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—Full Paper—

Comparison of the RNA Polymerase I-, II- and III-Dependent Transcript Levels Between Nuclear Transfer and *In Vitro* Fertilized Embryos at the Blastocyst Stage

Toru SUZUKI[1)], Naojiro MINAMI[1)], Tomohiro KONO[2)] and Hiroshi IMAI[1)]

[1)]Laboratory of Reproductive Biology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502 and [2)]Department of Bioscience, Tokyo University of Agriculture, Tokyo 156-8502, Japan

Abstract. Cloned animals have been produced in several mammalian species so far, although success rates to term are very low. Aberrations in gene expression derived from abnormal epigenetic status have been thought to be a cause of developmental abnormalities in clones, and several abnormalities in gene expression have already been detected in cloned animals and embryos. In this study, we examined the hypothesis that the poor survival rates of nuclear transfer (NT) embryos are partly due to aberrations in the regulation of expression of genes transcribed by RNA polymerases I and III, in addition to polymerase II. We produced cloned and *in vitro* fertilized mouse embryos that developed to the blastocyst stage, and the amounts of several genes were analyzed using individual embryos. We found that the amounts of mature 18S ribosomal RNA (rRNA) transcribed by RNA polymerase I were lower in NT embryos than in IVF embryos, but that the amounts of 47S rRNA and intermediates of mature rRNAs were higher in NT embryos. In addition, the amount of 7SK RNA transcribed by RNA polymerase III was lower in NT embryos than in IVF embryos. The transcripts of all but one of the genes transcribed by RNA polymerase II were not noticeably different between NT and IVF embryos. These results suggest that some of the transcripts produced by RNA polymerases I, II and III are aberrantly regulated in NT embryos.

Key words: Blastocyst, Gene expression, Mouse, Nuclear transfer, RNA polymerase I, II and III

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Reprogramming of the nuclei of differentiated cells introduced into mammalian oocytes has been reported, and the reconstructed embryos have been reported to acquire the potential to sustain further development. Using this technique, it is possible to produce viable cloned animals, including mice [1, 2], from somatic cells. However, the success rate of cloning is very low in all species. Nuclear transferred (NT) embryos usually develop serious problems during pre- and post-implantation development that lead to the gradual cessation of development. A high percentage of NT blastocysts stop their development around the period of implantation, even if they are morphologically normal. Thus, analysis of blastocyst stage embryos can provide insights into the failure of NT embryos. Genetic disorders, such as the karyotypic aberrations reported primarily by Di Berardino and King in a frog nuclear transfer study [3], are a candidate cause of the low viability of NT embryos. However, abnormal epigenetic regulation has often been considered to be the main cause of the developmental problems of NT embryos. In fact, DNA methylation, a common type of epigenetic regulation, has been reported to

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Correspondence: H. Imai (e-mail: imai@kais.kyoto-u.ac.jp)

be different between NT and fertilized embryos [4–6]. Furthermore, it has been reported that the abnormal phenotypes detected in cloned animals are not transmitted to the descendants of viable clones [7], implying that some of the abnormal phenotypes of clones are derived from restorable aberrations, which suggests that they are epigenetic abnormalities. Finding common abnormalities related to epigenetic regulation in NT embryos and elucidating the cause of these abnormalities could help in understanding nuclear reprogramming and its failure during somatic cloning.

