大韓獸醫學會誌 (1990) 第30卷 第4號
Korean J Vet Sci (1990) 30(4):355~360

Studies on nuclear transplantation in mouse embryos

Ⅱ. Developmental potential of nuclei from embryos of different developmental stages

Choong-saeng Park, Sang-yong Choe*, Hyo-jong Lee*, Hee-sung Park
College of Agriculture, College of Veterinary Medicine, Gyeongsang National University*
(Received Sept 3, 1990)

생쥐수정란의 핵이식에 관한 연구

Ⅱ. 발달단계별 수정란 핵의 이식후 생존성

박충생 · 최상용* · 이효종* · 박희성
경상대학교 농과대학, 수의과대학*
(1990. 9. 3 접수)

초록 : 포유동물의 초기 발생단계에서 핵의 분화와 전능성(totipotency)을 규명하고, 수정란의 cloning technique를 개발하여 우량유전자로 조성된 개체를 복제함으로써 효과적인 종축개량 기법으로 응용하기 위하여 생쥐 수정란을 모델로 하여 미세조작기법과 Sendai virus를 이용한 핵융합기술을 이용하여 인위적으로 동일한 유전자를 가진 복제 수정란을 작출하고 이들의 작출효과, 체외발달능력 및 체내 이식후 개체발생여부 등을 조사하였다.

2-세포기, 4-세포기 및 8-세포기의 수정란으로부터 핵을 채취하여 이들을 탈핵된 2-세포기의 수정란에 이식하였을 때, 이들의 핵융합 성공율은 각각 88.6%, 87.1% 및 84.7%이었다. 나아가서 이들 핵융합된 수정란을 체외에서 96시간 배양한 결과, 2-세포기, 4-세포기 및 8-세포기의 핵이 이식된 수정란은 각각 76.5%, 68.4% 및 48.3%가 배반포로 발달하였다. 핵이식 후 체외에서 배반포로 발달된 수정란을 골라 수란생쥐에 이식하였던 바, 2-세포기의 핵이 이식된 수정란 156개 중 58개(37.1%)가 발달하여 신생자로 생산되었으며, 4-세포기의 핵이 이식된 수정란 135개 중 40개(29.6%)가, 그리고 8-세포기의 핵이 이식된 92개의 수정란 중 15개(16.3%)가 신생자로 생산되었다.

Key words: nuclear transfer, *in vitro* development, embryo transfer, mouse embryo.

Introduction

The development of a technique for the efficient production of large numbers of genetically identical offspring in animals will be of great potential value for the multiplication, selection and evaluation of genotypes of superior economic value and for the reducton of the number of animals needed in experiments. For traits such as milk production, one number of an identical pair could replace more than 20 random animals in an expriment[1]. Also the genetically identical twins could be very beneficially used for the research on organ transplantation because they show least rejection reaction between identical twin individuals.

Nuclear transplantation is known as the most potential method for producing a large number of genetically identical animals. It is generally believed

*Supported by grant-in-aid for genetic engineering research of 1989 from the Ministry of Education.

that single blastomeres from cleavage stage embryos are totipotent to develop to the blastocyst stage and subsequently into viable young. On the theoretical basis each of the nuclei in an embryo at cleavage stage contains the same genetic material and, therefore, should be capable of giving rise to a complete individual of the same genetic constitution. For the large-scale production of genetically identical mammals with early stage donor embryos, it would be necessary to do serial transfer of nuclei by recycling method to achieve extensive multiplication of the original embryo.

When the nuclei from cleavage-stage embryos were transplanted into enucleated pronuclear-stage recipient embryos in mouse[2] and cow[3], very limited number of nuclear transplant embryos were developed *in vitro* and none of them developed to offspring. Development was more successful when the two-cell embryos were used as recipients.[4,5] In this experiment, therefore, two-cell embryos were used as nuclear recipients.

In this study, the developmental potential of single nuclei, from two-to eight-cell mouse embryos which were transplanted into the enucleated two-cell embryos was monitored by examining their development *in vitro* to form blastocyst and *in vivo* to term after transfer to recipients.

Materials and Methods

Preparation of embryos: Immature ICR and C_{57}BL/6J mice were superovulated by intra-peritoneal injection of 5 IU PMSG(Sigma, USA), followed 48 hours later by injection of 5 IU hCG(Sigma, USA). Pseudopregnant recipient females were obtained by mating superovulated ICR females to vasectomized ICR males. From C_{57}BL/6J females mated with males of the same strain, nuclear recipient two-cell embryos were collected 44~45 hours after hCG injection by flushing the excised oviducts with Hepes-BMOC-3 supplemented with 0.5% bovine serum albumin. Nuclear donor embryos of two-cell, four-cell and eight-cell were collected 44~45 hours, 55~56 hours and 65~66 hours after hCG injection as described above from C_{57}BL/6J females mated with males of the same strain respectively. Four-and eight-cell donor embryos were stored at 4°C for 4~10 hours in Hepes-BMOC-3 until used.

