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Factors Affecting the Development of Somatic Cell Nuclear Transfer Embryos in Cattle

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Abstract. Nuclear transfer is a complex multistep procedure that includes oocyte maturation, cell cycle synchronization of donor cells, enucleation, cell fusion, oocyte activation and embryo culture. Therefore, many factors are believed to contribute to the success of embryo development following nuclear transfer. Numerous attempts to improve cloning efficiency have been conducted since the birth of the first sheep by somatic cell nuclear transfer. However, the efficiency of somatic cell cloning has remained low, and applications have been limited. In this review, we discuss some of the factors that affect the developmental ability of somatic cell nuclear transfer embryos in cattle.
Key words: Cattle, Cloning, Embryo, Reprogramming, Somatic cell nuclear transfer

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The somatic cell nuclear transfer (SCNT) technology is expected to be useful for farm animal breeding and research, the production of transgenic animals for biomedical purposes, and the conservation of endangered species. Cattle are probably the most widely used species for SCNT experiments [1, 2]. Successful production of clones of elite bulls [3, 4], cows with high milk performance [5] and an endangered breed of cattle [6] has been reported. Furthermore, transgenic cattle such as calves lacking the prion protein [7] and cows overexpressing casein proteins in their milk [8] have been produced by SCNT. However, the efficiency of bovine cloning remains low, despite the numerous studies that have been conducted. Nuclear transfer (NT) is a complex multistep procedure including oocyte maturation, cell cycle synchronization of donor cells, enucleation, cell fusion, oocyte activation, and embryo culture. Therefore, many factors are believed to contribute to the success of embryo development following SCNT. In this review, we discuss some of the factors that affect the developmental ability of bovine SCNT embryos based on our studies as well as other previous reports.

Oocyte Source and Quality

Oocytes are usually collected from slaughterhouse-derived ovaries or live cows by ovum pick-up (OPU) and used for bovine SCNT after *in vitro* maturation. We examined the developmental ability of NT embryos derived from the cumulus cells of a Japanese black cow using slaughterhouse-derived and OPU-derived *in vitro* matured oocytes. As shown in Table 1, no significant differences in the cleavage and blastocyst formation rates were observed between oocyte sources (OPU and slaughterhouse). Japanese black cows (same breed as donor cells) and Holstein cows were used as the OPU donors, but the breed of oocyte donors did not affect the *in vitro* developmental ability of SCNT embryos. Sugimura *et al*. also reported no difference in the blastocyst formation rates of SCNT embryos between oocytes from a slaughterhouse and OPU, but follicle-stimulating hormone (FSH) pretreatment of OPU donor cows improved oxygen consumption and *OCT4* and *IFN-τ* expression of SCNT embryos to levels similar to fertilized embryos [9], suggesting that FSH pretreatment of OPU donor cows has a positive effect on oocyte quality. Furthermore, *in vivo*-matured oocytes can be collected by OPU from hormone-treated cows [10, 11]. *In vivo*-matured oocytes are more developmentally competent after *in vitro* fertilization (IVF) than *in vitro*-matured oocytes [10–12]. We examined the development of *in vivo*- and *in vitro*-matured oocytes after SCNT [13]. *In vivo*-matured oocytes collected by OPU from heifers treated with FSH, prostaglandin-F2α and gonadotropin hormone-releasing hormone, and *in vitro*-matured oocytes collected from slaughterhouse-derived ovaries were used as recipient cytoplasts. In accordance with the bovine IVF results [10–12], the blastocyst formation rate of *in vivo*-matured oocytes after SCNT was significantly higher than that of *in vitro*-matured oocytes. The pregnancy rate did not differ between *in vivo*- and *in vitro*-matured oocytes. However, a high abortion rate (75% of pregnancies) was observed in SCNT fetuses from *in vitro*-matured oocytes, whereas no subsequent abortions were observed from *in vivo*-matured oocytes. These results suggest that inappropriate

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Table 1. Development of nuclear transfer (NT) embryos derived from cumulus cells of a Japanese black cow using ovum pick-up (OPU)-derived and slaughterhouse-derived oocytes

Recipient oocytes		No. of NT embryos	No. of cleaved embryos (%)	No. of blastocysts (%)	No. of embryos transferred	No. of calves (%)	No. surviving > 60 days
Source	Breed						
Slaughterhouse	Unknown	89	70 (78.7)	32 (36.0)	20	5 (25)	3 (15)
OPU	Total	112	101 (90.1)	33 (29.5)	10	5 (50)	1 (20)
	Japanese black	70	64 (91.4)	19 (27.1)	7	2 (28)	0 (0)
	Holstein	42	37 (88.1)	14 (33.3)	3	3 (100)	1 (33)

oocyte maturation of recipient cytoplasts is one of the factors causing embryonic or fetal loss after NT in cattle.

