In Vitro Development and Mitochondrial Fate of Macaca–Rabbit Cloned Embryos

CAI-XIA YANG, ZHI-MING HAN, DUAN-CHENG WEN, QING-YUAN SUN, KE-YING ZHANG, LI-SHENG ZHANG, YU-QI WU, ZHAO-HUI KOU, AND DA-YUAN CHEN*
State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China

ABSTRACT   Interspecies cloning may be used as an effective method to conserve highly endangered species and to support the development of non-human primate animal models for studying therapeutic cloning and nuclear–cytoplasm interaction. The use of the monkey model for biomedical research can avoid legal, ethical, and experimental limitations encountered in a clinical situation. We describe in this study the in vitro development of macaca–rabbit embryos produced by fusing macaca fibroblasts with enucleated rabbit oocytes and examine the fate of mitochondrial DNA in these embryos. We show that macaca–rabbit cloned embryos can develop to the blastocyst stage when cultured in vitro in $HECM_{10}$ +10% FBS and that mitochondrial DNA derived from donor somatic cells was detectable in cloned embryos throughout pre-implantation development. These results suggest that (1) macaca fibroblast nuclei can dedifferentiate in enucleated metaphase II rabbit oocytes; (2) $HECM_{10}$ +10% FBS can break through the development block and support the development of macaca–rabbit cloned embryos to blastocysts; and (3) donor-cell-derived mitochondrial DNA is not eliminated until blastocyst stage. *Mol. Reprod. Dev. 65: 396–401, 2003.*
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Key Words: *Macaca mulatta*; interspecies cloning; in vitro development; mitochondria

INTRODUCTION

During the past several years, remarkable progress in mammal somatic cloning has been achieved. Many kinds of intraspecies somatic cloned mammals including sheep, mouse, cattle, goat, pig, rabbit, and cat have been obtained (Wilmut et al., 1997; Wakayama et al., 1998; Kato et al., 1998; Baguisi et al., 1999; Polejaeva et al., 2000; Chesne et al., 2002; Shin et al., 2002). At the same time, interspecies somatic cloned gaur and mouflon (Lanza et al., 2000; Loi et al., 2001) have been successfully obtained. Recently, we reported the successful implantation of panda–rabbit cloned embryos in the uterus of a third species, the domestic cat (Chen et al., 2002). Interspecies cloning is considered to be an effective method to conserve highly endangered species and also can support the development of non-human primate models for studying therapeutic cloning and nuclear–

cytoplasm interaction. This model also can avoid legal, ethical, and experimental limitations encountered in a clinical situation.

Rhesus monkeys produced by nuclear transfer (NT) from embryonic blastomeres have been reported (Meng et al., 1997) and intraspecies somatic cell cloned monkey embryos can develop to the blastocyst stage in vitro (Mitalipov et al., 2002). Two other reports have confirmed the ability of bovine and sheep oocyte cytoplasm to support early development under the direction of differentiated somatic cell nuclei of various mammals (Dominko et al., 1999; White et al., 1999). In our previous study, we reported that giant panda somatic cell nuclei could dedifferentiate in rabbit ooplasm and support early development following NT (Chen et al., 1999). At this point in time, it is unclear whether or not NT embryos reconstructed between primate adult somatic cell and enucleated rabbit oocyte can develop to advanced embryonic stages, and whether or not interspecies cloned macaca–rabbit embryos would harbor only the mitochondria from recipient oocytes. In the present study, we describe the production of interspecies cloned monkey embryos derived from adult fibroblasts, and examine mitochondrial fate of macaca–rabbit cloned embryos prior to implantation.

MATERIALS AND METHODS
Animals

Mature female Japanese Big Eared white rabbits were purchased from the Laboratory Animal Center, Institute of Zoology, Chinese Academy of Sciences and housed in stainless steel cages. Fodder and water were provided ad libitum. The collection and use of Taihang macaca (*Macaca mulatta*) ear tissues were approved by the ethical committee of the State Key Laboratory of

Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences.

