Nuclear transfer technique affects mRNA abundance, developmental competence and cell fate of the reconstituted sheep oocytes

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Abstract

The effect of technical steps of somatic cell nuclear transfer (SCNT) on different aspects of cloned embryo development was investigated in sheep. *In vitro*-matured oocytes were enucleated in the presence or absence of zona and reconstituted by three different SCNT techniques: conventional zona-intact (ZI-NT), standard zona-free (ZF-NT) and intracytoplasmic nuclear injection (ICI-NT). Stepwise alterations in nuclear remodeling events and in mRNA abundances, throughput and efficiency of cloned embryo development and cell allocation of the resulted blastocysts were assessed. Early signs of nuclear remodeling were observed as soon as 2 h post-reconstitution (hpr) for fusion-based methods of nuclear transfer (ZI-NT and ZF-NT) but were not observable until 4 hpr with the ICI-NT method. The relative mRNA abundances of *HSP90AA1* (*HSP90*), *NPM2* and *ATPase* genes were not affected by i) presence or absence of zona, ii) oocyte enucleation method and iii) nuclear transfer method. After reconstitution, however, the relative mRNA contents of *POU5F1* (*OCT4*) with the ZI-NT and ZF-NT methods and of *PAPOLA* (*PAP*) with ZF-NT were significantly lower than those for the ICI-NT method. Zona removal doubled the throughput of cloned blastocyst development for the ZF-NT technique compared with ZI-NT and ICI-NT. Cleavage rate was not affected by the SCNT protocol, whereas blastocyst yield rate in ICI-NT technique ($17.0 \pm 1.0\%$) was significantly ($P < 0.05$; ANOVA) higher than in ZF-NT ($7.1 \pm 1.5\%$) but not in the ZI-NT group ($11.2 \pm 3.3\%$). Despite the similarities in total cell number, SCNT protocol changed the distribution of cells in the blastocysts, as ZF-NT-cloned blastocysts had significantly smaller inner cell mass than ZI-NT. These results indicate that technical aspects of cloning may result in the variety of cloning phenotypes.

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Introduction

Although conceptually simple, the technique of somatic cell nuclear transfer (SCNT) has proven a demanding multistep process in practice. Furthermore, the overall efficiency of SCNT technique, in terms of live birth rate per transferred embryos, is low with potential perturbation in development occurring at all steps (Wells *et al.* 2004). Importantly, the investigation of biological factors underlying low cloning efficiency has been hampered by technical aspects of the cloning procedure (Oback *et al.* 2003).

In order to increase the efficiency and throughput of cloning process a wide range of cloning techniques have been developed and introduced over the time (for review, see Verma & Trounson (2006)). Importantly, a growing body of evidence indicates that certain SCNT techniques may be more efficient in certain species.

For example, Wakayama *et al.* (1998) demonstrated that employing a non-fusion piezo-actuated method of embryo reconstitution and prolonged exposure of donor nuclei (1–6 h) to the recipient cytoplasm rich in metaphase-promoting factor (MPF) were their keys to success in cloning mice. High MPF activity induces nuclear envelope breakdown (NEBD) via p34^{cdc2} kinase-related phosphorylation of lamin, which is a nuclear membrane protein (Peter *et al.* 1990). This in turn facilitates subsequent nuclear remodeling changes including premature chromosome condensation (PCC), nuclear swelling and microtubule organization (Latham 2005, Sung *et al.* 2007).

While the role of nuclear remodeling in successful reprogramming and clonal development is well documented (Mitalipov *et al.* 2007), there is ongoing serious debate about whether MPF activity is an absolute necessity for oocyte-mediated

reprogramming (Sung *et al.* 2007). In this regard and despite some studies emphasizing the essentiality of MPF for reprogramming of donor nuclei (Wakayama *et al.* 1998, Zhou *et al.* 2006), there are indications that high MPF activity is not important, as viable offspring have been produced after nuclear transfer into cytoplasm of preactivated oocytes (Sung *et al.* 2007), and even early zygotes (Schurmann *et al.* 2006). Even though, this contradiction may be a reflection of either different SCNT techniques used or species-specific variations in the nuclear remodeling events. Therefore, a clear understanding of how SCNT technique may affect reprogramming and cloned embryo development in a given species may shed some light on this ambiguity.

It has been demonstrated that the protocol used for SCNT in terms of fusion-activation timing affects the pattern of gene expression and developmental competence of cloned embryos (Wrenzycki *et al.* 2001). Our recent studies on epigenetic modification of donor cells and cloned embryos well showed that any change in compartmentalization of the chromatin leads to manifest changes in the transcription status and

developmental competence of bovine cloned embryos (Jafari *et al.* 2011a, 2011b, Jafarpour *et al.* 2011). In primates, Zhou *et al.* (2006) showed that monkey oocytes are particularly vulnerable to premature activation and MPF degradation. Accordingly, they developed a modified one-step micromanipulation protocol to prevent premature cytoplasm activation during SCNT, which resulted in robust NEBD and PCC with high cloned blastocyst development for the first time in non-human primates.

