cloned animals have been produced in several species [6–28], success rates remain low in all species, with published data showing that only 1% to 5% of reconstructed embryos result in live births (see Roslin Institute web site [www.roslin.ac.uk/public/cloning.html]). Many cloned offspring die late in pregnancy or soon after birth [8, 29, 30], often from respiratory or cardiovascular dysfunction [31–33]. Abnormal placental development [34–41] is common and this is probably the major cause of fetal loss earlier in pregnancy [42]. Many of the cloned cattle and sheep that are born are much larger [29, 30, 43, 44] than normal and apparently normal clones may carry unrecognized abnormalities [45].

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*To whom correspondence should be addressed.
e-mail: seiya@affrc.go.jp

Differences Between Embryonic and Somatic Clones

Oocyte cytoplasmic factor(s) can reprogram either embryonic or somatic cell genome during cloning. It is clear from cloning experiments that an embryonic/somatic nucleus can be reprogrammed in the enucleated unfertilized egg and can form the entire body of an animal. When an embryonic cell (blastomere) is used as a donor nucleus source, either activated or unactivated ooplasm receiving a donor nucleus can develop to term as a result of nuclear transfer [46, 47]. But when a somatic nucleus is transferred into activated ooplasm, the reconstructed egg is arrested during several cell divisions, although unactivated ooplasm receiving a somatic nucleus can develop to term [29, 48]. It is suggested that an oocyte plasmic factor(s), which is essential for reprogramming the somatic genome, but not for an embryonic genome, exists in an MII oocyte, and the factor(s) or its activity dissipates several hours after oocyte activation.

The conception rate of somatic NT embryos is not much lower than that of embryonic NT embryos, but a greater number of fetal losses and abortions occur during the gestation of somatic clones than of embryonic clones [44]. Neonatal death and large offspring are also common in somatic clone calves, therefore reprogramming of the genome in somatic NT embryos is insufficient for fetal and placental development or neonatal maturation during gestation. Somatic NT embryos can develop to the blastocyst stage and the developed blastocysts can be successfully implanted. But incomplete or inappropriate reprogramming leads to dysregulation of gene expression and phenotypes [32, 36, 49–55]. Even if it is hypothesized that 'difficulty' in reprogramming the somatic nucleus is closely related to totipotency that dissipates with the cell differentiation process, no clear

proof has been established.

Epigenetic Modification of DNA in Cloned Embryo

Epigenetic modification of DNA by methylation in mammals occurs predominantly at CpG dinucleotides and is involved in a number of key genome functions. These include roles in imprinting, X chromosome inactivation, genome stability, silencing of endogenous retrovirus, inactivation of genes related to cancers, and embryonic development [56]. Normal embryo development is dependent on the methylation state of the DNA contributed by the sperm and egg and on the appropriate reconfiguration of the chromatin structure after fertilization.

Genome-wide reprogramming of DNA methylation occurs in two steps: the first during germline development, when DNA methylation imprints are erased [57, 58], and the second during fertilization and preimplantation development [59–61]. Fertilization of sperm and oocyte achieve paternal genome introduction into the oocyte, where the oocyte cytoplasm quickly replaces protamines in sperm chromatin with oocyte histones, and active demethylation of paternal DNA (not of maternal DNA, asymmetric methylation) occurs before the onset of the first DNA replication [62]. Active demethylation is displayed in most mammalian species (mouse, rat, pig, and cattle) [63], with the exception of sheep [33, 64] and rabbits [65]. Passive demethylation of the maternal genome is observed in preimplantation embryos [60, 61]. Genome-wide de novo methylation (remethylation) occurs at the blastocyst stage in mouse (at 8- to 16-cell stage in cattle), preferentially in the inner cell mass [61, 63]. In marked contrast to the mouse, male pronuclear demethylation does not occur in sheep and rabbit embryos [64, 65].

In somatic cloning, however, the donor cell genome is compelled to bypass gametogenesis. Somatic cells have a very different chromatin structure from that of sperm and 'reprogramming' of the transferred nuclei must occur in the reconstructed embryos in order to achieve totipotency. Nevertheless the extent to which epigenetic reprogramming is conserved in cloned embryo development is unknown, and inefficient reprogramming of DNA methylation patterns may be partly responsible for the low birth rates and developmental abnormalities that often result from nuclear transfer [66]. As well as investigating the conservation of methylation reprogramming events in early mammalian development, Dean *et al.* [63] analyzed demethylation events in cloned bovine embryos. Interestingly, the initial demethylation event appears to be conserved in cloned 1 cell embryos, with some loss of methylation in the somatic donor nucleus occurring shortly after fusion with the enucleated oocyte. Further demethylation is observed at the 2 cell stage, but spurious *de novo* methylation is found in many 4–8 cell stage embryos. The observations of Dean *et al.* [63] show that methylation reprogramming in somatic nuclei is partially conserved in cloned embryos, with different extents of inappropriate methylation in individual embryos. But more detailed information on which sequences are aberrantly methylated in cloned embryos is required to understand the observed phenotypes and low success rates of cloning [67]. Another study reports a change in nuclear methylation in early stage somatic NT embryos. With the 5-mC antibody, Bourc'his *et al.* [68] reported that bovine cloned embryos fail to reproduce distinguishable paternal-chromosome methylation after fusion and to maintain their somatic pattern during subsequent stages. They were able to observe the methylation status of euchromatic and heterochromatic sequences in IVF and cloned bovine preimplantation embryos [68]. Compared to their normal counterparts, cloned bovine blastocysts show increased levels of methylation in centromeric heterochromatin, but comparable levels in euchromatin. In agreement with Dean *et al.* [69], this study found inefficient passive demethylation during the initial embryonic cell divisions. In contrast however, Bourc'his *et al.* [68] could find no evidence for the active demethylation of somatic nuclei after fusion. This seems to conflict with the two previous reports; the former report shows that active demethylation occurs in somatic NT embryos, but not in the latter report, although the same anti-5-mC antibody is used in both studies. In fact, differences may result from the immunostaining procedure, intensity comparison and/or microscopic resolution [70, 71]. Passive demethylation occurs during the cleavage stage in normal embryos, leading to global hypomethylation until implantation. After passive demethylation, *de novo* DNA methylation occurs in normal blastocysts [61]. Bisulphite mutagenesis of bovine embryos shows that heterochromatic Bov-B LINE (long interspersed repeat sequence) and unique sequences in epidermal cytokeratin and beta-lactoglobulin are normally demethylated and remain that way until the blastocyst stage [72, 73]. In bovine cloned blastocysts, the Bov-B LINE and satellite I sequence—a major sequence