Culture and Cycle Analysis of Donor Adult Fibroblasts

The method of cell culture and assessment has been described previously (Han et al., 2001). Briefly, skin biopsy specimens derived from a 6-year-old male and two 7-year-old female macaque were finely chopped into pieces measuring about 1 mm^2 and digested with 0.25% (w/v) trypsin (Gibco BRL, Grand Island, NY) for 12 hr at 4°C and then for 30 min at 37°C. The digested cells and tissues were seeded into 75-cm^3 cell culture flasks containing Dulbecco's modified Eagle's medium/F12 (DMEM/F-12; Gibco) supplemented with 20% fetal bovine serum (FBS; Gibco) and cultured in a 5% CO$_2$ incubator at 37°C. After reaching 75–85% confluency, monolayers of the primary cells with spindle-shaped morphology were disaggregated for further culture. Cells at passage 4–10 were used as donors. After reaching 90–100% confluency, the cells were disaggregated with 0.25% (w/v) trypsin and then resuspended in DMEM; one part of which was isolated for NT, another was used for flow cytometric analysis of the cell cycle, and for further culture.

Cells for cell cycle analysis were pelleted by centrifugation (5 min at 130g), resuspended in 0.1 ml of Ca^{2+} and Mg^{2+} Dulbecco PBS (PBS; Invitrogen), and slowly mixed with 3 ml of 100% ethanol (4°C). After ethanol fixation (at least 12 hr at 4°C), cells were pelleted, washed once with PBS, treated with PBS containing 0.2 mg/ml RNase A for 20 min at 37°C, and stained in PBS containing 0.1% (v/v) Triton X-100 and 10 µg/ml propidium iodide for 10 min at room temperature. Stained cells were filtered through a 30-µm nylon mesh (Spectrum, Los Angeles, CA) just prior to flow cytometry. Cycle analysis of stained cells was performed using a fluorescence-activated cell sorting (FACS) Calibur flow analysis (Boquest et al., 1999).

Preparation of Recipient Oocytes

Mature female Japanese Big Eared white rabbits were superovulated by administering PMSG and hCG (Institute of Zoology, Chinese Academy of Sciences). Each rabbit was injected with 150 IU PMSG and with 100 IU hCG 4 days after the PMSG injection. Rabbits were killed 14 hr after the hCG injection. Mature MII oocytes were collected by flushing the oviducts with M$_2$ medium (Sigma Chemical Company, St. Louis, MO). After exposure to 300 IU/ml hyaluronidase (Sigma) for 3–5 min (Sigma), cumulus cells were stripped from the oocyte by repeated gentle pipetting (Li et al., 2002).

NT Procedure

NT was conducted as previously described (Chen et al., 1999, 2002). Cumulus-free eggs were incubated for 15 min in M$_2$ medium containing 7.5 µg/ml cytochalasin B (CB; Sigma), 7.5 µg/ml Hoechst 33342 (Sigma), and 10% FBS before enucleation. The first polar body and a small amount of the underlying cytoplasm containing the meiotic spindle was aspirated using a 20–25 µm enucleation pipette. The aspirated karyoplast was examined with a fluorescence microscope to confirm the presence of metaphase II chromosomes. Only the oocytes from which all the chromosomes were removed were used for NT. A single adult macaque somatic cell was selected and transferred into the perivitelline space of the enucleated oocyte. Fusion of the fibroblast–oocyte pair was induced by two 80-µsec DC pulses of 1.4 kV/cm or 1.2 kV/cm (ECM2001 Electrocell Manipulator; BTX, Inc., San Diego, CA) in 0.25 M D-Sorbitol buffer containing 0.1 mM calcium acetate, 0.5 mM magnesium acetate, 0.5 mM Hepes, and 100 mg/ml of BSA (Mitalipov et al., 1999).

Activation and In Vitro Culture of Reconstructed Eggs

Fused complexes were activated either by two DC pulses of 1.4 kV/cm, two DC pulses of 1.2 kV/cm, or by exposure to 2.5 mM dimethylaminopurine for 5 hr. Activated NT embryos were placed in TCM199 medium or HECM$_{10}$ medium supplemented with 10% FBS. Embryonic development was observed daily.