These observations further indicate that each mammalian species may respond differently to a given technique. However, owing to the multistep nature of the cloning process, it is believed that the exact contribution of each step to overall cloning success must be determined in comparative studies (Oback & Wells 2002). This requires strict comparison between different steps in a single study, and application of stringent identification criteria in order to obtain a final conclusion. In this study, we investigate how different steps of three SCNT techniques may affect different aspects of *in vitro* clone embryo development in sheep.

Figure 1 Representative images showing different organizations of microtubules (A, B, C and D), chromosomes (A', B', C' and D'), and the merged images showing the organizations of both microtubules and chromosomes (A'', B'', C'' and D''). (A, A' and A'') No sign of nuclear swelling or spindle formation, (B, B' and B'') PCC with early developing spindle, (C, C' and C'') bipolar spindle (C) and complete chromosome (C') organization, (D, D' and D'') multipolar spindle (D) and aberrant chromosome (D') organization. Bars represent 100 μ m in all images.

Results

Nuclear remodeling

As shown in Figs 1 and 2, the SCNT protocol had a time-dependent effect on kinetic of nuclear remodeling. Accordingly, at 2 h post-reconstitution (hpr), the majority of the nuclei used in the ICI-NT approach remained intact with minor signs of bipolar spindle formation. At the same time, however, reasonable proportions of oocytes reconstituted via the ZI-NT and ZF-NT approaches showed signs of remodeling and had either swollen (28.5 and 30.0% respectively) or prematurely condensed (17.1 and 40.0% respectively) nuclei with bipolar spindles. The PCC rate of oocytes reconstituted by the ZF-NT approach was also significantly higher than that for the ZI-NT technique. At 4 hpr, however, the overall incidence rates of nuclear remodeling were not different between the SCNT protocols. Furthermore irrespective of the SCNT protocol, the majority of the reconstituted oocytes had prematurely condensed

chromosomes on well-structured bipolar spindles. Even though PCC rates of ZI-NT and ZF-NT were significantly higher than that for the ICI-NT method. At 6 hpr the overall percentages of nuclear remodeling were not significantly different between the SCNT groups and were comparable to nuclear remodeling events observed at 4 hpr as well. However, the frequencies of abnormal nuclear remodeling significantly increased at 6 hpr. Accordingly, approximately half of the reconstituted oocytes with remodeled nuclei revealed chromosomal aberrations and multipolar spindles.

Quantitative competitive real-time PCR (qcRT-PCR)

As shown in Fig. 3, qcRT-PCR showed that almost all oocyte manipulations have decreasing effects on relative abundances of maternal mRNA, though the effects of different SCNT techniques and different steps of each technique were not the same. Even though, the relative mRNA abundances of three genes (*HSP90AA1* (*HSP90*),

Figure 2 Time-dependent effect of SCNT technique on remodeling events of the reconstituted oocytes. Values with different letters are significantly different at $P < 0.05$.

Figure 3 Effect of different steps of SCNT on relative mRNA abundance of *in vitro* matured sheep oocytes. Values with at least one common letter are not significantly different at $P < 0.05$.

nucleoplasmin 2 (*NPM2*), and *ATPase*) remained nearly constant during SCNT and were not affected by i) presence or absence of zona, ii) cytochalasin-B (CB) treatment of oocytes (in ICI-NT and ZI-NT techniques), iii) oocyte enucleation method and iv) nuclear transfer method.

The most important decreases in mRNA were observed during the nuclear transfer step. In this sense, fusion-based methods of nuclear transfer (ZI-NT and ZF-NT) had overall more reductions in relative abundances of mRNA compared with non-fusion-based method (ICI-NT). For example, in ZF-NT oocytes, the relative mRNA abundances of five genes (*Geminin* (*GMNN*), *POU5F1* (*OCT4*), poly-A polymerase (*PAPOLA* (*PAP*)), cyclin-B, and *GJA1* (*CX43*)) were significantly lower than those in zona-intact (control) oocytes. Moreover, the relative abundances of *POU5F1*, *PAPOLA* and *GJA1* in ZI-NT oocytes were significantly lower than those in control oocytes. Importantly, the relative mRNA contents of *POU5F1* in ZI-NT and ZF-NT oocytes and of *PAPOLA* in ZF-NT oocytes were

significantly lower than those in ICI-NT oocytes. None of the manipulations affected the relative abundances of housekeeping genes (*GAPDH* and β -actin).