consisting of centrometric heterochromatin—are abnormally methylated. In addition, individual variation in the DNA methylation pattern is observed among different cloned blastocysts [72]. The same method demonstrates that differential demethylation events take place between tissue-specific gene (cytokeratin and beta-lactoglobulin) sequences and the satellite I DNA sequences, and also inner cell mass and trophoectoderm in cloned blastocysts [73]. Surprisingly, cloned porcine embryos do not appear to show highly aberrant methylation patterns, with cloned blastocysts retaining similar methylation levels to their normal counterparts in centromeric satellite and PRE-1 SINE sequences [74]. Overall, these studies present a picture of partial, but incomplete methylation reprogramming in cloned embryos. The details of the reprogramming failure differ among the three studies. The reasons for this are not entirely clear, but may lie in the methods—donor cell type, evidence of serum starvation, the oocyte source, and embryo culture conditions [75]—used in the different studies [67].

Santos *et al.* [76] examined histon H3 lysin 9 (H3-K9) methylation and acetylation, because links between DNA methylation and H3-K9 methylation have been established in other organisms [77–83]. They found that the majority of cloned embryos exhibit H3-K9 hypermethylation and hyperacetylation associated with DNA hypermethylation, although H3-K9 methylation and acetylation are reprogrammed in parallel with DNA methylation in normal bovine embryos [76].

Can Cloning Efficiency Be Improved?

Aberrant epigenetic profiles in NT embryos and cloned fetuses/offspring [84, 85] suggest that most somatic nuclei fail to be reprogrammed [70, 86]. It is clear that gene expression patterns and phenotypes can be disrupted in both somatic and extra-embryonic tissues of cloned animals [36, 87–92]. At present, to improve the success rate of normal offspring production, the only way to assess the 'quality' of embryos is to view them under a microscope and it is clear that the majority of embryos classified as 'normal' do not develop properly after implantation. If only 'healthy' embryos are selected from a number of junk embryos based on gene expression profiles etc., it may be a great tool to improve cloned animal production. A substantial effort is being made to use technological advances in genomics to screen the expression patterns of genes to identify differences between the development of cloned embryos, fetus, and offspring and those produced by *in vivo* or *in vitro* fertilization [39, 55, 93, 94]. Several studies with mouse clones show abnormal expression of Oct4 in the blastocysts [89], and imprinted genes and other non-imprinted genes in later stages [32, 36, 49–55]. Boiani *et al.* [95] reported that aggregated mouse cloned embryos express Oct4 normally and have higher rates of fetal and postnatal development. They propose that the regulation of gene expression and, possibly, reprogramming of nuclei in clones has a non-cell-autonomous component that can be complemented by clone-clone embryo aggregation [95].

Another possibility is to identify systematic ways of improving reprogramming in cloned embryos. Sullivan *et al.* [96] have developed a novel system for remodeling mammalian somatic nuclei *in vitro* prior to cloning. The system involves permeabilization of the donor cells and treatment in a mitotic cell extract to condense somatic nuclei. The condensed chromosomes are transferred into enucleated oocytes prior to activation. Cloned calves have been produced by this new method [96]. Santos *et al.* [76] reported that the precise epigenotype in cloned embryos depends on the donor cell type, and the proportion of embryos with normal epigenotypes correlates with the proportion of embryos developing to the blastocyst stage. This suggests that it may be possible through characterization of a donor population and the assessment of NT embryos at the blastocyst stage to begin to test manipulations of donor cells that in turn could improve cloning efficiency [76]. As a manipulation of donor cell epigenotype, pre-treatment of donor cells with trichostain A (TSA, a histone deacetylase inhibitor) or 5-aza-2'-deoxycytidine (5-aza-dC, a DNA methyltransferase inhibitor) was examined [97]. Adult fibroblast cells treated with TSA have elevated histone H3 acetylation compared with untreated controls. Cells treated with 5-aza-dC have decreased methylation. Development to blastocysts for embryos cloned from donor cells after 5-aza-dC treatment is lower than in untreated controls, whereas TSA treatment of donor cells increased blastocyst development compared to controls. The results indicate that partial erasure of preexisting epigenetic marks of donor cells improves subsequent *in vitro* development of cloned embryos [97].