Chromosome and Mitochondrial DNA Analysis

Chromosome analysis of donor and recipient somatic cells was conducted according to a method described previously (Lanza et al., 2000). Macaque–rabbit reconstructed blastocysts were treated with colcemid (0.04 µg/ml) for 5 hr in a 5% CO$_2$ incubator at 37°C, transferred to 0.075 M KCl for 40 min and then placed on slides. Single drops of freshly prepared fixative (methanol:glacial acetic acid 3:1) were added to the slides. After air-drying,

TABLE 1. Cell Cycle of Macaca Adult Fibroblasts at Different Passages in Culture

Degree of confluency	Passage number of cultured cells	Cell cycle phase (mean ± SD)		
		G$_0$ + G$_1$ (%)	S (%)	G$_2$ + M (%)
90–100%	4	95.9950 ± 0.0354[a]	3.5950 ± 0.6010[a]	0.4050 ± 0.5728[a]
	5	95.5500 ± 1.4142[a]	3.7050 ± 0.4031[a]	0.7450 ± 1.0253[a]
	6	93.3650 ± 1.2799[a]	6.6500 ± 1.2589[a]	0 ± 0[a]
	8	92.4233 ± 5.9521[a]	6.4400 ± 6.1072[a]	1.1400 ± 0.2000[a]
	9	97.0000 ± 1.0748[a]	1.9650 ± 0.2899[a]	1.0300 ± 1.3576[a]
	10	94.3900 ± 1.1464[a]	4.3100 ± 0.3900[a]	1.3000 ± 1.4117[a]

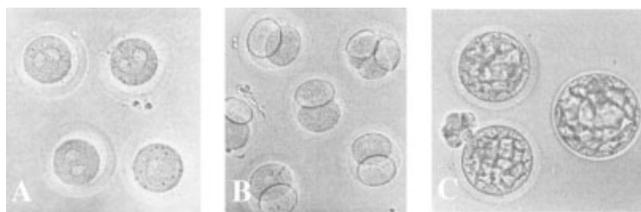Percentages with different superscripts within a column differ significantly ($P < 0.05$).

Fig. 1. In vitro development process of interspecies NT macaca embryos reconstructed with adult somatic cell. (**A**) Somatic cell NT embryos at the pronuclear stage (×200); (**B**) somatic cell NT embryos at the 2-cell to 4-cell stage (×200); (**C**) somatic cell NT embryos at the blastocyst stage (×400).

slides were stained in Giemsa stain and observed with a light microscope.

A region of the mitochondrial DNA cytochrome b (*cytb*) gene was analyzed by PCR with two sets of primers. The macaca primers were: forward 5′AAT-CCAACCCAATCATAAAAATAA3′, and reverse 5′GAA-GGGTAGGATAAAGTGTAAGGT3′. Amplification was performed at 94°C for 5 min, 94°C for 1 min, 58°C for 1 min, 72°C for 1.5 min for 35 cycles. The rabbit primers were: forward 5′TCTACATACACGTAGGCCGCGGA-A3′, and reverse 5′GAGGAGAAGAATGGCTACAAGG-AAA3′. Amplification was performed at 94°C for 5 min, 94°C for 1 min, 65°C for 1 min, 72°C for 1.5 min for 35 cycles. The final PCR products were separated by agarose electrophoresis and purified. The purified PCR products were sequenced by an automatic DNA sequencer (ABI377, Perkin Elmer).

Statistical Analysis

Results expressed as mean ± SD or percentage were analyzed using SAS software package. $P < 0.05$ was considered statistically significant difference.

RESULTS
Cell Cycle of Donor

In this study, we analyzed the cell cycle of donor cells that were at 90–100% confluency but not treated with serum starvation or any reagents. The percentage of G_0/G_1 cells was over 90% and the difference between any two passages at 90–100% confluency was not significant ($P > 0.05$; Table 1).

In Vitro Development of Reconstructed Embryos

Macaca fibroblast nuclei were transferred into enucleated metaphase II rabbit oocytes. About half of the fibroblast–oocyte pairs could be fused. Six hr following

activation, pronuclear formation was observed, and after being cultured for 5 days, 5.38–11.00% of the reconstructed embryos developed to the blastocyst stage (Fig. 1). No significant difference of fusion rate (53.96% vs. 43.67%) and development rate was observed between the two different fusion voltages employed here (1.20 kV/cm and 1.40 kV/cm). When macaca–rabbit cloned embryos were activated with three activation treatments (1.20 kV/cm, 1.40 kV/cm, 6-DMAP), higher 2-cell development rate was observed in 6-DMAP group compared to the other two groups. Subsequent development of embryos to the blastocyst stage was similar in all three groups. No significant difference for the rate of development from the 2-cell to the blastocyst stage was observed ($P > 0.05$; Table 2).