Yield and quality of embryo development

As shown in Table 1, within the SCNT groups, $14.9 \pm 1.2\%$ of oocytes in the ICI-NT group were found to be degenerated/fragmented, a rate that was significantly higher than that for the ZF-NT method ($7.2 \pm 1.9\%$), and non-significantly higher than that for the ZI-NT method ($9.4 \pm 1.6\%$). Cleavage rates were not affected by the SCNT protocol, and the percentages of cleaved embryos advanced to morula were not significantly different between the three SCNT techniques, except for morula rate of ZF-NT, which was significantly lower than that for the ZI-NT group. However, blastocyst yield using ICI-NT ($17.0 \pm 1.0\%$) was significantly higher than for ZF-NT ($7.1 \pm 1.5\%$), but not ZI-NT ($11.2 \pm 3.3\%$). Importantly, the relative efficiencies of cloned blastocyst development, based on the total number of oocytes used, were not significantly different between the SCNT groups.

Table 1 Effect of method of embryo production (SCNT, PA and IVF), technique of SCNT (ICI-NT, ZI-NT and ZF-NT), and presence/absence of zona in parthenogenetic embryos on yield, efficiency and quality of *in vitro* embryo development in sheep.

Experiments	Oocyte (n)	Frag/deg (% $\pm$ S.E.M.)	Number (% $\pm$ S.E.M.) of embryos developed			Cloning efficiency ^b	Differential staining		
			Cleavage	Morula	Blastocyst ^a		TCN (n $\pm$ S.E.M.)	ICM/TCN (% $\pm$ S.E.M.)	TE/TCN (% $\pm$ S.E.M.)
SCNT									
ICI-NT	290	44 (14.9 $\pm$ 1.2) [§]	162 (55.6 $\pm$ 5.1) ^{*†}	40 (25.2 $\pm$ 2.5) ^{*†}	28 (17.0 $\pm$ 1.0) [†]	9.5 $\pm$ 1.3 [*]	120.2 $\pm$ 7.8 [*]	19.4 $\pm$ 1.8 ^{†,‡}	80.5 $\pm$ 1.8 ^{*†,‡}
ZI-NT	220	22 (9.4 $\pm$ 1.6) ^{†,‡,§}	112 (51.3 $\pm$ 3.0) [*]	46 (40.6 $\pm$ 4.6) ^{†,‡}	12 (11.2 $\pm$ 3.3) ^{*†}	4.4 $\pm$ 2.2 [*]	116.3 $\pm$ 7.8 [*]	24.1 $\pm$ 1.8 [‡]	75.8 $\pm$ 1.8 ^{*†}
ZF-NT	260	20 (7.2 $\pm$ 1.9) ^{†,‡}	164 (63.2 $\pm$ 3.3) ^{*†,‡}	32 (18.9 $\pm$ 2) [*]	12 (7.0 $\pm$ 1.5) [*]	4.6 $\pm$ 1.2 [*]	100.8 $\pm$ 5.1 [*]	12.9 $\pm$ 1.2 ^{*†}	87.1 $\pm$ 1.2 ^{‡,§}
PA									
ZI-PA	215	10 (4.8 $\pm$ 0.8) ^{*†}	188 (87.2 $\pm$ 2.9) [§]	116 (62.5 $\pm$ 4.7) [§]	66 (35.7 $\pm$ 3.1) [†]	31.0 $\pm$ 2.2 [†]	190 $\pm$ 13.0 [†]	21.8 $\pm$ 2.9 ^{†,‡}	75.2 $\pm$ 2.2 [*]
ZF-PA	177	11 (6.3 $\pm$ 0.8) ^{*†,‡}	133 (76.6 $\pm$ 5.6) ^{†,‡,§}	77 (57.8 $\pm$ 4.5) ^{†,§}	45 (33.8 $\pm$ 1.9) [‡]	25.7 $\pm$ 1.7 [†]	114.3 $\pm$ 7.7 [*]	10.7 $\pm$ 1.4 [*]	89.2 $\pm$ 1.4 [§]
IVF									
ZI-IVF	173	4 (2.4 $\pm$ 0.6) [*]	124 (73.7 $\pm$ 5.4) ^{*†,‡,§}	78 (62.4 $\pm$ 3.4) [§]	53 (42.1 $\pm$ 2.3) [‡]	30.8 $\pm$ 0.5 [†]	149.5 $\pm$ 19.3 ^{*†}	21.0 $\pm$ 1.8 ^{†,‡}	78.9 $\pm$ 1.8 ^{*†}
ZF-IVF	167	19 (11.4 $\pm$ 0.3) ^{†,§}	140 (84.1 $\pm$ 3.7) ^{†,§}	76 (53.3 $\pm$ 3.9) ^{†,§}	43 (30.7 $\pm$ 1.2) [‡]	25.9 $\pm$ 2.1 [†]	125.0 $\pm$ 10.0 [*]	16.3 $\pm$ 1.3 ^{†,‡}	83.6 $\pm$ 1.3 ^{†,‡,§}

Cleavages were assessed based on total number of oocytes. Values with at least one common symbol are not significantly different at $P < 0.05$.

^aMeasured as the percentages of blastocysts developed based on the total number of cleaved oocytes. ^bEfficiency of blastocyst development, calculated based on the number of blastocyst developed per total number of oocytes used in three replicates in each group.