In SCNT, donor cells are electrically fused with enucleated recipient oocytes containing a large amount of foreign cytoplasm. Cloned calves with mixed mitochondrial DNA from the donor cell and the recipient oocyte (heteroplasmy) have been reported [14–17], although the influence of heteroplasmy on the development of SCNT embryos is unclear. Cloned calves can be produced using both oocytes and somatic cells derived from the same cow to avoid cytoplasmic contribution from foreign oocytes (autologous SCNT [18]). Cloned calves produced in this manner do not exhibit heteroplasmy. Yang *et al*. showed that autologous SCNT embryos resulted in higher developmental rates *in vitro* and *in vivo* compared with heterologous SCNT embryos (donor cell not related to recipient cytoplasm) [18]. In contrast, reports by other laboratories [19–21] have indicated no such positive effect of autologous SCNT. This discrepancy may be because of the influence of individual oocyte donors. The oocyte donor influences the production of blastocysts in bovine IVF [22] and SCNT [23]. We examined the developmental ability of autologous SCNT embryos using cumulus cells and oocytes collected from six cows by OPU [24]. The developmental rates of autologous SCNT embryos to the blastocyst stage varied widely among individual cows (range, 19–64%) [24]. We produced four cloned calves by autologous SCNT. However, two of the calves were stillborn, and the remaining two died 13 days and 150 days after birth and had anomalies at the postmortem examination. These results suggest that it is difficult to improve the birth rate of healthy cloned calves only using both oocytes and somatic cells derived from the same cow.

Cell Cycle Combination

The cell cycle of the donor cells is an important factor affecting the development of SCNT embryos, because cell cycle co-ordination of donor cells and recipient oocytes is essential to maintain ploidy and prevent DNA damage [25]. Nonactivated metaphase II (MII) oocytes have been primarily used as recipient cytoplasts for bovine SCNT [26]. Accordingly, G0- or G1-phase cells of the cell cycle have been used in almost all successful reports [27], although M-phase cells can also be reprogrammed in MII oocytes [28]. The efficiency of blastocyst and full-term development was compared between SCNT embryos derived from fibroblast cells at the G0 and G1 phases in several studies [29–32]. No significant difference was observed in *in vitro* development between G0- and G1-phase cell SCNT embryos. However, the *in vivo* developmental ability of SCNT embryos tended to be higher for G1-phase cells than that for G0-phase cells [29–32]. One study suggested that homogeneous expression among all blastomeres of SCNT embryos derived from G1-phase cells at embryonic gene activation contributes to a higher success rate [33]. The development of SCNT embryos using pre-activated oocytes has been examined in several studies [28, 34–37]. Oocytes activated 6 h before NT stopped developing at the 8-cell stage after NT, regardless of the cell cycle of the donor cells [28]. However, oocytes within a few hours after activation appear to have a capacity to reprogram the somatic cell nucleus, and this capacity may be largely dependent on the cell cycle stage of the donor cells. Successful production of cloned calves was reported with SCNT embryos using S/G2-phase cells and oocytes activated 2.5 h before NT [35]. In contrast, no cloned calves were obtained with oocytes activated 2 h before NT when we used G0- and G1-phase cells [38].

Cell Type and *In Vitro* Culture of Donor Cells

Cloned cattle have been produced from various somatic cell types. However, it is still unclear which cell type is the most appropriate for bovine SCNT [27, 39]. Moreover, the differentiation status of somatic cells may have no relationship with cloning efficiency [40]. Bovine SCNT embryos can develop to the blastocyst stage at a rate similar to that of embryos produced by IVF (approximately 30–50%) [41], although the electric conditions for fusion of enucleated oocytes differs among donor cell types [42]. However, high embryonic and fetal losses occur after embryo transfer regardless of donor cell type. Because the efficiency of bovine cloning is low, it may be difficult to show significant differences among donor cell types [43].