The development capacity of NT embryos cultured in two different culture media (M199 +10% FBS and $HECM_{10}$ +10% FBS) was compared. NT embryos cultured in M199 +10% FBS, which was used to culture the recipient rabbit eggs, were arrested at 4- to 8-cell stage and no 8-cell stage embryos were obtained. Whereas, reconstructed embryos cultured in $HECM_{10}$ +10% FBS could break through the development block and 8.30% developed to blastocyst stage (Table 3).

Chromosome Analysis of Donor Cell, Recipient Somatic Cell, and Reconstructed Blastocysts

Five reconstructed blastocysts were randomly selected for chromosome examination. Chromosome number of a reconstructed blastocyst was the same as that of macaca somatic cell (2n = 42) and different from that of rabbit somatic cell (2n = 44), indicating the origin of the genetic material of reconstructed embryos (Fig. 2).

Fate of Mitochondrial DNA in Reconstructed Embryos

In order to evaluate the fate of mitochondrial DNA in macaca–rabbit cloned embryos, specimens at the 1-cell to the blastocyst stages were examined by PCR amplification. Mitochondrial DNA from the macaca could be detected in all reconstructed embryos at various developmental stages (Fig. 3). PCR products of macaca somatic cells and those macaca–rabbit cloned embryos were sequenced and shown to be identical. Rabbit mitochondrial DNA was observed in reconstructed embryos as well (Fig. 4).

DISCUSSION

In this study, we described the development potential of macaca–rabbit embryos produced by NT from adult

TABLE 2. In Vitro Development of Macaque–Rabbit NT Embryos Treated With Different Activation Protocols*

Activation	Nuclear transfer (NT) units	Fused n (%)	2-Cell n (%)	4-Cell n (%)	8-Cell n (%)	Morulae n (%)	Blastocysts n (%)
1.20 kV/cm	244	130 (53.28)[a]	84 (64.62)[a]	41 (31.54)[a]	19 (14.62)[a]	14 (10.77)[a]	7 (5.38)[a]
1.40 kV/cm	229	100 (43.67)[a]	61 (61.00)[a]	37 (37.00)[a]	20 (20.00)[a]	14 (14.00)[a]	11 (11.00)[a]
6-DMAP	84	47 (55.95)[a]	36 (76.60)[a]	13 (27.66)[a]	9 (19.15)[a]	7 (14.89)[a]	5 (10.64)[a]

*Fused (%), fused eggs/NT units; development rate of embryos at different stages, number of embryos/number of fused eggs. Percentages with different superscripts within a column differ significantly ($P < 0.05$).

TABLE 3. In Vitro Development of Macaca–Rabbit NT Embryos Cultured in Two Different Culture Media*

Culture medium	NT units	Fused n (%)	2-Cell n (%)	4-Cell n (%)	8-Cell n (%)	Morulae n (%)	Blastocysts n (%)
M199 + 10% FBS	80	40 (50.00)[a]	17 (42.50)[a]	4 (10.00)[a]	0	0	0
HECM$_{10}$ + 10% FBS	557	277 (49.73)[a]	181 (65.34)[a]	91 (32.85)[b]	38 (13.72)	35 (12.64)	23 (8.30)

*Fused (%), fused eggs/NT units; development rate of embryos at different stages, number of embryos/number of fused eggs.
[a,b]Percentages with different superscripts within a column differ significantly ($P < 0.05$).

macaca somatic cells. We have shown that macaca–rabbit cloned embryos can develop to the blastocyst stage in vitro, suggesting that macaca fibroblast nuclei can dedifferentiate in enucleated metaphase II rabbit oocytes.

The cell cycle synchrony between donor nucleus and recipient cytoplast is regarded to be important for reprogramming of nucleus and successful NT. The production of live offspring from adult and embryonic sheep cell lines was reported to be possible only when cells at the G_0 phase of the cell cycle were employed (Wells et al., 1997; Wilmut et al., 1997). More recent studies, however, have demonstrated that the cell nuclei at the G_2/M-stage can direct reconstructed embryos to develop to the blastocyst stage (Lai et al., 2001). In the present study, adult somatic cells of 90–100% confluency without any treatments were used as donors. Macaca–rabbit reconstructed embryos could develop to blastocyst stage, which suggests that it is unnecessary to arrest the donor cells at G_0/G_1 stage in macaca–rabbit interspecies somatic cloning protocol.