Zona removal did not affect the potential of parthenogenetic (PA) and IVF embryos to cleave and further develop to blastocysts. Irrespective of the presence (ZI)/absence (ZF) of zona, total blastocyst development and efficiency of blastocyst development in IVF (ZI/ZF-IVF) and PA (ZI/ZF-PA) groups were significantly higher than in all SCNT groups (Table 1). Moreover, comparing between IVF, PA and the three SCNT methods, the highest survival rate of oocytes was observed in ZI-IVF oocytes, which was significantly higher than for all the other groups, except the ZI-PA and ZF-PA groups.

Total cell number (TCN) of cloned embryos was not significantly different between the SCNT protocols, but all were significantly lower than that for ZI-PA (Table 1). Importantly, SCNT protocol had a significant effect on the distribution of cells in the blastocysts (Table 1). Accordingly, the proportion of cells classified as ICM and TE in zona-intact cloned blastocysts derived using the ZI-NT method were significantly different from those for ZF-NT (Table 1 and Fig. 4D₁₋₃). Moreover, ZF-PA blastocysts had smaller ICM compared with ZI-PA and both ZI-IVF and ZF-IVF blastocysts. In contrast, zona removal significantly increased TE:TCN ratio of ZF-PA vs ZI-PA and ZI-IVF embryos (Table 1).

SCNT throughput

The accuracies of zona-free and zona-intact oocyte enucleation were similar (100.0 vs 89.6 $\pm$ 7.4% respectively). Table 2 summarizes the gains in throughput of reconstructed oocytes per person using different SCNT techniques. As shown, the mean number of oocytes enucleated within 1 h *per se* was 132 using zona-free oocytes, which was approximately double than the number of zona-intact oocytes, which could be enucleated (68) by the same person at the same time. Moreover, both nuclear transfer and bulk fusion were quicker to perform using ZF-NT rather than ICI-NT and ZI-NT techniques.

Discussion

Nuclear transfer protocol affects mRNA abundances of the reconstituted oocytes

Early embryonic events are orchestrated through posttranscriptional control of mRNAs that have been accumulated during oogenesis (Zuccotti *et al.* 2008, Vigneault *et al.* 2009). These maternal mRNA and their related proteins serve the distinct purpose of reprogramming and facilitate early mitotic divisions of the fertilized embryos until broad embryonic genome activation that initiates, for example, at eight-cell stage in sheep (Hosseini *et al.* 2012b). Importantly, comparative analysis of oocyte transcript profiles has indicated a high degree of conservation even between species with distinct taxonomic relationships (Vallée *et al.* 2008). Without a doubt these transcripts are pivotal for reprogramming of terminally differentiated somatic cells (Sylvestre *et al.* 2010) and hence cloning efficiency.

Accordingly, the first point highlighted in this study was that SCNT protocol affects relative mRNA abundances of the recipient oocytes. Given the multistep nature of the SCNT process, we followed stepwise variations occurred in the relative abundances of eight developmentally important genes that are characteristic of different cell functions including pluripotency and reprogramming (*POU5F1*, *GMNN* and *NPM2*), metabolism (*ATPase*), stress (*HSP90AA1*), gap-junction and compaction (*GJA1*), nuclear remodeling (cyclin-B), and *PAPOLA* as an important protector of RNA degradation. The results showed that i) enzymatic zona digestion, ii) CB treatment and iii) method of oocyte enucleation did not significantly affect the mRNA pool of the oocyte, whereas NT method did. Given that the only difference between these NT methods was the application of electrical pulse in ZI-NT and ZF-NT methods, one may consider that electrofusion may cause these reductions in mRNA abundances. Gradual consumption of maternal mRNA during embryo development is well documented in mammals (Vigneault *et al.* 2009).

Figure 4 (A_{1–3}) H33342-stained *in vitro*-matured ovine oocytes before zona removal under u.v. light (A₁), and after zona removal under normal (A₂) or u.v. (A₃) light. Arrowheads indicate the cytoplasmic extrusion cone. Steps involved in zona-intact (B_{1–3}) and zona-free (C_{1–3}) methods of oocyte enucleation. Representative images of cloned blastocysts developed by zona-intact (D₁) and zona-free (D₂) methods of SCNT and differential staining of a ZI-NT blastocyst (D₃) are shown. Bar represents 100 μ m.

Moreover, abrupt reduction of oocyte mRNA in response to intensive stresses such as cryopreservation has been established (Asgari *et al.* 2011, Hosseini *et al.* 2012a). However, there is no, to our knowledge, previous information about the effect of electric pulses on oocyte mRNA. Therefore, whether this reduction in mRNA is an early direct effect of electrofusion or a subsequent indirect effect of oocyte activation remains to be elucidated.