Many studies concerning gene expression of NT embryos have been performed at the blastocyst stage. In NT blastocysts, *Oct4*, whose expression is a marker of pluripotency, has been shown to be incompletely reactivated [8, 9]. *Oct4* transcripts, which normally localize to the inner cell mass (ICM) at the blastocyst stage, have been detected ectopically in a trophoblast cell lineage of NT embryo [10]. Some imprinted genes have been found to be aberrantly expressed [6]. Smith *et al*. compared the global gene expression profiles of individual bovine NT blastocysts with their somatic donor cells and fertilized control embryos using cDNA microarray technology and demonstrated that 2–3% of about 20,000 genes were aberrantly regulated in NT embryos [11]. Although these studies have mainly focused on RNA polymerase (RNA pol) II-transcribed genes, two other RNA polymerases, namely RNA pol I and III, are also involved in RNA synthesis in eukaryotes. Like RNA pol II, RNA pol I and III produce specific types of RNA that play crucial roles in cellular processes, such as protein synthesis, protein transport, and regulation of transcription [12–14]. Mammalian RNA pol I synthesizes a 47S ribosomal RNA (rRNA) long precursor, which is thereafter processed into the mature 18S, 5.8S and 28S rRNAs through several rRNA intermediates [15]. RNA pol III synthesizes relatively short RNAs, including tRNA, 5S rRNA, 7SK RNA, 7SL RNA and some repetitive elements in mice, such as *SINE B1* and *B2* [16, 17]. The expression levels of some RNA pol I- and III-dependent transcripts are regulated according to a specific cell state [13, 16, 18, 19]. Epigenetic regulation of gene expression, such as regulation by DNA methylation and histone modification, affects the abundance of not only RNA pol II-dependent transcripts but also some RNA pol I- and III-dependent transcripts [20–22]. In view of the fact that abnormal epigenetic regulation is a major cause of death of NT embryos, it is possible that there are some abnormalities in NT embryos in expression of RNA pol I- and III-dependent transcripts. Despite their biological importance, the roles of RNA pol I- and III-dependent transcripts in NT embryos have received little attention.

In this study, we investigated transcripts produced by all three RNA polymerases and compared the expression of these transcripts between nuclear transfer and *in vitro* fertilized (IVF) blastocysts using RT-PCR.

Materials and Methods

Experimental animals

All animal experiments were performed in accordance with the Institutional Animal Care and Use Committee of Kyoto University.

Medium

CZB medium [23] supplemented with 5.5 mM glucose (CZB-G) was used to culture oocytes and embryos in a humidified atmosphere containing 5% CO_2 and 95% air at 37 C. Chemical activation of oocytes was carried out in CZB-G without Ca^{2+}. HEPES-buffered CZB-G (CZB-H) was used for micromanipulation of oocytes outside the incubator. Enucleation of oocytes was performed in CZB-G containing 5 μg/ml cytochalasin B. TYH medium [24] was used for preincubation of sperm and *in vitro* fertilization. For electrofusion during the NT procedure, 275 mM D(–)-mannitol (pH 7.2) supplemented with 1 mM $MgSO_4$, 0.1 mg/ml of polyvinyl alcohol and 3 mg/ml of BSA was used.

NT and IVF embryo production and collection

NT and IVF embryo production were performed as described previously [25]. Unfertilized oocytes were collected from superovulated 8- to 10-week-old BDF1 female mice treated with eCG and hCG. For NT embryos, unfertilized MII oocytes were enucleated and electrofused with cultured BDF1 male tail tip cells at the G0/G1 phase using an ECM 2001 electro cellmanipulator (BTX, San Diego, CA, USA). Fusion was performed using two DC pulses of 16 V for 10 μsec. At least 1 h after electrofusion, the reconstructed oocytes were activated using 10 mM SrCl without Ca^{2+} for 5 h. For IVF embryos,