In bovine SCNT, donor cells are usually cultured *in vitro* before being used for NT [44–46]. Not only the nuclei of short-term cultured cells but also the nuclei of long-term cultured cells (cultured for 3 months) [47] or those close to the end of their life span [48] have the ability to generate live healthy calves after NT. We compared the developmental ability of SCNT embryos using bovine cumulus cells under four different culture conditions (non-culture, maturation culture for 20 h, cycling culture and serum-starved culture) to examine the effect of *in vitro* culture of donor cells on cloning efficiency [49]. The blastocyst formation rate and blastocyst cell number of SCNT embryos derived from cultured cumulus cells (cycling culture and serum-starved culture) were significantly higher than those of SCNT embryos derived from fresh (non-cultured) cells [49]. Cell cycle analysis using flow cytometer showed that the relative percentage of fresh cells in the G0/G1 phase of the cell cycle (89.7 ± 0.4%) was similar to that of serum-starved cells (90.6 ± 0.6%) but lower than that of cycling cells (76.0 ± 1.8%) [49], indicating that the

Fig. 1. Cloned calves produced by nuclear transfer using cumulus cells under 4 different conditions: (a) cells removed from cumulus-oocyte complexes (COCs) after aspiration of ovarian follicles; (b) cells removed from COCs after *in vitro* maturation; (c) cells cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) for 3 days after some subculture; and (d) cells cultured in DMEM with 0.5% FBS for an additional 5 days.

difference in *in vitro* development between fresh and cultured cells did not result from the cell cycle of the donor cells. The same results have also been obtained for goat [50] and rabbit SCNT [51]. These results suggest that culture of donor cells increases the efficiency of SCNT embryo production *in vitro*. However, the subsequent viability of blastocyst-stage embryos produced using fresh cells may not be different from that using cultured cells. No difference was observed in the *in vivo* developmental ability of SCNT embryos between fresh and cultured cells, and live calves were obtained from cumulus cells under all culture conditions (Fig. 1) [49].

Timing of Fusion and Activation

In SCNT, the lack of sperm-induced fertilization necessitates artificial activation to trigger further development. Direct exposure of chromosomes to nonactivated MII cytoplasm is effective for somatic cell nuclear reprogramming [28, 52], and nonactivated MII oocytes have been used in almost all successful bovine SCNT reports [26, 27]. The timing of activation of MII oocytes can be classified into two protocols as follows: (1) activation performed immediately after fusion (simultaneous fusion and activation method, FA) and (2) activation performed several hours after fusion (delayed activation method, DA). Successful production of cloned offspring using SCNT has been reported for both the FA [45, 47] and DA [44, 46] methods. Donor chromosomes are exposed to factors present in MII cytoplasm for only a short time in the FA method and for a longer time in the DA method. The DA method improves the *in vitro* development of bovine [6, 53] and mouse [54, 55] NT embryos derived from somatic cells at the G0/G1 stage compared with that of the FA method. We compared the developmental ability of bovine fibroblast cell NT embryos produced using different fusion and chemical activation timings to develop an efficient fusion and activation protocol for producing SCNT embryos [56]. As shown in Table 2, the *in vitro* development of SCNT embryos was affected by the timing of fusion and chemical activation, and the development of SCNT embryos to the blastocyst stage in the F21A24 group (fusion at 21 h and activation 24 h postmaturation) of the DA method was significantly higher than that in the other groups. However, the development of SCNT embryos activated 6 h after fusion (F21A27 and F24A30 groups) in the DA method was significantly lower than that in the FA method. In reports by Aston *et al*. [57] and Choi *et al*. [58], excessive exposure to MII cytoplasm resulted in abnormal chromatin morphology, but SCNT embryos activated less than 2.5 h after fusion resulted in improved nuclear morphology and increased development to the compacted morula/blastocyst stage. These reports and our results suggest that the exposure duration of somatic cell nuclei to oocyte cytoplasm before activation affects the *in vitro* development of SCNT embryos and that excessive exposure to MII cytoplasm results in a poor developmental rate to the blastocyst stage. However, no influence of the duration of exposure to oocyte cytoplasm on the *in vivo* developmental ability has been observed. When we examined the *in vivo* developmental ability of cumulus cell NT embryos and postnatal survivability of cloned calves produced by the DA (F21A24) and FA (F24A24) methods, the pregnancy and calving rates did not differ significantly between the two methods [59]. In addition, high rates of postnatal mortality were observed in both the methods [59]. Sung *et al*. obtained similar results using two types of donor cells (cumulus and fibroblast cells) [60]. In a report by Aston *et al*., the time interval between fusion and activation when using the DA method did not affect the *in vivo* development of SCNT embryos [57].