POU5F1 is the most important hallmark of reprogramming and pluripotency, and the presence of *POU5F1* mRNA has been demonstrated in oocytes of different species including human (Hansis *et al.* 2001), cattle

(Vigneault *et al.* 2009), but is already demonstrated in sheep. Importantly, Zuccotti *et al.* (2008) demonstrated that maternal pool of *POU5F1* regulates the cascade of molecular pathways that 'govern the establishment of the maternal legacy of MII oocytes required for the transition from a gametic to an embryonic control of development'. Their data provide some indications for maternal *POU5F1* as a potential key regulator of developmental competence in mice. *NPM2* is a major regulator of sperm and somatic cell chromatin decondensation (Tamada *et al.* 2006). Importantly, by *NPM2* injection into reconstituted bovine oocytes,

Table 2 Comparison in throughput between the three SCNT methods in sheep.

SCNT steps	Throughput per person (NT reconstructs/h)			Increased throughput of ZF-NT method vs	
	ICI-NT	ZI-NT	ZF-NT	ICI-NT	ZI-NT
Enucleation	68	68	132	1.9×	1.9×
Addition of donor cell	40	55	150	3.8×	2.7×
Fusion	—	60	120	—	2.0×
Total (steps 1–3)	25	20	44	2.0×	1.7×

Bethhauser *et al.* (2006) observed upregulation of over 200 genes in cloned embryos, which provided embryos with genetic profiles more similar to those of embryos produced by IVF and higher pregnancy rate compared with non-NPM2-injected oocytes. *GMNN* is another important gene that is highly expressed in oocytes with an important role in cell differentiation of early embryos (Gonzalez *et al.* 2006, Yang *et al.* 2011). Therefore, it is plausible to say that the maternal contribution of crucial transcripts and proteins that have been inherited in the oocyte may have critical role in developmental competence of the resulting embryo.

SCNT protocol affects developmental competence of the reconstituted sheep oocytes

In vitro, the ultimate readout of cloning efficiency is the ability of cloned embryos to develop into blastocysts. Accordingly, the results of this study indicated a marked effect of SCNT technique on developmental competence of the cloned embryos, as the blastocyst yield of ICI-NT was significantly higher than that of ZF-NT. By definition, the most important differences between ICI-NT and ZF-NT methods in this study are i) kinetics of remodeling events, ii) the absence of zona in ZF-NT, and iii) significantly reduced mRNA abundances of oocyte's *POU5F1* and *PAPOLA* in ZF-NT. Moreover, we previously showed that the most important challenge for zona-free embryos is blastomere loss during pre-compaction stage (Nasr-Esfahani *et al.* 2011). To evaluate this hypothesis here, *in vitro* development of zona-intact and zona-free PA and IVF embryos were compared, and interestingly, no significant difference was observed between the blastocyst yields of these groups. Therefore, while the exact reason for lower *in vitro* development of zona-free cloned embryos remains to be clearly elucidated, two suggested explanations are i) reduction in relative abundances of important maternal mRNA of *POU5F1* (Zuccotti *et al.* 2008) and ii) species-specific differences in technical preferences of SCNT protocol.

SCNT protocol affects cell fate determination of cloned sheep embryos

During mammalian embryo development, the first-cell lineage specification is the formation of ICM and TE in

the blastocyst (Marikawa & Alarcón 2009). These distinct cell fates distinguish pluripotent ICM cells, which ultimately generate all the tissues in the animal body, from differentiating TE cells that form fetal placenta (Zernicka-Goetz *et al.* 2009). While the importance of this initial cell differentiation for further development of the embryo is well documented, the exact time-window and mechanism governing the allocation of cells to ICM and TE is a matter of serious debate. Even though the results of this study indicated that SCNT protocols had variable effects on ICM:TE ratios of the developed blastocysts. However, considering some important studies on the possible effects of zona on cell fate determination of early embryo, we carried out further experiments using PA and IVF embryos to unravel the possible crucial importance of zona for cell fate determination. The results were not consistent and did not show the same trend of zona effect on cell fate for PA and IVF. While zona removal significantly switched the cell fate of PA embryos from ICM toward TE formation, the zona had a minor effect on cell fate of IVF embryos. The confusion increases when we observed reports that are for (Kurotaki *et al.* 2007) and against (Gardner 2007) the impact of zona on cell fate. For example, Katayama *et al.* (2010) believed that zona removal provided embryos with a high degree of plasticity in response to the orientation of their first two cleavage planes. On the other hand, using zona-intact and zona-free mouse embryos, Gardner (2007) demonstrated that embryonic–abembryonic axis of the mouse blastocyst is normally prepatterned and suggested no role for zona in its specification. Therefore, while the effect of zona on cell fate of sheep embryos remains to be obtained, in the absence of confirmative evidence of zona effect on cell fate, and considering the crucial impact of *POU5F1* in the formation of ICM in mice (Dietrich & Hiiragi 2007), one may argue that the reduction of maternal *POU5F1* in oocytes reconstituted using ZI-NT and particularly ZF-NT methods of SCNT may contribute to the lower number of ICM cells in the ZF-NT vs ICI-NT cloned embryos. Further studies to confirm this are necessary.

SCNT protocol, choosing throughput or efficiency?