Outlook for Applicability of Animal Cloning

Some of the more utilitarian opportunities provided by animal cloning are the ability to expand populations of

valuable, high-quality or endangered animals [19, 98]. The other opportunities provided by clone technology combined with gene engineering are production of transgenic animals producing valuable recombinant proteins [31, 99] and animals for xeno-transplantation [100–103]. Whether it is feasible to produce large numbers of clones, all of which express the same desirable phenotype (performance) as the nuclear donor, remains to be determined [104]. Cloning results to date reveal that, while many cloned offspring appear outwardly normal and healthy [105, 106], in many cases cloned animals suffer from epigenetic abnormalities [45], which may affect the degree to which desirable characteristics are obtained. Also, epigenetic abnormalities may affect the risk of endogenous retroviruses [107], carcinogenesis, and the instability of genomes or transgenes in cloned animals. It has been seen that cloning failures are 'epigenetic' in the mouse. The obese phenotype frequently observed in cloned mice is not transmitted to offspring generated by mating male clone and female clone mice [108], indicating that failures associated with cloning are epigenetic and not inheritable. This is, in fact, 'good news' to use progenies from clones for genetic applications.

Some questions persist: Why is cloning inefficient? How can cloning efficiency be improved? What is genome reprogramming? These are all important questions that must be addressed with NT technology, approaching valuable applications and developmental biology.

References

1) Willadsen, S.M. (1986): Nuclear transplantaion in sheep embryos. Nature, 320, 63–65.
2) Willadsen, S.M. (1989): Cloning of sheep and cow embryos. Genome, 31, 956–962.
3) Prather, R., Barnes, F.L., Sims, M.M., Robl, J.M., Eyestone, W.H. and First, N.L. (1987): Nuclear transplantation in the bovine embryo: assessment of donor nuclei and recipient oocytes. Biology of Reproduction, 37, 859–866.
4) Campbell, K.H., McWhir, J., Ritchie, W.A. and Wilmut, I. (1996): Sheep cloned by nuclear transfer from a cultured cell line. Nature, 380, 64–66.
5) Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J. and Campbell, K.H. (1997): Viable offspring derived from fetal and adult mammalian cells. Nature, 385, 810–813.
6) Wakayama, T., Rodriguez, I., Perry, A.C., Yanagimachi, R. and Mombaerts, P. (1999): Mice cloned from embryonic stem cells. Proc. Natl. Acad. Sci. USA., 96, 14984–14989.
7) Cibelli, J.B., Stice, S.L., Golueke, P.J., Kane, J.J., Jerry, J., Blackwell, C., Ponce de Leon, F.A. and Robl, J.M. (1998): Cloned transgenic calves produced from nonquiescent fetal fibroblasts. Science, 280, 1256–1258.
8) Kato, Y., Tani, T., Sotomaru, Y., Kurokawa, K., Kato, J., Doguchi, H., Yasue, H. and Tsunoda, Y. (1998): Eight calves cloned from somatic cells of a single adult. Science, 282, 2095–2098.
9) Wells, D.N., Misica, P.M. and Tervit, H.R. (1999): Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. Biol. Reprod., 60, 996–1005.
10) Kato, Y., Tani, T. and Tsunoda, Y. (2000): Cloning of calves from various somatic cell types of male and female adult, newborn and fetal cows. J. Reprod. Fertil., 120, 231–237.
11) Baguisi, A., Behboodi, E., Melican, D.T., Pollock, J.S., Destrempes, M.M., Cammuso, C., Williams, J.L., Nims, S.D., Porter, C.A., Midura, P., Palacios, M.J., Ayres, S.L., Denniston, R.S., Hayes, M.L., Ziomek, C.A., Meade, H.M., Godke, R.A., Gavin, W.G., Overstrom, E.W. and Echelard, Y. (1999): Production of goats by somatic cell nuclear transfer. Nat. Biotechnol., 17, 456–461.
12) Hill, J.R., Winger, Q.A., Long, C.R., Looney, C.R., Thompson, J.A. and Westhusin, M.E. (2000): Development rates of male bovine nuclear transfer embryos derived from adult and fetal cells. Biol. Reprod., 62, 1135–1140.
13) Kubota, C., Yamakuchi, H., Todoroki, J., Mizoshita, K., Tabara, N., Barber, M. and Yang, X. (2000): Six cloned calves produced from adult fibroblast cells after long-term culture. Proc. Natl. Acad. Sci. USA., 97, 990–995.
14) Ogura, A., Inoue, K., Ogonuki, N., Noguchi, A., Takano, K., Nagano, R., Suzuki, O., Lee, J., Ishino, F. and Matsuda, J. (2000): Production of male cloned mice from fresh, cultured, and cryopreserved immature sertoli cells. Biol. Reprod., 62, 1579–1584.
15) Ogura, A., Inoue, K., Takano, K., Wakayama, T. and Yanagimachi, R. (2000): Birth of mice after nuclear transfer by electrofusion using tail tip cells. Mol. Reprod. Dev., 57, 55–59.
16) 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., Colman, A. and Campbell, K.H. (2000): Cloned pigs produced by nuclear transfer from adult somatic cells. Nature, 407, 86–90.
17) Onishi, A., Iwamoto, M., Akita, T., Mikawa, S., Takeda, K., Awata, T., Hanada, H. and Perry, A.C. (2000): Pig cloning by microinjection of fetal fibroblast nuclei. Science, 289, 1188–1190.
18) Park, K.W., Kuhholzer, B., Lai, L., Machaty, Z., Sun, Q.Y., Day, B.N. and Prather, R.S. (2001): Development and expression of the green fluorescent protein in porcine embryos derived from nuclear transfer of transgenic granulosa-derived cells. Anim. Reprod. Sci., 68, 111–120.
19) Loi, P., Ptak, G., Barboni, B., Fulka, J. Jr., Cappai, P. and Clinton, M. (2001): Genetic rescue of an endangered mammal by cross-species nuclear transfer using post-mortem somatic cells. Nat. Biotechnol., 19, 962–964.
20) Zou, X., Chen, Y., Wang, Y., Luo, J., Zhang, Q., Zhang,