Table 2. *In vitro* development of somatic cell nuclear transfer (SCNT) embryos produced using different fusion and activation timings

Group	Hours post IVM		No. SCNT embryos	No. of cleaved embryos (%)	No. of blastocysts (%)
	Fusion	Activation			
F21A21	21	21	89	60 (67)^{cd}	25 (28)^{c}
F21A24	21	24	125	97 (78)^{bc}	79 (63)^{a}
F21A27	21	27	96	52 (54)^{d}	3 (3)^{e}
F24A24	24	24	150	123 (82)^{b}	59 (39)^{bc}
F24A27	24	27	134	122 (91)^{a}	63 (47)^{b}
F24A30	24	30	93	63 (68)^{cd}	13 (14)^{d}
F27A27	27	27	121	99 (82)^{b}	49 (41)^{bc}

^{a,b,c,d,e} Values without common characters in the same column of each group differ significantly ($P < 0.05$, chi-square test).

Histone Deacetylase Inhibitor (HDACi) Treatment

Abnormal epigenetic modifications such as aberrant DNA methylation and histone modification have been observed in SCNT embryos [61–64]. Therefore, preventing epigenetic errors is expected to lead to improved animal cloning success rates [65]. Several DNA methylation inhibitors and HDACis have been used to improve the developmental ability of bovine SCNT embryos [66]. Treatment of donor cells with 5-aza-2'-deoxycytidine (5-aza-dC), a DNA methylation inhibitor, did not improve the *in vitro* developmental ability of SCNT embryos [67–69], whereas treatment with trichostatin A (TSA) or sodium butyrate, an HDACi, increased the blastocyst formation rate [67–70]. However, improvement of full-term development following HDACi treatment of donor cells has not been demonstrated. It was reported from two laboratories in 2006 that TSA treatment of mouse SCNT embryos after NT improved the success rate of mouse cloning [65, 71]. In these reports, TSA treatments for 9–20 h led to a significant increase not only in the blastocyst formation rate but also in the full-term developmental rate [65, 71]. In addition, Kohda *et al*. showed that the gene expression profile of TSA-treated cloned mice came to resemble that of mice produced by intracytoplasmic sperm injection [72]. These reports suggest that inhibiting histone deacetylation in SCNT embryos during a short period of culture after NT promotes reprogramming of the donor nucleus in mice. We examined the effects of treatment with HDACis, TSA and scriptaid (SCR), on the *in vitro* development of bovine SCNT embryos using three fibroblast cell lines (L1, L2 and L3) [73]. As shown in Fig. 2, TSA treatment improved blastocyst formation rates of SCNT embryos derived from L1 and L3 but had no effect on the rate of embryos derived from L2. Furthermore, SCR treatment increased the blastocyst formation rates of SCNT embryos derived from L1 and L2, but no significant increase was observed in SCNT embryos derived from L3. These results suggest that HDACi treatment of bovine SCNT embryos improves the blastocyst formation rate; however, optimal treatment conditions may differ among donor cell lines. Four laboratories have recently reported the *in vivo* developmental ability of bovine SCNT embryos treated with TSA [74–77]. In contrast to the results in mice [65, 71], treatment of bovine SCNT embryos with TSA alone did not significantly improve the full-term developmental rate [74–76]. In contrast, a higher calving rate was observed following a combined treatment with a DNA methylation inhibitor [77]. Wang *et al*. reported that a combined treatment of both donor cells and SCNT embryos with 5-aza-dC and TSA reduced the methylation levels of the NT blastocyst satellite I sequence to levels similar to those in IVF embryos and increased the cloning efficiency from 2.6 to 13.4% [77]. However, it is difficult to correct epigenetic abnormalities completely only by treatment with epigenetic modifiers, as various abnormalities including large offspring syndrome have been observed in cloned calves after combined treatment of both donor cells and cloned embryos with 5-aza-dC and TSA as well as untreated cloned calves [77].