Table 1. Primers used for RT-PCR

Genes	Accession No.		Primer sequence (5′-3′)	Annealing temperature (C)
Oct4	NM_013633	Sense	TGCCGTGAAGTTGGAGAAGGTG	64
		Antisense	GCTGATTGGCGATGTGAGTGAT	
Cdx2	NM_007673	Sense	AAACCTGTGCGAGTGGATG	64
		Antisense	CTGCGGTTCTGAAACCAAAT	
β-actin	NM_007393	Sense	CCACCACAGCTGAGAGGGAA	64
		Antisense	AGCCACCGATCCACACAGAG	
IAP	M17551	Sense	CAGATCCTTCGGGAATTGAGACTTC	72
		Antisense	CAGTGCTTAGCCGTGCAACTCTCT	
LINE	AC107828	Sense	AGTGCAGAGTTCTATCAGACCTTC	66
		Antisense	AACCTACTTGGTCAGGATGGATG	
ETn	AC110544	Sense	CCAAGAGGCTGGGTCTCTAA	60
		Antisense	AACCTTGGGAAAGCAAAACC	
47S rRNA	V00850	Sense	CTCCTGTCTGTGGTGTCCAA	55
		Antisense	GCTGGCAGAACGAGAAGAAC	
*rRNAIM**	K01365	Sense	TGCAGGACACATTGATCATCGACA	55
		Antisense	AACCGCCCACACGTCTGAAC	
28S rRNA	X00525	Sense	GGGCGAAAGACTAATCGAAC	55
		Antisense	CCTGCTGTCTATATCAACCAAC	
18S rRNA	X00686	Sense	CGCGGTTCTATTTTGTTGGT	55
		Antisense	AGTCGGCATCGTTTATGGTC	
5.8S rRNA	J01871	Sense	CTCTTAGCGGTGGATCACTC	55
		Antisense	GATGATCAATGTGTCCTGCAA	
5S rRNA	M31319	Sense	ACGGCCATACCACCCTGAAC	55
		Antisense	CGGTCTCCCATCCAAGTACTAACC	
7SK RNA	M63671	Sense	GACATCTGTCACCCCATTGA	60
		Antisense	GCGCAGCTACTCGTATACCC	
7SL RNA	AC099934	Sense	GTTGCCTAAGGAGGGGTGA	60
		Antisense	GTGCAGTGGCTATTCACAGG	
SINE B1	Reference [17]	Sense	TGGTGGTGCATGCCTTTAAT	55
		Antisense	CCTGGTGTCCTGGAACTCACT	
SINE B2	Reference [17]	Sense	GGCTGGTGAGATGGCTCAGT	55
		Antisense	TACACTGTAGCTGTCTTCAGACA	
α-globin	V00875	Sense	GCAGCCACGGTGGCGAGTAT	64
		Antisense	GTGGGACAGGAGCTTGAAAT	

* Ribosomal RNA intermediates are referred to as rRNAIM.

unfertilized oocytes were inseminated using preincubated sperm from 10-week-old BDF1 male mice for 6 h. The activated and fertilized oocytes were transferred to fresh CZB-G medium and cultured for 4 days to the blastocyst stage. Ninety-six h after activation or fertilization, individual embryos were collected in 1.7 ml tubes and frozen at –80 C until use.

RNA extraction and RT-PCR

RNA extraction and RT-PCR were performed as described previously with minor modifications [25]. Twelve or 16 embryos were analyzed for each gene. Total RNA was extracted from individual blastocysts at 96 h after activation or fertilization and supplemented with 5 pg of rabbit α-globin RNA as an external control using a Totally RNA Kit (Ambion, Austin, TX, USA). Briefly, denatured samples were extracted by 1:1 acid phenol/chloroform and precipitated using isopropyl alcohol and 40 μg of glycogen. The RNA pellet was vacuum dried and dissolved in DEPC-treated water. After DNase I treatment, cDNA was synthesized from total RNA using random hexamer (Invitrogen, Carlsbad, CA, USA) and Superscript III (Invitrogen) according to manufacturer's instructions. PCR reactions were conducted using 0.1 to 1 μl (30 μl), 0.25 unit of Ex Taq (Takara, Ohtsu, Japan) and gene specific primers (Table 1). Each cycle of PCR consisted of 15 sec at 94 C for DNA denaturing, 30 sec at the gene specific temperature (Table 1) for primer annealing