X., Yang, Y., Ju, H., Shen, Y., Lao, W., Xu, S. and Du, M. (2001): Production of cloned goats from enucleated oocytes injected with cumulus cell nuclei or fused with cumulus cells. Cloning, 3, 31–37.
21) Keefer, C.L., Keyston, R., Lazaris, A., Bhatia, B., Begin, I., Bilodeau, A.S., Zhou, F.J., Kafidi, N., Wang, B., Baldassarre, H. and Karatzas, C.N. (2002): Production of cloned goats after nuclear transfer using adult somatic cells. Biol. Reprod., 66, 199–203.
22) Shin, T., Kraemer, D., Pryor, J., Liu, L., Rugila, J., Howe, L., Buck, S., Murphy, K., Lyons, L. and Westhusin, M. (2002): A cat cloned by nuclear transplantation. Nature, 415, 859.
23) Chesne, P., Adenot, P.G., Viglietta, C., Baratte, M., Boulanger, L. and Renard, J.P. (2002): Cloned rabbits produced by nuclear transfer from adult somatic cells. Nat. Biotechnol, 20, 366–369.
24) Yin, X.J., Tani, T., Yonemura, I., Kawakami, M., Miyamoto, K., Hasegawa, R., Kato, Y. and Tsunoda, Y. (2002): Production of cloned pigs from adult somatic cells by chemically assisted removal of maternal chromosomes. Biol. Reprod., 67, 442–446.
25) Ohkoshi, K., Takahashi, S., Koyama, S., Akagi, S., Adachi, N., Furusawa, T., Fujimoto, J., Takeda, K., Kubo, M., Izaike, Y. and Tokunaga, T. (2003): In vitro oocyte culture and somatic cell nuclear transfer used to produce a live-born cloned goat. Cloning Stem Cells, 5, 109–115.
26) Zhou, Q., Renard, J.P., Le Friec, G., Brochard, V., Beaujean, N., Cherifi, Y., Fraichard, A. and Cozzi, J. (2003): Generation of fertile cloned rats by regulating oocyte activation. Science, 302, 1179.
27) Galli, C., Lagutina, I., Crotti, G., Colleoni, S., Turini, P., Ponderato, N., Duchi, R. and Lazzari, G. (2003): Pregnancy: a cloned horse born to its dam twin. Nature, 424, 635.
28) Woods, G.L., White, K.L., Vanderwall, D.K., Li, G.P., Aston, K.I., Bunch, T.D., Meerdo, L.N. and Pate, B.J. (2003): A mule cloned from fetal cells by nuclear transfer. Science, 301, 1063.
29) Takahashi, S., Kubota, C., Shimizu, M., Tabara, N., Izaike, Y. and Imai, H. (2000): Production of cloned calves by somatic cell-nuclear transplantation. In: Cloned animal and placentation (Roberts, R.M., Yanagimachi, R., Kariya, T. and Hashizume, K. eds.), pp. 30–35, Yokendo Ltd., Tokyo.
30) Akagi, S., Adachi, N., Matsukawa, K., Kubo, M. and Takahashi, S. (2003): Developmental potential of bovine nuclear transfer embryos and postnatal survival rate of cloned calves produced by two different timings of fusion and activation. Mol. Reprod. Dev., 66, 264–272.
31) Schnieke, A.E., Kind, A.J., Ritchie, W.A., Mycock, K., Scott, A.R., Ritchie, M., Wilmut, I., Colman, A. and Campbell, K.H. (1997): Human factor ix transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science, 278, 2130–2133.
