A Potential Demerit of the Pronuclear Microinjection Technique

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Pronuclear microinjection (PMI) is a primary method for producing transgenic mice and offers a powerful tool for investigating gene function *in vivo*. The method has several reported advantages and disadvantages. Here, we report another potential shortcoming. The survival rate of fertilized one cell-stage embryos was significantly reduced after PMI procedure (65.4% (1202/1838)). In addition, the proportion of embryos developing to full-term was also significantly lower than that of embryos not undergoing PMI (26.5% (319/1202) vs 41.9% (57/136)). Moreover, 3 out of 21 (14.3%) founder control mice which were non-transgene-carrying littermates of transgenic founders showed histopathological changes in their liver, which was comparable to that in of transgenic lineages (4 out of 27 (14.8%)). In conclusion, the mechanical damages in chromosomes occurring during PMI procedure may be a potential factor influencing phenotypes of transgenic mice.

Key words – Nuclear damage, pronuclear microinjection, transgenic mice

Introduction

The mouse as experimental animals offers many advantages in research field of life sciences because of its small size, high breeding capacity, and relatively short generation time. In addition, the mouse is genetically similar to humans[11] and there are so many established mouse strains which serve as excellent animal models[7]. With recent development of molecular biology, making genetically modified mouse strains became a powerful tool for studying mammalian gene functions. Accordingly, producing transgenic mice is one of the most widely used methods for studying of mammalian gene functions. The microinjection of DNA directly into the pronucleus of a fertilized one-cell stage mouse egg is the most popular method to produce transgenic mouse[3].

Although producing and analyzing phenotypes of transgenic mice may provide much helpful information to study developmental biology, metabolism, human diseases, and so on, there are several difficulties in the detailed phenotyping. The nature of random insertion of transgenes into chromosomes, variable gene-expression patterns, requirement for multiple transgenic variants, heterogeneity of genetic backgrounds of mice used, and the resulting difficulties in cross-comparisons are common hurdles encountered in transgenic mouse investigations. Moreover, the survival rate of fertilized one cell-stage embryos and the proportion of embryos developing to full-term were much reduced after the pronuclear microinjection (PMI) procedure compared to that of embryos only undergoing embryo transfer (ET) procedure. Here, we offer the evidence that showed another potential demerit, mechanical genetic damages which may occur during PMI procedure and which might give birth of confusion in their phenotyping.

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Materials and Methods

Production and lineage establishment of transgenic mice

All the procedure for production of transgenic mice using PMI with expression construct for a target transgene was performed using standard protocols[4,9] (for the detailed information, refer to our previous paper[12]). The mouse embryos used in this study were from the hybrid BCF1 (C57BL/6J♀ × CBA/J♂). The transgenic founders

were backcrossed with C57BL/6J to establish lineages with genetic background of C57BL/6J. Transgenesis was confirmed by both PCR and Southern blotting using their genomic DNA. The mice analyzed in the present study were maintained in a controlled and specific pathogen-free environment, in accordance with the guideline of the Institutional Animal Care and Use Committee, Korea Research Institute of Bioscience and Biotechnology (KRIBB, Daejeon, Korea).

Data analysis

In order to determine whether PMI procedure influenced embryo development, the survival rate of embryos and the ratio of pups born were determined from our five-year cumulated records.

For the brief phenotyping, we performed histopathological examination for the liver of the mice. Liver samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin according to the standard methods. Histopathological diagnoses were based on the criteria described by Firth and Ward [2] and Maronpot *et al.*[5].

In order to determine whether PMI procedure contributes to any histopathological changes, the incidence of abnormal changes observed in the liver of founder control mice was compared with that of transgenic mice. *In vitro* fertilization and embryo transfer are techniques widely used in veterinary medicine and to treat human infertility. To present, no report describes a significant difference in the phenotypes of mammals before and after the embryo-transfer procedure. Therefore embryo transfer itself may not influence the phenotype of mice. Accordingly any abnormal phenotypes in founder control mice would be largely owing to the damages occurring during PMI procedure.