Increased potential applications of SCNT have resulted in a parallel increased interest in development of cloning methods that are improved in both efficiency and ease of operation (Oback *et al.* 2003). Accordingly, the most dramatic changes in cloning technologies were first achieved in cattle, in which zona-free-based methods of Oback *et al.* (2003) provided a twofold increase in throughput compared with conventional methods. Through modification of different steps, we could also develop a goat-specific zona-free SCNT protocol that improved both throughput and efficiency compared with

all previous studies (Nasr-Esfahani *et al.* 2011). In contrast, the ZI-NT approach in this study could not reach such a point of success in both throughput and efficiency. Indeed, zona removal considerably facilitated the enucleation step and significantly increased the speed and efficiency of donor cell addition and cell fusion compared with ICI-NT and ZI-NT methods. However, there was a marked reduction in the yield of zona-free-derived blastocysts compared with two other approaches. This contradiction between efficiency and throughput has raised the question of which criterion is more important: throughput or efficiency? Therefore, we investigated the efficacy of blastocyst production based on the total number of oocytes used in each approach. It was observed that despite higher blastocyst yields of ICI-NT rather than ZF-NT, the efficiency of cloned blastocyst development was not significantly different between the groups. This was due to the higher number of oocytes that were degenerated or fragmented during different steps of ICI-NT compared with ZF-NT. In this situation, one may prefer to choose ZF-NT as a simpler and faster technique of SCNT compared with conventional zona-intact methods. In agreement, Wilmut (2003) stated that the basic routines of zona-free SCNT can be mastered in 3 weeks by a person with no previous experience of micromanipulation, an advantage that is in stark contrast with the time and effort required to master the conventional zona-intact SCNT technique. By looking to these results and to the history of technical advancement of SCNT, one may argue that zona-free SCNT methods may be eventually considered the method of choice.

We obtained successful live birth from cloned sheep embryos derived using ICI-NT and ZI-NT (Kazemi-Ashtiani *et al.* 2008). Moreover, ZF-NT resulted in the live birth of transgenic and non-transgenic clones in cattle and goats (Nasr-Esfahani *et al.* 2011) and term pregnancy of an endangered wild sheep using interspecies cloning (Hajian *et al.* 2011). However, there is yet a place to compare the efficiency of the three techniques in a single study.

Concluding remarks

This study could provide a direct comparison between three common methods of SCNT in sheep. From a technical point of view, enucleation of zona-free oocytes was easy to perform in lesser time compared with zona-intact ones. Secondly, highly efficient fusion of large pool of oocytes reduced both the time and the number of oocytes, which subsequently resulted in similar cloning efficiency compared with zona-intact methods. These results lead to a final conclusion that SCNT protocols have disparate effects on different aspects of cloned embryo development *in vitro*, and the most important effect was the different phenotypes of cloned embryos.

Materials and Methods

Chemicals and media

Unless specified, all chemicals and media were obtained from Sigma Chemical Co. and Gibco respectively.

Recipient oocytes

Abattoir-derived ovine oocytes were used for *in vitro* maturation (IVM) as described previously (Hosseini *et al.* 2012a). In brief, cumulus–oocyte complexes (COCs) were aspirated from antral follicles (2–6 mm diameter) using 20 gauge needles attached to a vacuum pump (80 mmHg). COCs with more than three layers of cumulus cells were selected and washed first in HEPES tissue culture medium 199 (HTCM199) plus 10% self-prepared sheep serum and then in maturation medium before being cultured in the maturation droplets (10–15 COCs/100 µl) under mineral oil at 38.5 °C, 6% CO₂, and humidified air. Maturation medium was TCM199 containing 2.5 mM Na-pyruvate, 1 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 10% sheep serum, 10 µg/ml ovine FSH, 10 µg/ml ovine LH, 1 µg/ml estradiol-17β, 100 µg/ml EGF and 0.1 mM cysteamine. At 22–24 h post-IVM, oocytes were denuded from expanding cumulus cells and only the oocytes with homogenous cytoplasm and first polar body extruded were used for subsequent studies.

Somatic cells

A skin biopsy was prepared from one healthy adult ram and used for *in vitro* culture of fibroblasts as described previously (Hosseini *et al.* 2008). In brief, the biopsy was cut into small explants (2–3 mm²) before being cultured in DMEM/F-12 containing 10% sheep serum and 1% penicillin–streptomycin at 38.5 °C in a humidified atmosphere of 5% CO₂ until confluence. Fibroblast phenotype was verified via an immunostaining method described previously (Jafarpour *et al.* 2011). To obtain a presumptive G0/G1 population of cells for NT, cells were first cultured at 2.5×10^4 cells/cm² and on the next day, the cells were washed three times with PBS before being cultured in medium that contained 0.5% sheep serum for 4–5 days.