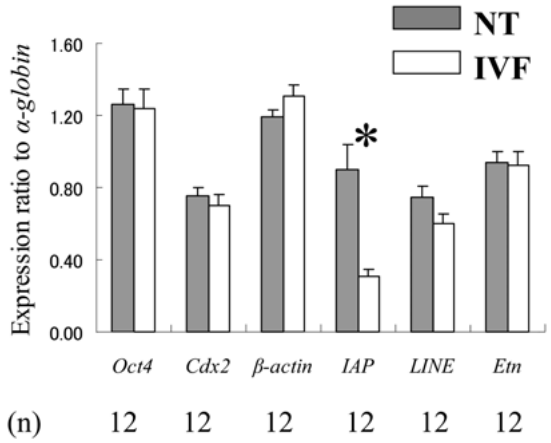

Fig.1. Expression levels of RNA polymerase II-dependent transcripts in individual NT and IVF embryos at the blastocyst stage. The intensities of the RT-PCR products for each transcript were normalized to that of rabbit α-globin as an external control. The asterisk indicates a significant difference (P<0.05) between the mean levels of transcripts in the NT and IVF embryos. The error bars indicate the SEM.

Table 2. Expression ratios of genes in the NT and IVF embryos

Gene	Standard	NT/IVαF ratio
Oct4	*α-globin*	1.02
Cdx2	*α-globin*	1.07
β-actin	*α-globin*	0.91
IAP	*α-globin*	3.00*
LINE	*α-globin*	1.25
ETn	*α-globin*	1.02
47S rRNA	*α-globin*	1.21*
rRNAIM	*α-globin*	1.44*
28S rRNA	*α-globin*	0.81
18S rRNA	*α-globin*	0.76*
5.8S rRNA	*α-globin*	0.80
rRNAIM	*47S rRNA*	1.27*
28S rRNA	*47S rRNA*	0.70*
18S rRNA	*47S rRNA*	0.70*
5.8S rRNA	*47S rRNA*	0.70*
5S rRNA	*α-globin*	0.93
7SK RNA	*α-globin*	0.74*
7SL RNA	*α-globin*	0.81
SINE B1	*α-globin*	1.26
SINE B2	*α-globin*	0.90

Asterisks show significant differences between the NT and IVF embryos (P<0.05). Ribosomal RNA intermediates are referred to as rRNAIM.

and 30 sec at 72 C for elongation. The number of PCR cycles was adjusted to the log phase of amplification of the PCR products for each gene. The PCR products amplified were electrophoresed in 2–3% agarose gel and stained with ethidium bromide for 10 min. Densitmetric analysis was performed using a model 6.0 Atto densitograph (Atto, Tokyo, Japan). Relative intensity was expressed as the ratio of the intensity of the PCR products relative to the intensity of α-globin.

Statistical analysis

The relative amount of gene expression was compared using the *t*-test following ANOVA. All analyses were conducted using the general linear model (GLM) procedure of Statistical Analysis Systems (SAS Institute, Cary, NC, USA). A P value of less than 0.05 was considered statistically significant.

Results

RNA pol II transcription

To examine the expressions of RNA pol II-dependent transcripts, we selected two genes critical for embryonic development (*Oct4* and *Cdx2*), a housekeeping gene (*β-actin*) and retrotransposons known to be expressed in mouse preimplantation stages (*IAP*, *LINE* and *ETn*) [26–28]. Each of these genes was detected in all NT embryos, although there were significantly more IAP transcripts in the NT embryos than in the IVF embryos (Fig. 1, Table 2).

RNA pol I transcription

To examine the expressions of RNA pol I-dependent transcripts, we used *47S rRNA*, rRNA intermediate (*rRNAIM*), *28S rRNA*, *18S rRNA* and *5.8S rRNA*. The expression level of unprocessed *47S rRNA* (Fig. 2) was significantly higher in the NT embryos than in the IVF embryos (Fig. 3A, Table 2). After transcription, 47S rRNA is processed to mature rRNA by two alternative pathways in mouse cells (Fig. 2B) [29, 30]). We designed PCR primers to amplify the partial sequence containing both *5.8S rRNA* and internal transcribed spacer 2 (ITS2) (Fig. 2A) in order to detect a semi-processed rRNAIM (Fig. 2B). Expression of rRNAIM was higher in the NT embryos than in the IVF embryos (Fig. 3A, Table 2). To examine whether the high expression of unprocessed 47S rRNA and rRNAIM in NT embryos affects the levels of three-processed