32) Hill, J.R., Roussel, A.J., Cibelli, J.B., Edwards, J.F., Hooper, N.L., Miller, M.W., Thompson, J.A., Looney, C.R., Westhusin, M.E., Robl, J.M. and Stice, S.L. (1999): Clinical and pathologic features of cloned transgenic calves and fetuses (13 case studies). Theriogenology, 51, 1451–1465.
33) Wilmut, I. and Paterson, L. (2003): Somatic cell nuclear transfer. Oncol. Res., 13, 303–307.
34) Wakayama, T. and Yanagimachi, R. (1999): Cloning of male mice from adult tail-tip cells. Nat. Genet., 22, 127–128.
35) Eggan, K., Akutsu, H., Loring, J., Jackson-Grusby, L., Klemm, M., Rideout, W.M. 3rd., Yanagimachi, R. and Jaenisch, R. (2001): Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. Proc. Natl. Acad. Sci. USA., 98, 6209–6214.
36) Inoue, K., Kohda, T., Lee, J., Ogonuki, N., Mochida, K., Noguchi, Y., Tanemura, K., Kaneko-Ishino, T., Ishino, F. and Ogura, A. (2002): Faithful expression of imprinted genes in cloned mice. Science, 295, 297.
37) Clark, A.J., Ferrier, P., Aslam, S., Burl, S., Denning, C., Wylie, D., Ross, A., de Sousa, P., Wilmut, I. and Cui, W. (2003): Proliferative lifespan is conserved after nuclear transfer. Nat. Cell Biol., 5, 535–538.
38) De Sousa, P.A., King, T., Harkness, L., Young, L.E., Walker, S.K. and Wilmut, I. (2001): Evaluation of gestational deficiencies in cloned sheep fetuses and placentae. Biol. Reprod., 65, 23–30.
39) Hashizume, K., Ishiwata, H., Kizaki, K., Yamada, O., Takahashi, T., Imai, K., Patel, O.V., Akagi, S., Shimizu, M., Takahashi, S., Katsuma, S., Shiojima, S., Hirasawa, A., Tsujimoto, G., Todoroki, J. and Izaike, Y. (2002): Implantation and placental development in somatic cell clone recipient cows. Cloning Stem Cells, 4, 197–209.
40) Lee, R.S., Peterson, A.J., Donnison, M.J., Ravelich, S., Ledgard, A.M., Li, N., Oliver, J.E., Miller, A.L., Tucker, F.C., Breier, B. and Wells, D.N. (2004): Cloned cattle fetuses with the same nuclear genetics are more variable than contemporary half-siblings resulting from artificial insemination and exhibit fetal and placental growth deregulation even in the first trimester. Biol. Reprod., 70, 1–11.
41) Ravelich, S.R., Breier, B.H., Reddy, S., Keelan, J.A., Wells, D.N., Peterson, A.J. and Lee, R.S. (2004): Insulin-like growth factor-i and binding proteins 1, 2, and 3 in bovine nuclear transfer pregnancies. Biol. Reprod., 70, 430–438.
42) Hill, J.R., Burghardt, R.C., Jones, K., Long, C.R., Looney, C.R., Shin, T., Spencer, T.E., Thompson, J.A., Winger, Q.A. and Westhusin, M.E. (2000): Evidence for placental abnormality as the major cause of mortality in first-trimester somatic cell cloned bovine fetuses. Biol. Reprod., 63, 1787–1794.
43) Lazzari, G., Wrenzycki, C., Herrmann, D., Duchi, R., Kruip, T., Niemann, H. and Galli, C. (2002): Cellular and molecular deviations in bovine in vitro-produced embryos are related to the large offspring syndrome. Biol. Reprod., 67, 767–775.
44) Heyman, Y., Chavatte-Palmer, P., LeBourhis, D., Camous,