When the donor somatic nucleus is transferred into an enucleated MII oocyte with high MPF activity, NEBD and PCC occur, while cytoplast activation results in a decline of MPF activity to basal levels. The development capacity of NT embryos activated with three different treatments (1.20 kV/cm, 1.40 kV/cm, 6-DMAP) was compared and similar results were obtained in each case, suggesting that different activation treatments used here do not affect the early development rates of macaca–rabbit cloned embryos.

An important factor governing early development of reconstructed embryos is the in vitro culture system. At the present time, however, it is unknown whether the culture medium for interspecies reconstructed embryos should be matched to the donor cell or to the recipient egg. Dominko et al. (1999) successfully cultured NT embryos reconstructed between somatic cells (sheep,

pigs, monkeys, and rats) and enucleated bovine oocytes using CR1$_{AA}$ medium, which is suitable for bovine embryos culture in vitro. In our previous report, M199 +10% FBS was also able to support the in vitro development of panda–rabbit cloned embryos to blastocyst stage (Chen et al., 1999, 2002). Macaca–rabbit NT embryos cultured in M199 +10% FBS, however, were arrested at 4-cell stage and could not break through the early development block. HECM$_{10}$ +10% FBS, normally used to culture macaca embryos (Schramm and Bavister, 1996; Zheng et al., 2001), could support 8.30% of the macaca–rabbit cloned embryos to develop to the blastocyst stage. The early embryo development block is related to embryo species, culture medium and culture conditions. At the first few mitotic divisions, the embryonic genome has little or no transcriptional activity and proteins and mRNAs derived from the cytoplasm of oocyte support development. The onset of embryonic transcription of monkey is at 4- to 8-cell stage (Schramm and Bavister, 1999). Our results show that HECM$_{10}$ +10% FBS can successfully support embryonic transcription and allow macaca–rabbit cloned embryos to develop to the blastocyst stage (38°C, 5% CO$_2$, saturated humidity).

Reports of the fate of mitochondria from donor cells and from recipient oocyts are controversial. In intraspecies cloning, mitochondria are exclusively oocyte-derived. Donor-derived mitochondria are eliminated from the cytoplasm of reconstructed embryos during the first few mitotic divisions and are hardly detectable by the blastocyst stage (Steinborn et al., 1998; Evans et al., 1999; Takeda et al., 1999). In other cases, mitochondrial DNA heteroplasmy in intraspecies cloned animals was found (Hiendleder et al., 1999; Steinborn et al., 2000; Do et al., 2002). In closely correlated interspecies cloned animals, mitochondria are also primarily from oocytes (Lanza et al., 2000; Loi et al., 2001; Meirelles et al., 2001). Our results of macaca–rabbit cloned embryos show that mitochondria derived from donor cells are not eliminated from the cytoplasm, but coexist with oocytes-derived mitochondria during preimplantation development. This result is consistent with our recent study (Chen et al., 2002), in which the mitochondria from donor panda cells and those from recipient rabbit oocytes coexisted in embryos before implantation.

In the present study, we used part of the mitochondrial *cytb* gene as target fragment and PCR products were analyzed by direct PCR product sequencing. In intraspecies and closely correlated interspecies cloning, the D-loop region is widely used to analyze the
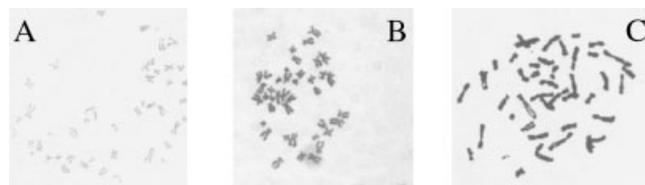

Fig. 2. Chromosomes of rabbit somatic cell, macaca somatic cell, and macaca–rabbit reconstructed blastocyst. (**A**) Rabbit somatic cell (2n = 44); (**B**) macaca somatic cell (2n = 42); (**C**) macaca–rabbit reconstructed blastocyst (2n = 42).
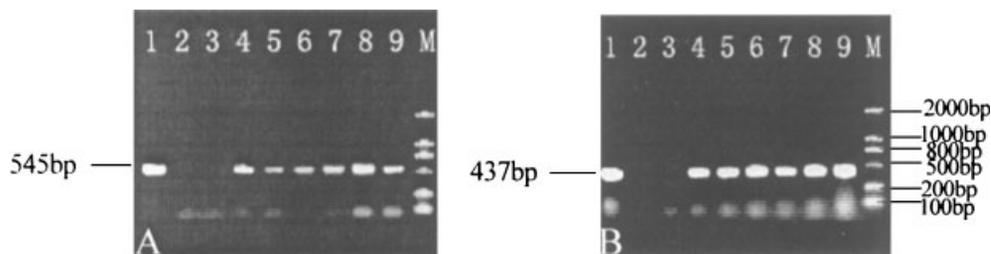
Fig. 3. A: Electrophoretic analysis of PCR-amplified product by using specific macaca cytochrome B (cytb) primer. **Lane 1**: macaca somatic cells; **lane 2**, rabbit somatic cells; **lane 3**, water (negative control); **lane 4**, 1-cell stage of macaca–rabbit reconstructed embryo; **lane 5**, 2-cell stage; **lane 6**, 4-cell stage; **lane 7**, 8-cell stage; **lane 8**, morula stage; **lane 9**, blastocyst stage; **lane 10**, DL 2000-marker.