SCNT experiments

Oocyte enucleation

Zona-intact oocytes. Denuded oocytes were incubated in HEPES synthetic oviductal fluid free of calcium and magnesium (HSOF[−]) containing CB (7.5 µg/ml) and H33342 for 15 min at normal culture condition of matured oocytes described earlier. As shown in Fig. 4B_{1–3}, enucleation was performed by aspirating a small quantity of cytoplasm directly beneath the first polar body using a beveled (5–10 µm outside diameter) glass pipette under an inverted microscope (Olympus IX71) equipped with Narishige micro-manipulator system. Successful enucleation was confirmed by brief exposure of the expelled cytoplasm to fluorescent light to observe MII chromosomes.

Zona-free oocytes. The procedure of zona-free enucleation was as described previously (Oback *et al.* 2003) with minor modifications. In brief, zona was removed by brief incubation (up to 2 min) of the oocytes with 2.5 mg pronase/ml dissolved in HSOF containing 5% sheep serum. Interestingly, as shown in Fig. 4A_{1–3}, after zona removal, a cone-like cytoplasmic extrusion was observed in 50% of zona-free oocytes. H33342 staining showed that this cytoplasmic extrusion contains metaphase plate, a sign that was not observed in zona-intact oocytes. Therefore, this cytoplasmic extrusion was used as an aid for enucleation. H33342 stain was used for confident enucleation of zona-free oocytes. As shown in Fig. 4C_{1–3}, the metaphase plate was removed using a blunt enucleation pipette (5–10 µm outer diameter, perpendicular break) and a blind separation needle. In each approach the mean volume of cytoplasm removed was recorded as described elsewhere (Nasr-Esfahani *et al.* 2011).

Nuclear transfer

Conventional (zona-intact) method (ZI-NT). Individual fibroblast cells were aspirated into the injection pipette and introduced through the same slit in the zona (performed during enucleation) and wedged between the zona and the cytoplasmic membrane, to facilitate close membrane contact between donor cell and cytoplasm.

Intracytoplasmic injection method (ICI-NT). Donor cells were picked up in HSOF[−] droplets containing 3% (w/v) polyvinylpyrrolidone using a transfer pipette that was narrow enough to isolate the nuclei. The method of cell insertion into the cytoplasm of the oocyte was similar to that described by Lee *et al.* (2003). Owing to critical susceptibility of ovine oocytes to spontaneous activation and fragmentation (Loi *et al.* 1998), care was taken to transfer the donor cell content with the minimum amount of the media into the oocyte.

Standard zona-free method (ZF-NT). As described by Oback *et al.* (2003), nuclear donor cells were suspended in HSOF in 5 ml centrifuge tubes, and through making serial dilutions in three to four droplets, a population of 10–20 individual cells was added to a drop of 10 mg/ml phytohemagglutinin (PHA-P) in HSOF. Oocytes were then individually picked up and gently pushed over a single cell to adhere to it.

Electrofusion, chemical activation, and embryo culture

The integration of donor cell cytoplasm was induced via electrofusion in ZI-NT and ZF-NT methods of SCNT as described

elsewhere (Hosseini *et al.* 2006). In brief, reconstituted oocytes were first equilibrated in fusion buffer (0.3 M mannitol, 100 µM MgSO₄, 50 µM CaCl₂, 500 µM HEPES and 0.05% BSA) for up to 1 min. Then, couplets were located in a fusion chamber (0.5 mm apart electrodes) filled with fusion medium. The couplets were first aligned manually and then using sinusoidal electric current (7 V for 30 s) and then fused with two direct currents of electric pulses (1.75 KV/cm for 80 µs and 1 s delay; Cryologic, Mulgrave, Victoria, Australia). Fused couplets were incubated for up to 4 h in modified synthetic oviductal fluid (mSOF) before chemical activation using an ovine-specific activation protocol described previously (Hosseini *et al.* 2012a). In brief, reconstituted oocytes were first incubated with 2.5 µM ionomycin for 1 min, inactivated by several washes in 30 mg/ml BSA dissolved in HSOF and finally incubated in 6-dimethylaminopurine (2 mM) for 2 h.

Activated couplets were cultured in groups of ten in 20 µl droplets of mSOF containing 5% sheep serum and 8 mg/ml BSA for 7 days. The basic formulation of SOF was based on that used in the study of Tervit *et al.* (1972), with further modifications described elsewhere (Hajian *et al.* 2011). To preclude aggregation of zona-free embryos to each other and also in order to facilitate their group culture, the wells of the wells system of zona-free embryo culture was applied (Vajta *et al.* 2000). Culture conditions were 39 °C, 5% O₂, 5% CO₂, and maximum humidity.

In this study, zona-intact and zona-free parthenogenesis and IVF were considered as controls. The method of PA was the same as the protocol used for artificial chemical activation of cloned embryos. The process of IVF was as described previously (Hosseini *et al.* 2012a). In brief, frozen ram semen straws were thawed in warm water bath (37 °C) and cryoprotectants were removed by centrifugation (10 min at 700 g at RT). The pellet was layered on 45:45 ratio of Puresperm (Nidacon, Gothenburg, Sweden) and centrifuged at 700 g for 15 min to isolate highly motile spermatozoa. Forty to 50 IVM COCs were then incubated with 4 × 10⁵ 200 µl spermatozoa in a droplet of IVF at 39 °C, 5% CO₂ in humidified air for 24 h. Presumptive zygotes in groups of ten were then cultured in 20 µl droplets of mSOF. Developed blastocysts in different groups were used for differential staining as described by Moulavi *et al.* (2006).