S., Vignon, X. and Renard, J.P. (2002): Frequency and occurrence of late-gestation losses from cattle cloned embryos. Biol. Reprod., 66, 6–13.
45) Latham, K.E. (2004): Cloning: questions answered and unsolved. Differentiation, 72, 11–22.
46) Campbell, K.H., Loi, P., Cappai, P. and Wilmut, I. (1994): Improved development to blastocyst of ovine nuclear transfer embryos reconstructed during the presumptive s-phase of enucleated activated oocytes. Biol. Reprod., 50, 1385–1393.
47) Kono, T., Sotomaru, Y., Aono, F., Takahashi, T., Ogiwara, I., Sekizawa, F., Arai, T. and Nakahara, T. (1994): Effect of ooplast activation on the development of oocytes following nucleus transfer in cattle. Theriogenology, 41, 1463–1471.
48) Shiga, K., Fujita, T., Hirose, K., Sasae, Y. and Nagai, T. (1999): Production of calves by transfer of nuclei from cultured somatic cells obtained from Japanese black bulls. Theriogenology, 52, 527–535.
49) Eggan, K., Akutsu, H., Hochedlinger, K., Rideout, W. 3rd, Yanagimachi, R. and Jaenisch, R. (2000): X-chromosome inactivation in cloned mouse embryos. Science, 290, 1578–1581.
50) Humpherys, D., Eggan, K., Akutsu, H., Friedman, A., Hochedlinger, K., Yanagimachi, R., Lander, E.S., Golub, T.R. and Jaenisch, R. (2002): Abnormal gene expression in cloned mice derived from embryonic stem cell and cumulus cell nuclei. Proc. Natl. Acad. Sci. USA., 99, 12889–12894.
51) Park, K.W., Lai, L., Cheong, H.T., Cabot, R., Sun, Q.Y., Wu, G., Rucker, E.B., Durtschi, D., Bonk, A., Samuel, M., Rieke, A., Day, B.N., Murphy, C.N., Carter, D.B. and Prather, R.S. (2002): Mosaic gene expression in nuclear transfer-derived embryos and the production of cloned transgenic pigs from ear-derived fibroblasts. Biol. Reprod., 66, 1001–1005.
52) Xue, F., Tian, X.C., Du, F., Kubota, C., Taneja, M., Dinnyes, A., Dai, Y., Levine, H., Pereira, L.V. and Yang, X. (2002): Aberrant patterns of x chromosome inactivation in bovine clones. Nat. Genet., 31, 216–220.
53) Bortvin, A., Eggan, K., Skaletsky, H., Akutsu, H., Berry, D.L., Yanagimachi, R., Page, D.C. and Jaenisch, R. (2003): Incomplete reactivation of oct4-related genes in mouse embryos cloned from somatic nuclei. Development, 130, 1673–1680.
54) Wrenzycki, C., Wells, D., Herrmann, D., Miller, A., Oliver, J., Tervit, R. and Niemann, H. (2001): Nuclear transfer protocol affects messenger rna expression patterns in cloned bovine blastocysts. Biol. Reprod., 65, 309–317.
55) Wrenzycki, C., Herrmann, D., Lucas-Hahn, A., Lemme, E., Korsawe, K. and Niemann, H. (2004): Gene expression patterns in in vitro-produced and somatic nuclear transfer-derived preimplantation bovine embryos: relationship to the large offspring syndrome? Anim. Reprod. Sci., 82–83, 593–603.
56) Bird, A. (2002): DNA methylation patterns and epigenetic memory. Genes Dev., 16, 6–21.
57) Lee, J., Inoue, K., Ono, R., Ogonuki, N., Kohda, T., Kaneko-Ishino, T., Ogura, A. and Ishino, F. (2002): Erasing genomic imprinting memory in mouse clone embryos produced from day 11.5 primordial germ cells. Development, 129, 1807–1817.
58) Hajkova, P., Erhardt, S., Lane, N., Haaf, T., El-Maarri, O., Reik, W., Walter, J. and Surani, M.A. (2002): Epigenetic reprogramming in mouse primordial germ cells. Mech. Dev., 117, 15–23.
59) Rougier, N., Bourc'his, D., Gomes, D.M., Niveleau, A., Plachot, M., Paldi, A. and Viegas-Pequignot, E. (1998): Chromosome methylation patterns during mammalian preimplantation development. Genes Dev., 12, 2108–2113.
60) Oswald, J., Engemann, S., Lane, N., Mayer, W., Olek, A., Fundele, R., Dean, W., Reik, W. and Walter, J. (2000): Active demethylation of the paternal genome in the mouse zygote. Curr. Biol., 10, 475–478.
61) Santos, F., Hendrich, B., Reik, W. and Dean, W. (2002): Dynamic reprogramming of DNA methylation in the early mouse embryo. Dev. Biol., 241, 172–182.
62) Mayer, W., Niveleau, A., Walter, J., Fundele, R. and Haaf, T. (2000): Demethylation of the zygotic paternal genome. Nature, 403, 501–502.
63) Dean, W., Santos, F., Stojkovic, M., Zakhartchenko, V., Walter, J., Wolf, E. and Reik, W. (2001): Conservation of methylation reprogramming in mammalian development: aberrant reprogramming in cloned embryos. Proc. Natl. Acad. Sci. USA., 98, 13734–13738.
64) Beaujean, N., Hartshorne, G., Cavilla, J., Taylor, J., Gardner, J., Wilmut, I., Meehan, R. and Young, L. (2004): Non-conservation of mammalian preimplantation methylation dynamics. Curr. Biol., 14, R266–267.
65) Shi, W., Dirim, F., Wolf, E., Zakhartchenko, V. and Haaf, T. (2004): Methylation reprogramming and chromosomal aneuploidy in in vivo fertilized and cloned rabbit preimplantation embryos. Biol. Reprod., 71, 340–347.
66) Young, L.E. and Fairburn, H.R. (2000): Improving the safety of embryo technologies: possible role of genomic imprinting. Theriogenology, 53, 627–648.
67) Fairburn, H.R., Young, L.E. and Hendrich, B.D. (2002): Epigenetic reprogramming: how now, cloned cow? Curr. Biol., 12, R68–70.
68) Bourc'his, D., Le Bourhis, D., Patin, D., Niveleau, A., Comizzoli, P., Renard, J.P. and Viegas-Pequignot, E. (2001): Delayed and incomplete reprogramming of chromosome methylation patterns in bovine cloned embryos. Curr. Biol., 11, 1542–1546.
69) Dean, W.L., Kelsey, G. and Reik, W. (2001): Generation of monoparental embryos for investigation into genomic imprinting. Methods Mol. Biol., 181, 1–19.
70) Kang, Y.K., Lee, K.K. and Han, Y.M. (2003): Reprogramming DNA methylation in the preimplantation stage: peeping with dolly's eyes. Curr. Opin. Cell Biol., 15, 290–295.
71) Young, L.E. and Beaujean, N. (2004): DNA methylation in the preimplantation embryo: the differing stories of the mouse and sheep. Anim. Reprod. Sci., 82–83, 61–78.
72) Kang, Y.K., Koo, D.B., Park, J.S., Choi, Y.H., Chung,