Results and Discussion

After PMI procedure, only 65.4% of the embryos were survived and transferred into surrogate mother. The results indicate that PMI itself may damage embryo to death. In addition, only 26.4% of transferred embryos with PMI have developed to full-term in their surrogate mothers, which was significantly lower than that of embryos developed in the surrogate mothers without PMI (41.9%)(Table 1). This is consistent with previous reports showing that the microinjected zygotes were less viable than those not undergone experimental manipulation[1,8]. Typically, 50-80% of the embryos survive after PMI. 10-30% of the embryos transferred into the oviduct develop to full-term[4]. This might indicate that damages in the embryos occur by the microinjection. This damage might be the main cause of the embryonic death after microinjection and during developmental period in the oviducts or uteri. There should be mechanical damages in genetic materials in nuclear and/or cytoplasm. There may be also some genes those are altered in their expression resulting from the loss of gene function owing to a random insertion of the transgene or DNA fragment. No reports, however, have examined whether the non-lethal damage by PMI has any correlation with any phenotypes.

Table 2 shows the results of histopathological examination of mice born by the standard transgenic mice production procedure. Each lineage for the founder transgenic mice was established. The histopathological observations for the liver revealed hepatic tumors in four of 27 (14.8%) transgenic founder mice. The tumors observed in transgenic founders (H-ras12V and NS5A, Table 2) were attributed to the functions of the transgenes, because all of the established lineages from these transgenic founders showed the same phenotype and because the litter mates without transgene had no live tumor. Unexpectedly, hepatic tumors were observed in some of the founder control mice developed from embryos that underwent PMI, but without transgene. In addition, even in non-transgenic mice originated from transgenic founders (2 in Core and 1 in Core 116, Table 2) showed inheritable hepatic tumor le-

Table 1. Developmental rates in embryos born with or without pronuclear microinjection

	No. of embryos collected	No. of embryos survived after PMI and transferred(%)	No. of pregnant/surrogate mothers(%)	No. of pups born(%)
PMI and ET	1,838	1,202 (65.4%)	42/44 (95.3%)	319 (26.5%)
ET only	136	136	5/6 (83.3%)	57 (41.9%)*

Notes: All the embryos were collected from superovulated BCF1 female mice. ICR mice were used as surrogate mothers. *Indicates a significant difference within the same column. $P < 0.01$. **PMI**: Pronuclear MicroInjection, **ET**: Embryo Transfer.

Table 2. Summary of histopathological findings in mice born by PMI procedure

Transgenes	Non-transgenic founder control [a]	Transgenic founder [b]	Selected transgenic founder for lineage establishment [c]	Established transgenic lineage [d]	Transgenic lineages with non-specific histological changes [e]
NS5A	2/4	1/8	3	3	0
NS4B	1/4	0/4	3	3	0
H-ras12V	0/2	3/3	3	2	0
HDAC1	0/3	0/3	2	2	0
Core	0/3	0/4	4	4	2
Core116	0/3	0/3	3	3	1
Core99	0/2	0/2	2	2	0
Total	3/21 (14.3%)	4/27 (14.8%)	20	19 (95%)	3 (15.8%)

Notes: Description of transgenes: NS5A, hepatitis C virus nonstructure 5A; NS4B, hepatitis C virus nonstructure 4B; HDAC1, histone deacetylase 1; Core, hepatitis C virus core; Core116, core mutant (Ser116 to isoleucine); Core99, core mutant (Ser99 to glutamine).

The tissues for histopathological examination were sampled at between 10 and 15 months of age.

The histopathological changes detected in these transgenic mice included hepatic foci, hepatic tumors, and lymphomas.

[a]The mice were born through PMI procedure, but do not carry the transgenes. The number in parenthesis is expressed as (number with histopathological changes/total number obtained).

[b]The mice were born through PMI procedure and carried the transgenes. The number in parenthesis is expressed as (number with histopathological changes/total number obtained).

[c]Selected number of transgenic founder for lineage establishment among transgenic founders.

[d]Number of successfully established transgenic lineage.

[e]Number of transgenic lineages with non-specific histological changes which is not correlated with the target transgene. Abnormal lineages in which histopathological changes were detected in both transgenic mice and control littermates. For the detailed histopathological changes observed, see Table 3.

sions that were appeared by 15 months of age. This indicates that the histopathological changes limited to transgenic mice in transgenic lineages are owing to the functions of the transgenes. By contrast, the causes of the abnormal findings detected in some transgenic lineages (15.8%) with non-specific histopathological changes were clearly not attributable to the transgenes.