Nuclear transplantation of embryos: Nuclear transplantation was performed as described by McGrath and Solter[6,7]. Recipient embryos were prepared by removing nuclei from both blastomeres of two-cell embryos. Single nucleus from two-, four- and eight-cell embryos were fused into one of the enucleated blastomeres of two-cell recipient embryos using inactivated Sendai virus(Fig A,B,C and D).

***In vitro* culture and transfer of nuclear transplant embryos:** Culture of nuclear transplant embryos *in vitro* for 96 hours and transfer of nuclear transplant blastocysts into recipient mice were done as described previously.[8]

Statistical analysis: The proportions of embryos that fused or developed to blastocysts *in vitro* and developed to new-born young *in vivo* after transfer into recipients were compared between enucleation cell stages of donor embryos by Chi-square test.

Results and Discussion

Nuclear transplantation into enucleated two-cell embryos: The results of enucleation of the recipient embryos and the proportions of embryos that a nucleus from two- to eight-cell donor embryos was successfully injected and subsequently fused into the cytoplasm of recipient embryos are shown in Table 1.

The proportion of embryos that successfull enucleated, injected and fused into the recipient cytoplasm was observed to be greater than 90%, 92% and 84%, respectively. We found no significant ($p<0.05$) difference in micromanipulation for enucleation, injection and fusion between the cell stages of nuclear donor. Two-cell mouse embryos are capacious enough as recipient cytoplasm for advanced nuclei than do zygotes[2,4] perhaps because the transition from maternal to embryonic genome control of mouse embryos begins at the two-cell stage. The asynchrony between donor embryos at four- and eight-cell stage and recipient embryos at two-cell stage was overcome by storing the donor embryos for 4~10 hours prior to nuclear transplantation at 4°C.

Table 1. Successful injection and fusion of nuclei from mouse embryos at different development stages

Stage of nuclear donor	Recipient embryos	No. and(%) of recipient embryos enucleated/used	No. and(%) of embryos injected/ enucleated	No. and(%) of embryos fused/injected	Overall success rate(%)
2-cell	Enucleated 2-cell	285/313(91.0)	273/285(95.7)	242/273(88.6)	77.3
4-cell	Enucleated 2-cell	281/305(92.1)	263/281(93.6)	229/263(87.1)	75.1
8-cell	Enucleated 2-cell	262/288(90.9)	242/262(92.3)	205/242(84.7)	71.2

There are no significant ($p<0.05$) differences between the cell stages.

Table 2. Preimplantation *in vitro* development of nuclear transplant mouse embryos by stages of nuclear donor

Stage of nuclear donor	No. of nuclear transplant embryos	No. and(%) of embryos developed to		
		4-cell	Morula	Blastocyst
2-cell	230	204(88.7)[b]	182(79.1)[b]	176(76.5)[b]
4-cell	222	186(83.8)[b]	161(72.5)[b]	152(68.4)[b]
8-cell	203	132(65.0)[a]	109(53.7)[a]	98(48.3)[a]

The numbers with the different superscript denote significant($p<0.05$) difference between the cell stages of nuclear donor.

***In vitro* development of nuclear transplant embryos:** The fused embryos which were received a nucleus from two-, four- and eight-cell donor embryos were cultured *in vitro* for 96 hours. Their developmental potential of forming blastocyst in 96 hours is shown in Table 2. When single nuclei from two-, four- and eight-cell embryos transplanted into enucleated recipient embryos, 76.5%(176/230), 68.4%(152/222) and 48.3%(98/203) of which developed to blastocysts, respectively(Fig E). The proportion of embryos which developed to the blastocyst stage was not significantly($p<0.05$) different between two-cell and four-cell stage of nuclear donors, but the proportion was significantly ($p<0.05$) diminished to less than 50% when a nucleus from eight-cell embryos was introduced.

The present study confirmed that the results of Robl et al,[4] who reported that 51% of enucleated two-cell embryos that received a nucleus from eight-cell embryos developed to blastocysts *in vitro* and the results of Tsunoda et al,[5] who reported that 72% and 35% of the embryos that received a nucleus from four- and eight-cell embryos developed to blastocysts *in vitro*.