A.S., Lee, K.K. and Han, Y.M. (2001): Aberrant methylation of donor genome in cloned bovine embryos. Nat. Genet., 28, 173–177.
73) Kang, Y.K., Park, J.S., Koo, D.B., Choi, Y.H., Kim, S.U., Lee, K.K. and Han, Y.M. (2002): Limited demethylation leaves mosaic-type methylation states in cloned bovine pre-implantation embryos. EMBO J., 21, 1092–1100.
74) Kang, Y.K., Koo, D.B., Park, J.S., Choi, Y.H., Kim, H.N., Chang, W.K., Lee, K.K. and Han, Y.M. (2001): Typical demethylation events in cloned pig embryos. Clues on species-specific differences in epigenetic reprogramming of a cloned donor genome. J. Biol. Chem., 276, 39980–39984.
75) Young, L.E., Fernandes, K., McEvoy, T.G., Butterwith, S.C., Gutierrez, C.G., Carolan, C., Broadbent, P.J., Robinson, J.J., Wilmut, I. and Sinclair, K.D. (2001): Epigenetic change in igf2r is associated with fetal overgrowth after sheep embryo culture. Nat. Genet., 27, 153–154.
76) Santos, F., Zakhartchenko, V., Stojkovic, M., Peters, A., Jenuwein, T., Wolf, E., Reik, W. and Dean, W. (2003): Epigenetic marking correlates with developmental potential in cloned bovine preimplantation embryos. Curr. Biol., 13, 1116–1121.
77) Tamaru, H. and Selker, E.U. (2001): A histone h3 methyltransferase controls DNA methylation in neurospora crassa. Nature, 414, 277–283.
78) Jackson, J.P., Lindroth, A.M., Cao, X. and Jacobsen, S.E. (2002): Control of cpnpg DNA methylation by the kryptonite histone h3 methyltransferase. Nature, 416, 556–560.
79) Fuks, F., Hurd, P.J., Deplus, R. and Kouzarides, T. (2003): The DNA methyltransferases associate with hp1 and the suv39h1 histone methyltransferase. Nucleic Acids Res., 31, 2305–2312.
80) Silva, J., Mak, W., Zvetkova, I., Appanah, R., Nesterova, T.B., Webster, Z., Peters, A.H., Jenuwein, T., Otte, A.P. and Brockdorff, N. (2003): Establishment of histone h3 methylation on the inactive x chromosome requires transient recruitment of eed-enx1 polycomb group complexes. Dev. Cell, 4, 481–495.
81) Dodge, J.E., Kang, Y.K., Beppu, H., Lei, H. and Li, E. (2004): Histone h3-k9 methyltransferase eset is essential for early development. Mol. Cell Biol., 24, 2478–2486.
82) Espada, J., Ballestar, E., Fraga, M.F., Villar-Garea, A., Juarranz, A., Stockert, J.C., Robertson, K.D., Fuks, F. and Esteller, M. (2004): Human DNA methyltransferase 1 is required for maintenance of the histone h3 modification pattern. J. Biol. Chem., 279, 37175–37184.
83) He, H. and Lehming, N. (2003): Global effects of histone modifications. Brief Funct. Genomic Proteomic, 2, 234–243.
84) Ohgane, J., Wakayama, T., Kogo, Y., Senda, S., Hattori, N., Tanaka, S., Yanagimachi, R. and Shiota, K. (2001): DNA methylation variation in cloned mice. Genesis, 30, 45–50.
85) Cezar, G.G., Bartolomei, M.S., Forsberg, E.J., First, N.L., Bishop, M.D. and Eilertsen, K.J. (2003): Genome-wide epigenetic alterations in cloned bovine fetuses. Biol. Reprod., 68, 1009–1014.
86) Mann, M.R., Chung, Y.G., Nolen, L.D., Verona, R.I., Latham, K.E. and Bartolomei, M.S. (2003): Disruption of imprinted gene methylation and expression in cloned preimplantation stage mouse embryos. Biol. Reprod., 69, 902–914.
87) Daniels, R., Hall, V.J., French, A.J., Korfiatis, N.A. and Trounson, A.O. (2001): Comparison of gene transcription in cloned bovine embryos produced by different nuclear transfer techniques. Mol. Reprod. Dev., 60, 281–288.
88) Daniels, R., Hall, V. and Trounson, A.O. (2000): Analysis of gene transcription in bovine nuclear transfer embryos reconstructed with granulosa cell nuclei. Biol. Reprod., 63, 1034–1040.
89) Boiani, M., Eckardt, S., Scholer, H.R. and McLaughlin, K.J. (2002): Oct4 distribution and level in mouse clones: consequences for pluripotency. Genes Dev., 16, 1209–1219.
90) Niemann, H., Wrenzycki, C., Lucas-Hahn, A., Brambrink, T., Kues, W.A. and Carnwath, J.W. (2002): Gene expression patterns in bovine in vitro-produced and nuclear transfer-derived embryos and their implications for early development. Cloning Stem Cells, 4, 29–38.
91) Chung, Y.G., Ratnam, S., Chaillet, J.R. and Latham, K.E. (2003): Abnormal regulation of DNA methyltransferase expression in cloned mouse embryos. Biol. Reprod., 69, 146–153.
92) Ohgane, J., Wakayama, T., Senda, S., Yamazaki, Y., Inoue, K., Ogura, A., Marh, J., Tanaka, S., Yanagimachi, R. and Shiota, K. (2004): The sall3 locus is an epigenetic hotspot of aberrant DNA methylation associated with placentomegaly of cloned mice. Genes Cells, 9, 253–260.
93) Dindot, S.V., Farin, P.W., Farin, C.E., Romano, J., Walker, S., Long, C. and Piedrahita, J.A. (2004): Epigenetic and genomic imprinting analysis in nuclear transfer derived bos gaurus/bos taurus hybrid fetuses. Biol. Reprod., 71, 470–478.
94) Singh, U., Fohn, L.E., Wakayama, T., Ohgane, J., Steinhoff, C., Lipkowitz, B., Schulz, R., Orth, A., Ropers, H.H., Behringer, R.R., Tanaka, S., Shiota, K., Yanagimachi, R., Nuber, U.A. and Fundele, R. (2004): Different molecular mechanisms underlie placental overgrowth phenotypes caused by interspecies hybridization, cloning, and esx1 mutation. Dev. Dyn., 230, 149–164.
95) Boiani, M., Eckardt, S., Leu, N.A., Scholer, H.R. and McLaughlin, K.J. (2003): Pluripotency deficit in clones overcome by clone-clone aggregation: epigenetic complementation? EMBO J., 22, 5304–5312.
96) Sullivan, E.J., Kasinathan, S., Kasinathan, P., Robl, J.M. and Collas, P. (2004): Cloned calves from chromatin remodeled in vitro. Biol. Reprod., 70, 146–153.
97) Enright, B.P., Kubota, C., Yang, X. and Tian, X.C. (2003): Epigenetic characteristics and development of embryos cloned from donor cells treated by trichostatin a or 5-aza-