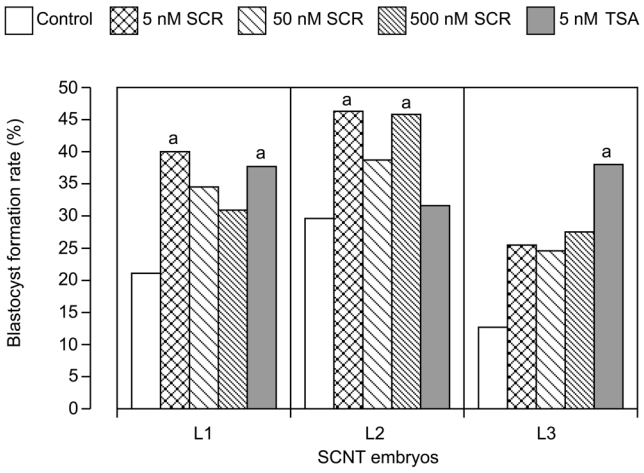

Fig. 2. Development to the blastocyst stage of somatic cell nuclear transfer (SCNT) embryos treated with 5, 50 and 500 nM scriptaid (SCR) or 5 nM trichostatin A (TSA). Three fibroblast cell lines (L1, L2 and L3) were used as somatic cell donors. [a]Significant difference compared with the control ($P < 0.05$, chi-square test). Reproduced with permission of the Society for Reproduction and Development from Akagi S, *et al*.: Treatment with a histone deacetylase inhibitor after nuclear transfer improves the preimplantation development of cloned bovine embryos. *J Reprod Dev* 2011; 57: 120–126.

Table 3. *In vivo* development of bovine fibroblast cell-nuclear transfer (NT) embryos and aggregates after embryo transfer

Embryo (time of aggregation)	No. of embryos/aggregates transferred	No. of pregnancies			No. of calves (%)
		Day 30	Day 60	Day 90	
Single NT, zona-intact	10	3 (30)	0 (0) [a]	0 (0)	0 (0)
Aggregate (8-cell stage)	11	7 (64)	6 (55) [b]	3 (27)	2 (18)
Aggregate (16- to 32-cell stage)	7	4 (57)	4 (57) [b]	1 (14)	1 (14)

[a,b] Values without common characters in the same column differ significantly ($P < 0.05$, Fisher's exact test). Reproduced with permission of the Society for Reproduction and Development from Akagi S, *et al*.: Developmental ability of somatic cell nuclear transferred embryos aggregated at the 8-cell stage or 16- to 32-cell stage in cattle. *J Reprod Dev* 2011; 57: 500–506.

Embryo Aggregation

The cell number in blastocysts has been used as an indicator of embryo quality [78]. The cell number in SCNT embryos is lower than that in *in vivo*-derived embryos [79, 80]. This poor blastocyst quality appears to contribute to the decreased survival rate of SCNT embryos after embryo transfer. Embryo aggregation is a method that enables an increase in the cell number in embryos [80, 81]. In addition, several studies have indicated that embryo aggregation affects SCNT embryo gene expression [82–84]. In mice, aggregation of SCNT embryos at the 4-cell stage led not only to an increase in cell number but also improved *Oct 4* expression, and resulted in eight times higher full-term development compared with single embryos [80]. In cattle, a high pregnancy rate was observed in embryos aggregated at 4 days after SCNT (day 4) [85, 86], whereas aggregation of 1-cell stage SCNT embryos did not improve *in vivo* development [82, 87]. Aggregation of 1-cell stage SCNT embryos resulted in reduced *OCT4* expression compared with IVF embryos [82], whereas no significant difference in *OCT4* expression was observed between IVF embryos and aggregates of day 2 embryos [88]. Thus, the timing of aggregation may be important for producing high-quality SCNT embryos. We examined the effect of the timing of aggregation on the development of SCNT embryos [89]. One-cell stage embryos after activation, 8-cell stage embryos on day 2 or 16- to 32-cell stage embryos on day 4 were used for embryo aggregation after removing the zona pellucida. Irrespective of the timing of aggregation, aggregates of the three SCNT embryos developed to the blastocyst stage at a high rate (aggregates of 1-cell stage embryos, 17/19, 89%; aggregates of day 2 embryos, 23/23, 100%; aggregates of day 4 embryos, 22/22, 100%). Furthermore, a significant increase in cell number was observed in aggregates of three day 2 and day 4 embryos (223 ± 30 and 163 ± 18, respectively) compared with single SCNT embryos (89 ± 6). A significantly higher pregnancy rate was observed after embryo transfer at 60 days of gestation in aggregates of three day 2 or day 4 embryos than in single SCNT embryos; however, a high incidence of abortion and stillbirth was subsequently observed in aggregates (Table 3). These results suggest that aggregation of SCNT embryos may improve the pregnancy rate after embryo transfer but that it cannot reduce the high incidence of fetal loss and stillbirth, which is often observed in bovine SCNT.

Conclusion

NT is a complex multistep procedure, and there are many biological and technical factors affecting the development of bovine SCNT embryos. Numerous studies have led to significant improvements in SCNT protocols [90]; however, the cloning efficiency in terms of healthy cloned calves born still remains low. Failure to reprogram the donor genome is believed to be the main reason for the low cloning efficiency [91]. Further studies to optimize each step of SCNT together with a better understanding of the reprogramming mechanism are necessary in order to improve the efficiency of bovine cloning.

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