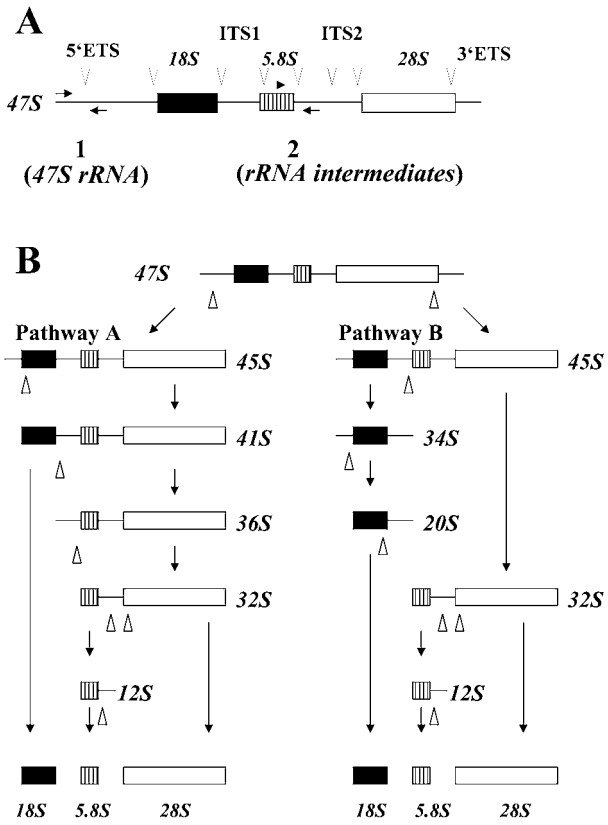

Fig. 2. Major pre-rRNA processing pathways in mouse cells. (A) The structure of the primary *47S rRNA* transcript and positions of the major processing sites are shown. The positions of the primer pairs used in RT-PCR to amplify 47S rRNA and the rRNA intermediates are indicated (arrows). Arrowheads indicate major processing sites. ETS is the external transcribed region. ITS is the internal transcribed spacer. (B) Flowchart of mouse pre-rRNA processing. The 47S pre-rRNA is processed through intermediates into mature 18S, 28S and 5.8S rRNAs.