Nuclear remodeling

Stepwise assessment of nuclear remodeling was assessed at 2–6 hpr as described previously (Asgari *et al.* 2011). In brief,

Table 3 Specific primers used in this study.

Gene	Primer sequences (forward and reverse; 5'–3')	Length (bp)	Annealing temperature (°C)
GJA1 (1)	TCGTGTCGTTGGTGTCTCTTGGAGGAGCAGCCATTGAAATAAGC	175	61
Cyclin-B (2)	GATTGGAGAGGTTGATGTTGAGAGGTAATGCTGTAGAGTTGGTG	172	62
Na/K ATPase	GCTGACTTGGTCATCTGCAACATTCAGGGCAGTAGGAAA	128	58
HSP90AA1	GCATTCTCAGTTCATTGGCTATCCTTCTATCTCGGGCTTGTCATCAG	175	61
POU5F1	AGAAGGGCAAACGATCAAGCGAATGGGACCGAAGAGTACAGAGT	169	62
GMMN	TGGCAGAGATGATACAGAGGGAACACTACAGCGGAGGTAAAC	223	58
NPM2	GAGGAAGTGAAGAGGAGGATGCAGGGCTCCCTTTAAGATGGTG	202	61
PAPOLA	GAAC TTGCCAGCTTATCCATTGTGGGTATGCTGGTGTA	201	54
GAPDH	TGCCGCTGGAGAAACCTGAAGTCGCAGGAGACAACC	121	60
β-Actin	CCATCGGCAATGAGCGGTCTGTGTTGGCGTAGAGGTC	146	59

reconstituted oocytes were fixed in 4% paraformaldehyde for 15 min. Microtubules were immunostained with anti- β -tubulin monoclonal primary antibody (1:100) and FITC-labeled anti-mouse-IgG secondary antibody (1:100). The chromosomes were counterstained with 2 μ g H333242/ml and then samples were washed and mounted on glass slides in glycerol droplets to be observed using epifluorescence microscope (Olympus BX51) at 400 $\times$ magnification. Upon exposure, a digital image of each sample was taken with a high sensitive camera (Olympus DP-72) operated on DP2-BSW Software.

qRT-PCR

Relative mRNA abundances of eight developmentally important genes (Table 3) were analyzed. For this purpose, 20 oocytes were collected 30–40 min after each manipulation (described in Fig. 3) in each replicate. After several washes in PBS, oocytes in minimum volume of PBS were transferred to RLT (Qiagen) buffer and stored in -70°C until use. Total RNA of oocytes was extracted using the RNeasy Micro Kit (Qiagen). Then 300 ng of each RNA samples was used for the first-strand cDNA synthesis with RevertAid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). cDNA synthesis reactions contained 1 μ l random hexamer primer (0.2 $\mu\text{g}/\mu\text{l}$) 1 μ l RNase inhibitor (20 U/ μl), 4 μ l 5 $\times$ reaction buffer, 2 μ l dNTP mix (10 mM) and 1 μ l M-MuLV reverse transcriptase (200 U/ μl). Final volume was adjusted up to 20 μ l by adding diethylpyrocarbonate (DEPC)-treated water. cDNA synthesis was performed by incubation for 5 min at 25°C followed by 60 min at 42°C and terminated by heating at 70°C for 5 min. The real-time RT-PCR was carried out using the Corbett Rotor Gene 6000 (Qiagen). Each reaction mix contained 25 ng cDNA, 10 μ l SYBR Premix Ex Taq II (2 $\times$; Takara, Otsu, Shiga, Japan), and 1 μ l of each the forward and reverse primers (5 μM) adjusted to a total volume of 20 μ l using nuclease-free water. All reactions were performed in triplicate and normalized to two housekeeping genes (*GAPDH* and β -actin). All data were analyzed by $\Delta\Delta\text{Ct}$ method. For each of the cDNA samples, three replicate PCRs were carried out.

Statistical analysis

Percentage data were modeled to the binomial model of parameters by ArcSin transformation and the transformed data were analyzed by one-way ANOVA model of SPSS-17. Differences were compared by Tukey multiple comparison *post hoc* test. All data were presented as means $\pm$ S.E.M. and differences considered as significant at $P < 0.05$. Additional experiments were carried out in this study to provide adequate blastocysts for differential staining.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

S M Hosseini and M H Nasr-Esfahani conceived and designed the experiments and wrote the paper and F Moulavi, S M Hosseini, M Hajian, M Forouzanfar, S Ostadhosseini, P Abedi and V Asgari performed the experiments.

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