***In vivo* development of nuclear transplant embryos:** Full-term development of nuclear transplant embryos after *in vitro* culture and transfer to pseudopregnant recipient mice was achieved(Fig F). As shown in Table 3, the proportion of recipient mice that became pregnant was significantly ($p<0.05$) decreased to 24% when 8-cell nuclei were transferred to enucleatd two-cell embryos comparing to 59.4% in two-cell nuclei, and 53.3% in four-cell nuclei.

The proportions of embryos which developed to term were significantly($p<0.05$) higher in two-cell nuclei(37.1%) and in four-cell nuclei(29.6%) than in

Table 3. Production of live young after transfer of nuclear transplanted embryos in recipient mice

Stage of nuclear donor	No. of pregnant/ No. of recipients used(%)	No. of young/ No. of embryos transferred(%)
Intact blastocyst	22/35(62.8)[b]	89/182(48.9)[c]
2-cell	19/32(59.4)[b]	58/156(37.1)[b]
4-cell	16/30(53.3)[b]	40/135(29.6)[b]
8-cell	6/25(24.0)[a]	15/ 92(16.3)[a]

The proportions with the different superscript denote significant ($p<0.05$) difference between the cell stages of nuclear donor.

eight-cell nuclei(16.3%). These data indicate that the proportions of pregnant recipients that became pregnant and the proportions of transferred embryos that developed to term are lower in nuclear transplant embryos than in intact blastocysts.

The results of this experiment clearly indicate that the enucleated two-cell embryos which received nuclei from two-, four- and eight-cell embryos could develop to blastocysts *in vitro* and to full term in the recipient mouse, as demonstrated by others[4,5,9].

Although the rates of success in nuclear transplantation, *in vitro* culture to the blastocyst stage and production of live young with nuclear transplant embryos were gradually reduced when the more developed embryos were used for nuclear donor, the total number of new-born offspring per donor embryo and the overall efficiency in producing identical offspring are greater when eight-cell embryos are used for nuclear donor than two-or four-cell embryos are used.

For the large scale production of cloned animals it would be unlimitedly desirable that the complete sets of nuclear transplant embryos can develop to blastocysts and eventually to live young or nuclear transplant embryos can be recycled to serve as nuclear donors.

Summary

Single nuclei from two-, four- and eight-cell mouse embryos were transplanted into enucleated two-cell embryos by micromanipulation and Sendai virus mediated fusion. The developmental potential of these reconstituted embryos *in vitro* and *in vivo* was examined. It was found that the single nuclei which were transplanted to enucleated two-cell embryos were not only able to develop to the blastocyst stage *in vitro*(two-cell nuclei, 76.5%; four-cell nuclei, 68.4%; eight-cell nuclei, 48.3%), but also able to develop to full term *in vivo* after transfer to recipient mice(two-cell nuclei, 37.1%; four-cell nuclei, 29.6%; eight-cell nuclei, 16.3%).

Although the proportion of live young produced after transfer of nucler of nuclear transplant embryos which received eight-cell nuclei was significantly ($p<0.05$) reduced, it would be suggested that the overall efficiency in producing identical offspring is greater when eight-cell embryos were selected for nuclear donor than two- or four-cell embryos were selected.

Legends for figures

Fig A. Eucleation of karyoplasts from a mouse embryo at two-cell stage(×200).