As embryo transfer itself do not significantly influence the phenotype of the animals, the pathological changes in the founder controls imply that some non-lethal damage occurred by PMI and caused hepatic phenotypes. Such damages are induced probably in the genome or genes by mechanical PMI procedure, because 1) no transgene insertion was identified in the founder controls, 2) tumorigenesis was largely correlated with genetic alterations, and 3) the hybrid BCF1 strain showed vary rare incidence of tumorigenesis until about 28 months of age[10], as we also have detected rare histopathological changes in normal BCF1 strain mice used in this (data not shown). Another possibility is that genetic disruption had occurred by a random insertion of transgene fragments that were not detected. However the incidence of that kind disruption of a gene is very rare.

On the other hand, non-transgene specific histopathological changes observed in three transgenic lineages also support that inheritable damages occur by PMI procedure (Table 3). These transgenic lineages are useless in studying the phenotypes of the target transgenes. Considering that the tissues for histological examination were obtained between 12 and 14 months of age, the tumorigenic rate was extremely higher compared with that of wild BCF1 in which the rates at 28 months of age were 5.8% and 8.7% for hepatoma and lymphoma, respectively. These non-transgene specific histopathological changes were not attributable to environmental factors, because that was observed in mice reared in separated facilities by two independent pathologists.

In addition, one founder mice that was used for establishment of transgenic lineage was infertile. This infertility may result from genetic damage caused by mechanical procedure during PMI; it is not attributable to the action of the transgene because there was no other infertile

Table 3. Summary of the histopathological changes observed in the transgenic lineages with non specific histological changes

Transgenes	Altered hepatic foci		Hepatic tumors		Lymphoma	
	non-Tg	Tg	non-Tg	Tg	non-Tg	Tg
Core (K51)	1/4	1/9	1/4	1/9	1/4	1/9
Core (K52)	2/4	2/10	1/4	0/10	1/4	0/10
Core 116 (166-2)	1/4	1/14	1/4	1/14	1/4	0/14

Notes: The tissues analyzed were sampled at between 12 and 14 months of age in two generations. K51, K52, and 166-2 are the established transgenic lineages from independent founders.
Tg: transgenic mice; non-Tg: non-transgenic littermates.

founder (data not shown). The infertility in transgenic lineage without clear causes was also reported in other research group[6].

In this study, we only examined the histological changes in the liver of mice undergone PMI and found altered phenotypes, irrespective of their transgenesis. Other abnormal phenotypes, such as physiological or behavioral phenotypes may exist. Thus, it should be cautious in the analysis of phenotypes in a transgenic mice which experienced PMI procedure. During PMI procedure, when the tip of injection pipette reaches the pronucleus through the egg plasma membrane, there must be a mechanical touch of the micropipette with chromosomes and may cause damage in the genetic materials. Even though this possibility was not directly demonstrated, our data suggest that it may occur at a considerable rate. As the tumorigenic phenotypes in mice undergone PMI were inheritable, irrespective of the presence of transgenes, we postulate that damages occurring during PMI procedure were the main causes of the abnormal histopathological changes detected in our founder controls lineages. This may also be the cause of embryonic death after embryo transfer when the damage is severe.

In conclusion, potential nuclear damage occurring during PMI procedure may influence the phenotype in transgenic mice. As more and more transgenic mice are being used in areas of life sciences, we suggest that, when establishing a transgenic mouse lineage, non-transgene carrying littermates should be maintained and examined as a real control group to verify the phenotypes coming up from transgenic littermates for minimizing the false positive phenotyping.

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초록 : 형질전환 마우스 생산 및 표현형에 pronuclear microinjection 이 미치는 영향 연구

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현재, 유전자의 *in vivo* 기능을 연구하기 위해 가장 많이 이용되고 있는 transgenic mice를 생산하기 위한 기본적으로 이용되고 있는 방법이 one cell-stage embryo에 pronuclear microinjection (PMI)이다. 그러나, 이 PMI 후에 one cell-stage embryo들의 생존율은 현저히 감소 (65.4%)할 뿐만 아니라 PMI 후의 embryo의 출생률(26.4%)이 PMI 처리를 하지 않은 것 (41.9%) 보다 현저히 낮다. 더욱이, PMI 방법에 의해 태어난 transgenic founder들의 간 조직에 병리학적 변화가 14.8% 정도에 대해서 같은 한배의 새끼 non-transgenic founder들의 경우도 간 조직에 병리학적 변화가 14.3%로 나타났다. 결론적으로, 이 PMI 방법에 의한 염색체에 물리적 손상은 형질전환 마우스의 생산 및 표현형에 영향을 미치는 잠재적 요소로 생각된다.