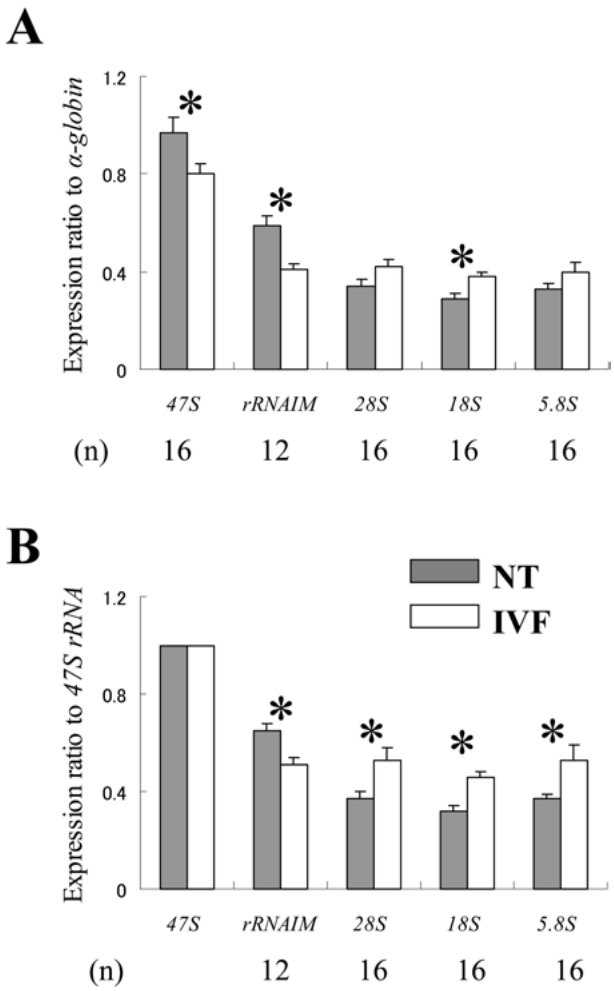

Fig. 3. Expression levels of RNA polymerase I-dependent transcripts in individual NT and IVF embryos at the blastocyst stage. (A) The intensities of the RT-PCR products for each transcript were normalized to that of rabbit α-globin as an external control. Asterisks indicate significant differences ($P<0.05$) between the mean levels of transcripts in the NT and IVF embryos. (B) The intensities of the RT-PCR products for each transcript were normalized to that of the *47S rRNA* transcript. Asterisks indicate significant differences ($P<0.05$) between the mean levels of transcripts in the NT and IVF embryos. Ribosomal RNA intermediates are referred to as rRNAIM. The error bars indicate the SEM.

rRNA (28S, 18S and 5.8S rRNA), we performed semi-quantitative RT-PCR for these 3 mature rRNA. The expression of 18S rRNA was lower in the NT embryos than in the IVF embryos and the expressions of 28S and 5.8S rRNA in the two types of embryos were the same (Fig. 3A, Table 2). When unprocessed 47S rRNA was used as a standard, the expressions of the 3 mature rRNAs were lower in the NT embryos than in the IVF embryos (Fig. 3B, Table 2).

RNA pol III transcription

We used *5S rRNA*, *7SK*, *7SL*, *SINE B1* and *SINE B2* to examine the expressions of RNA pol III-dependent transcripts. Among the transcripts examined, the expression level of *7SK* was lower in the NT embryos than in the IVF embryos (Fig. 4, Table 2). No significant differences were observed in the expression levels of the other transcripts between the NT and IVF embryos (Fig. 4, Table 2).

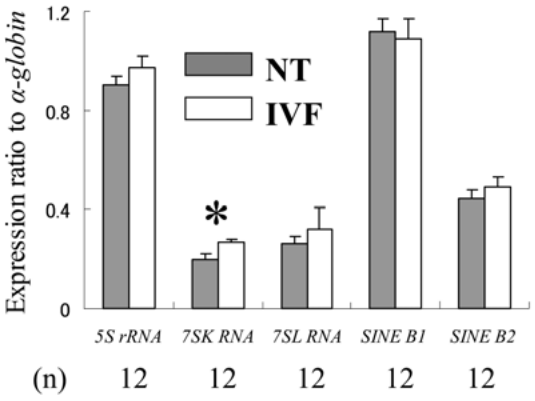

Fig. 4. Expression levels of RNA polymerase III-dependent transcripts in individual NT and IVF embryos at the blastocyst stage. The intensities of the RT-PCR products for each transcript were normalized to that of rabbit α-globin as an external control. The asterisk indicates a significant difference ($P<0.05$) between the mean levels of transcripts in the NT and IVF embryos. The error bars indicate the SEM.

Discussion

The success rates of viable cloned animals have been very low for all species reported [31]; this is probably due to abnormal gene expression caused by insufficient reprogramming of donor nuclei [32]. To elucidate the viability of NT embryos in terms of gene expression, it is important to detect the cause of the abnormality by comparative analysis between NT and fertilized embryos.