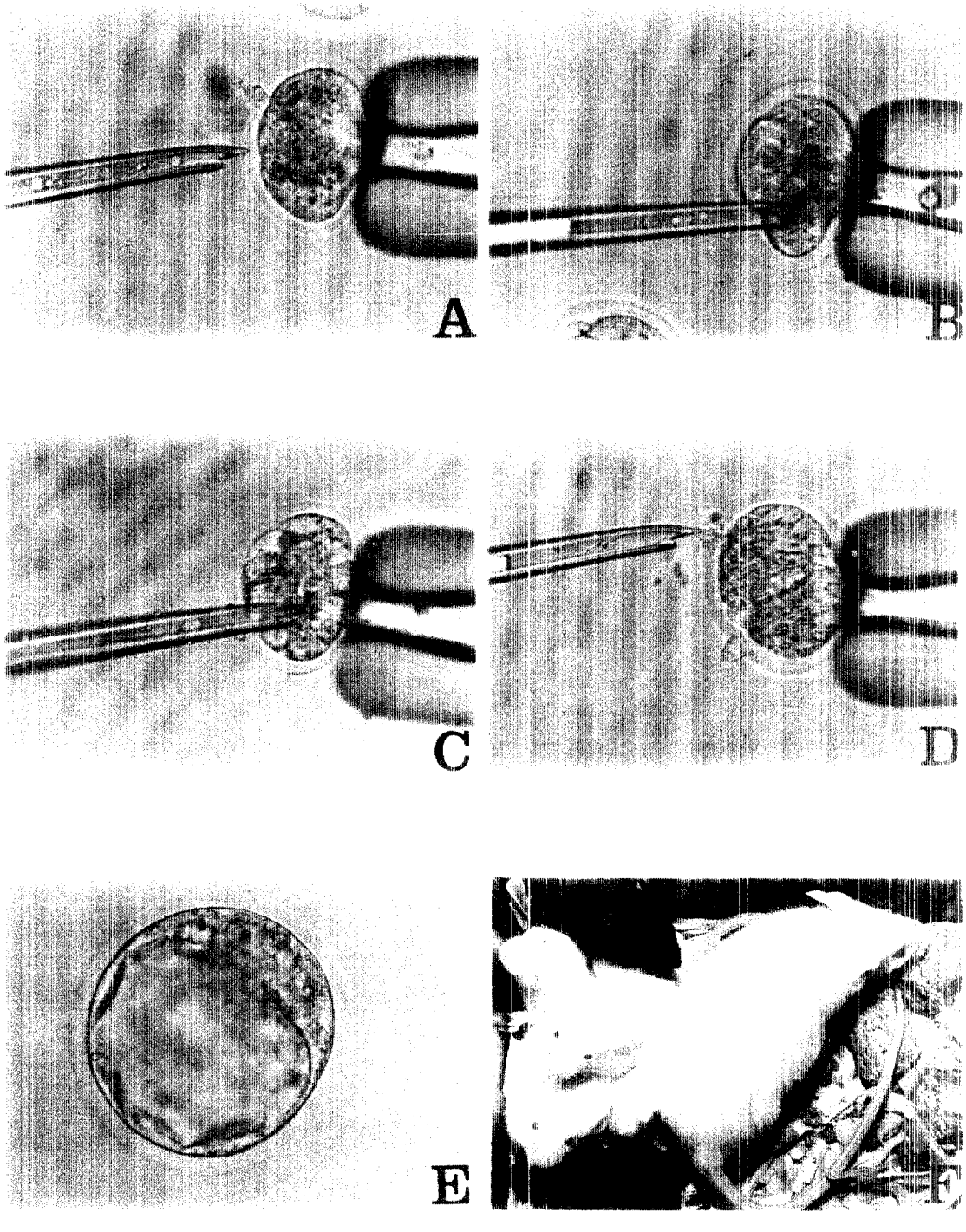
Fig B. Enucleation of karyoplasts from a mouse embryo at four-cell stage(×200).

Fig C. Enucleation of karyoplasts from a mouse embryo at eight-cell stage (×200).

Fig D. Injection of a karyoplast into an enucleated two-cell mouse embryo(×200).

Fig E. A blastocyst produced by *in vitro* culture of a nuclear transplant two-cell embryo(×200).

Fig F. Two $C_{57}BL$ mice(black-coat) produced by *in vitro* culture and *in vivo* transfer of nuclear transplant two-cell embryos.


A
B
C
D
E
F

References

1. Biggers JD. The potential use of artificially produced monozygotic twins for comparative experiments. *Theriogenology* 1986;26:1-25.
2. McGrath J, Solter D. Inability of mouse blastomere nuclei transferred to enucleated zygotes to support development *in vitro*. *Science* 1984;236:1317-1319.
3. Robl JM, Prather R, Eyestone W, et al. Nuclear transplantation in bovine embryos. *J Anim Sci* 1987;64:642-647.
4. Robl JM, Gilligan B, Crister ES. Nuclear transplantation in mouse embryos: Assessment of recipient cell stage. *Biol Reprod* 1986;34:733-739.
5. Tsunoda Y, Yasui R, Shioda Y. Full-term development of mouse blastomere nuclei transplanted into enucleated two-cell embryos. *J Exp Zool* 1987;242:147-151.
6. Mc Grath J, Solter D. Nuclear transplantation in mouse embryos. *J Exp Zoology* 1983a;228:355-362.
7. McGrath J, Solter D. Nuclear transplantation in mouse embryos by microsurgery and cell fusion. *Science* 1983b;220:1300-1302.
8. Choe SY, Park CS, Lee HJ, et al. Studies on nuclear transplantation in mouse embryos: I. Functional differences between maternal and paternal genomes. *Korean J Vet Res* 1990;30(2):123-127.
9. Kono T, Tsunoda Y. Development of single blastomeres from four- and eitht-cell mouse embryos fused into the enucleated half of a two-cell embryo. *Gamete Res* 1989;22:427434.