2'-deoxycytidine. Biol. Reprod., 69, 896–901.
98) Wells, D.N., Misica, P.M., Tervit, H.R. and Vivanco, W.H. (1998): Adult somatic cell nuclear transfer is used to preserve the last surviving cow of the enderby island cattle breed. Reprod. Fertil. Dev., 10, 369–378.
99) Polejaeva, I.A. and Campbell, K.H. (2000): New advances in somatic cell nuclear transfer: application in transgenesis. Theriogenology, 53, 117–126.
100) Phelps, C.J., Koike, C., Vaught, T.D., Boone, J., Wells, K.D., Chen, S.H., Ball, S., Specht, S.M., Polejaeva, I.A., Monahan, J.A., Jobst, P.M., Sharma, S.B., Lamborn, A.E., Garst, A.S., Moore, M., Demetris, A.J., Rudert, W.A., Bottino, R., Bertera, S., Trucco, M., Starzl, T.E., Dai, Y. and Ayares, D.L. (2003): Production of alpha 1,3-galactosyltransferase-deficient pigs. Science, 299, 411–414.
101) Dai, Y., Vaught, T.D., Boone, J., Chen, S.H., Phelps, C.J., Ball, S., Monahan, J.A., Jobst, P.M., McCreath, K.J., Lamborn, A.E., Cowell-Lucero, J.L., Wells, K.D., Colman, A., Polejaeva, I.A. and Ayares, D.L. (2002): Targeted disruption of the alpha1,3-galactosyltransferase gene in cloned pigs. Nat. Biotechnol., 20, 251–255.
102) Kolber-Simonds, D., Lai, L., Watt, S.R., Denaro, M., Arn, S., Augenstein, M.L., Betthauser, J., Carter, D.B., Greenstein, J.L., Hao, Y., Im, G.S., Liu, Z., Mell, G.D., Murphy, C.N., Park, K.W., Rieke, A., Ryan, D.J., Sachs, D.H., Forsberg, E.J., Prather, R.S. and Hawley, R.J. (2004): Production of alpha-1,3-galactosyltransferase null pigs by means of nuclear transfer with fibroblasts bearing loss of heterozygosity mutations. Proc. Natl. Acad. Sci. USA., 101, 7335–7340.
103) Lai, L., Kolber-Simonds, D., Park, K.W., Cheong, H.T., Greenstein, J.L., Im, G.S., Samuel, M., Bonk, A., Rieke, A., Day, B.N., Murphy, C.N., Carter, D.B., Hawley, R.J. and Prather, R.S. (2002): Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science, 295, 1089–1092.
104) Archer, G.S., Dindot, S., Friend, T.H., Walker, S., Zaunbrecher, G., Lawhorn, B. and Piedrahita, J.A. (2003): Hierarchical phenotypic and epigenetic variation in cloned swine. Biol. Reprod., 69, 430–436.
105) Pace, M.M., Augenstein, M.L., Betthauser, J.M., Childs, L.A., Eilertsen, K.J., Enos, J.M., Forsberg, E.J., Golueke, P.J., Graber, D.F., Kemper, J.C., Koppang, R.W., Lange, G., Lesmeister, T.L., Mallon, K.S., Mell, G.D., Misica, P.M., Pfister-Genskow, M., Strelchenko, N.S., Voelker, G.R., Watt, S.R. and Bishop, M.D. (2002): Ontogeny of cloned cattle to lactation. Biol. Reprod., 67, 334–339.
106) Norman, H.D., Lawlor, T.J., Wright, J.R. and Powell, R.L. (2004): Performance of holstein clones in the united states. J. Dairy Sci., 87, 729–738.
107) Yao, S., Sukonnik, T., Kean, T., Bharadwaj, R.R., Pasceri, P. and Ellis, J. (2004): Retrovirus silencing, variegation, extinction, and memory are controlled by a dynamic interplay of multiple epigenetic modifications. Mol. Ther., 10, 27–36.
108) Tamashiro, K.L., Wakayama, T., Akutsu, H., Yamazaki, Y., Lachey, J.L., Wortman, M.D., Seeley, R.J., D'Alessio, D.A., Woods, S.C., Yanagimachi, R. and Sakai, R.R. (2002): Cloned mice have an obese phenotype not transmitted to their offspring. Nat. Med., 8, 262–267.