The objective of this study was to compare the expression patterns of genes transcribed by each of the three RNA polymerases in individual mouse NT and IVF embryos in order to detect abnormalities in the NT embryos at the blastocyst stage. The results demonstrate that some of the transcripts produced by RNA pol I, II and III are aberrantly regulated in mouse NT embryos. Among the genes transcribed by RNA pol II that were examined, expression of *IAP* retrotransposon was stronger in NT embryos than in IVF embryos. Upregulation of the retrotransposon sequence appears to be specific to *IAP* in this study because the expression levels of other retrotransposons, such as *LINE* and *ETn*, were similar in the NT and IVF blastocysts. Expression of *IAP* has been reported to be regulated by DNA methylation during mouse development [33]. Some type of epigenetic misregulation may be involved in the abnormal expression of *IAP* in NT embryos that we observed in the present study.

We were unable to detect any differences in the expression of *Oct4* and *Cdx2* between NT and IVF blastocysts. The expression of *Cdx2* in NT blastocysts has been shown to be normal compared with those of in a previous study [34]. In contrast to *Cdx2*, it has been reported that the amounts of *Oct4* transcript and protein are reduced in NT blastocysts compared with those of fertilized blastocysts [8, 9, 34]. Wrenzycki *et al*. reported that the nuclear transfer protocol affects the messenger RNA expression patterns of cloned bovine blastocysts [35]. Differences in the methods of producing NT embryos between this and other reports may lead to different results for the gene expression pattern of *Oct4*.

We also observed that the amount of 18S rRNA decreased and that the amount of unprocessed 47S rRNA and semi-processed rRNA intermediates increased in the NT embryos. In addition, the expression ratios of three mature rRNAs (28S, 18S and 5.8S) relative to unprocessed 47S rRNA in the NT embryos were significantly lower than those in the IVF embryos. These results suggest that the accumulation of unprocessed 47S rRNA and rRNA intermediates is due to failure of RNA processing, and as a result, some mature rRNAs, such as 18S RNA, are not sufficiently produced in NT blastocysts. NT embryos may also have some defects in protein synthesis as a result of abnormal rRNA production because rRNA plays an important role in translation. In addition, it has been reported that rRNA plays an important role in cell proliferation [13]. The reduced production of mature rRNA detected in this study may explain the low cell numbers in mouse NT blastocysts [25, 36].

Transcription of rRNA, pre-rRNA processing and ribosome assembly occur in nucleoli. Ribosomal RNA genes are transcribed by RNA pol I to produce the 47S rRNA precursor, which is processed into three mature rRNAs (28S, 18S and 5.8S) soon after transcription. These mature rRNA and RNA pol III-dependent 5S rRNA form ribosomes with ribosomal proteins. Ribosomal RNA processing requires many proteins and RNAs. It is possible that the expression and function of these types of proteins and RNAs are unregulated in NT embryos, resulting in

dysfunction of the rRNA production mechanism. Based on the observation that rRNA transcription and pre-rRNA processing are coupled [37], these two processes may not be properly coordinated in NT embryos.

The small nuclear RNA 7SK RNA is transcribed by RNA pol III. It is implicated in repression of RNA pol II-dependent transcription by inhibiting positive transcriptional elongation factor b (P-TEFb), which consists of a CDK9/cyclin T1 heterodimer [38, 39]. By binding to 7SK RNA, HEXIM1 protein becomes capable of interacting with P-TEFb [40]. Although P-TEFb stimulates transcription by phosphorylating RNA polymerase II, it becomes inactivated when associated with HEXIM1/7SK [40]. The suppression of 7SK in the NT embryos in this study may cause abnormal expression of other genes in NT embryos.

Based on the reports that most NT embryos cease development at the pre- and peri-implantation stages [1, 41], it is probable that the accumulation of different abnormalities observed in NT embryos at various developmental stages, such as failure to erase some of the features of donor cells [42–44], abnormal gene expression [4, 8, 10, 25, 45–47], abnormal DNA methylation [4–6], abnormal formation of placentae [1, 41, 48–50], and the abnormalities detected in this study, leads to gradual loss of NT embryos. Abnormalities that have not been detected in previous studies and/or small differences in gene expression may seriously affect embryonic development. Examinations of abnormal gene expression in NT embryos should consider not only genes transcribed by RNA pol II but also those transcribed by RNA pol I and III.

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