B: Electrophoretic analysis of PCR-amplified product using specific rabbit *cytb* gene primer. **Lane 1**: rabbit somatic cells; **lane 2**, macaca somatic cells; **lane 3**, water (negative control); **lane 4**, 1-cell stage of macaca–rabbit reconstructed embryo; **lane 5**, 2-cell stage; **lane 6**, 4-cell stage; **lane 7**, 8-cell stage; **lane 8**, morula stage; **lane 9**, blastocyst stage; **lane 10**, DL 2000-marker.

mitochondrial DNA heteroplasmy because it is a high variable region of mitochondrial DNA. In interspecies cloning, however, some genes fragments that are conserved in interspecies and mutable in interspecies, such as *cytb* gene, can be used for studying the fate of mitochondrial DNA. Our results verify the suitability of this method to study the fate of mitochondrial DNA in macaca–rabbit cloned embryos.

In summary, our results suggest that (1) enucleated rabbit oocytes containing macaca somatic cell nuclei are able to dedifferentiate and develop to blastocyst stage; (2) macaca–rabbit clones incubated in $HECM_{10} + 10\%$ FBS can break through the 4-cell development block and develop to the blastocyst stage; (3) donor cell-derived mitochondria are not eliminated from macaca–rabbit clones during preimplantation development.

ACKNOWLEDGMENTS

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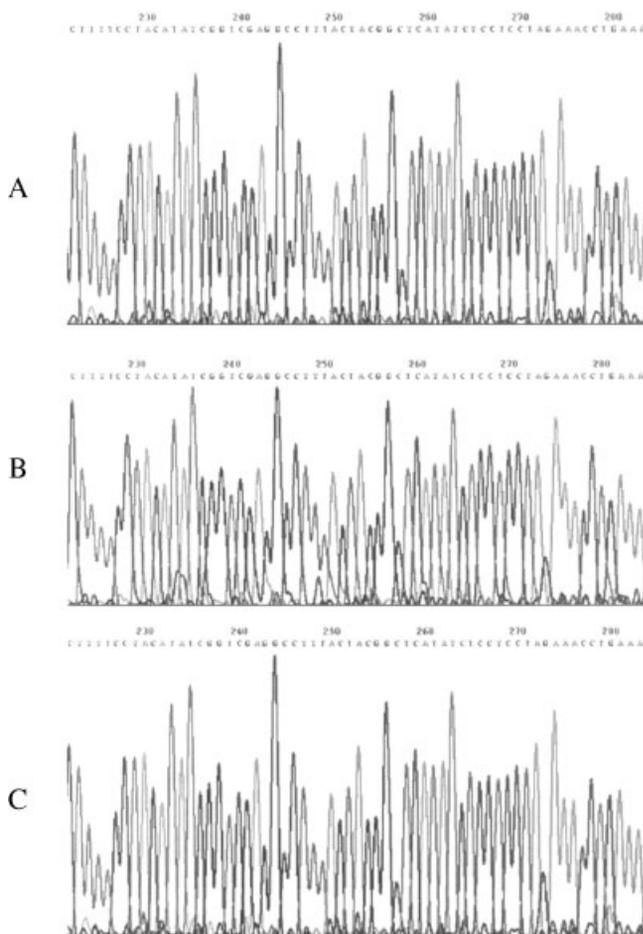

Fig. 4. Sequences of PCR products by using specific macaca *cytb* gene primer. **A**: Macaca somatic cells; (**B**) 4-cell stage of macaca–rabbit reconstructed embryo; (**C**) blastocyst stage NT embryo.

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