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Preselection of Bovine Blastocysts Expressing Exogeneous Gene Following Microinjection

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외래유전자를 주입한 소 수정란에서 형질전환가능 수정란의 선발

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요 약

본 연구는 수정란을 수란우에 이식하기 이전에 형질전환가능 수정란을 선발할 수 있다면 형질전환동물의 생산에 크게 도움이 되므로 3.2 kb β-actin promoter (lacZ/neo) DNA를 미세주입하여 배반포배에서의 발현을 확인하여 이들을 선발할 수 있는가를 규명하고자 하였다. 채란된 난포란은 10% FBS, 5 μg/ml LH, 0.5 μg/ml FSH, 100 unit/ml penicillin 및 100 μg/ml streptomycin이 함유된 TCM199에 22~24 시간동안 체외성숙을 유도후 5 μg/ml heparin으로 수정능획득을 유도한 1×10^6 sperm/ml의 정자로 체외수정을 시켰다. 체외수정후 18~20시간째에 vortexing에 의해 과립막세포를 제거하고 원심분리시켜 자/웅전핵이 확인되는 수정란의 핵에 3~4 ng/μl lacZ/neo DNA를 미세주입하였다. 모든 수정란의 배양은 3 mg/ml BSA, 20 μl/ml NEM amino acids 및 40 μl/ml BME amino acids가 함유되어 있는 CR1aa 배양액에 neo/DNA로 transfected 된 BRL 단층에서 실시하였다.

G418에 대한 적정농도를 찾기 위하여 정상적인 수정란에 0, 50, 100 및 200 μg/ml G418를 첨가하여 배양한 결과 8일째에 30.3% (44/145), 8.7% (13/150), 0.7% (1/151) 및 0% (0/134)의 수정란이 배반포기까지 발달하였다. 그래서 본 실험에서는 일정하게 100 μg/ml G418을 첨가하여 배양하였다. 총 1,127개의 수정란을 미세주입후 G418 없는 배양액에서 710개 (63.0%)가 분할하였다. 미세주입후 48시간째에 2-세포기이상 분할된 수정란을 대조구 및 100 μg/ml G418처리구를 무작위로 할당하여 배양하였으며, 또한 740개의 정상수정란도 같은 반복수로 배양을 실시하였다. 미세주입한 수정란은 8일 후 11.6% (26/225) 및 5.2% (14/267)가 대조구 및 G418 처리구에서 배반포기까지 발달하였으며 정상수정란은 27.2% (151/740)가 배반포기 배까지 발달하였다. 미세주입후 대조구에서는 23.1±2.6/70.7±4.7 (32.7%)의 할구가 β-Gal 활력을 보였고, 반면에 100 μg/ml G418 처리구에서는 40.3±4.1/48.8±7.5 (82.6%)가 β-Gal 활력을 보였다. 비록 mosaic 형태로 외래유전자가 발현되었지만 대조구에서 87.0% (26/30개) 배반포기가 β-Gal 활력을 보인 반면, G418 처리구에서는 모든 배반포기가 β-Gal 활력을 보였다 ($P<0.05$). 그러나 대조구 및 G418 처리구의 ICM colony에서는 영양배엽과 내배엽을 제외한 epiblast에서는 확인되지 않았다. 그러나 이 결과로부터 β-actin promoter/lacZ gene이 integration되지 않는 것인지 또는 다만 염색 확인이 되지 않는 것인지를 판단할 수는 없다.

이상의 결과는 미세주입후 G418에서 배양한 배반포기배에서는 대부분의 할구에서 주입된 gene을 발현하고 있었으나 ICM colony에서는 특히 epiblast에서는 발현되지 않거나 침묵하고 있었다. 비록 G418 처리구

에서 훨씬 더 높은 비율로 주입된 gene 이 발현되고 있으나 총세포수는 유의적으로 감소하여 이후 형질전환동물의 생산과 ES like-cells의 설립에는 감소될 것으로 사려된다. 그러나 형질전환 수정란의 선발 및 형질전환동물의 생산 능력에 관해서는 더 많은 연구가 필요하다고 사려된다.
(Key words : β-actin /neo gene, Microinjection, G418 preselection, Bovine IVMFC)

Ⅰ. INTRODUCTION

Here are two important steps that must occur following microinjection of DNA into pronuclear embryos which will result in the production of useful transgenic animal. The first is integration of the transferred DNA (transgene) into the host genome of the developing embryo; the second is gene integration in a position that will allow expression of the transgene. If the first step of integration fails to occur then there is no possibility of expression of the transgene. Production of transgenic farm animals has been costly do to the maintain of large numbers of recipient animals which ultimately carry microinjected embryos to term which are subsequently identified as non-transgenic. These non-transgenic offspring result from a failure of the early embryonic genome to integrate the injected transgene. If it is possible to preselect embryos during culture which have integrated the transgene, this would reduce the cost of production of transgenic farm animals. Pronuclear microinjection of foreign DNA has been the most commonly used technique to produce transgenic animals (Gordon et al., 1980). Brinster et al. (1985) found that integration efficiencies were optimal when about 750 copies of linear DNA fragments in 10 mM Tris-HCl and 0.1 mM EDTA at pH 7.5 were injected into the male pronucleous of mouse zygotes. Even with optimized injection conditions, pronuclear microinjection produces transgenic animals in only 10 ~30% of live births. In addition, many founder transgenic animals contain a mosaic pattern of integration, which may average less than one copy of the construct per cell. In principle, a gene transfer programme in farm animals consists of the following steps: 1) cloning of gene constructs and preparation of DNA solution for microinjection; 2) collection of embryos and visualization of pronuclei; 3) DNA microinjection and transfer of embryos to recipients; and 4) integration, breeding and expression analysis in offspring.

In vitro maturation /*in vitro* fertilization procedures have been used to provide a large number of bovine zygotes from abattoir ovaries for pronuclear microinjection (Thomas et al., 1993; Hill et al., 1992; Krimpenfort et al., 1991). Moreover, the efficiency of transgene integration and producing live transgenic offspring from microinjected bovine zygotes has been very low (Krimpenfort et al., 1991; Bowen et al., 1994). *In vitro* development of murine, ovine and bovine species' ova to develop normally *in vitro* is thought to be caused by the mechanical process of microinjection (Walton et al., 1987; Peura et al., 1993).

This study was carried out to evaluate the potential for preselection of transgenic embryos and to detect the expression of injected DNA in blastocyst or ICM colonies.

Ⅱ. MATERIALS AND METHODS

1. Preparation of bovine oocytes

Bovine ovaries were obtained from a local slaughter house. Cumulus-oocyte complexes were aspirated from antral follicles (2 to 8 mm in diameter) with a hypodermic needle (18 G), se-

lected for evenly granulated cytoplasm and a compact cumulus oophorus, and washed three times in TL-Hepes medium supplemented with 3 mg/ml fatty acid free bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO), 0.2 mM pyruvate (Sigma), 50 μg/ml gentamicin (Sigma), 5 mM glucose (Fisher Scientific Co., Fair Lawn, NJ), and pH adjusted to 7.4 prior to *in vitro* maturation.

2. Oocyte maturation (IVM) and fertilization (IVF)

The oocytes were matured in Ham's F-10 medium supplemented with 10% fetal bovine serum (FBS), 5 μg/ml LH, 0.5 μg/ml bovine FSH, 100 units/ml penicillin, and 100 μg/ml streptomycin in 4-well culture dishes and cultured at 39℃ in a humidified atmosphere of 5% CO_2 and air for 23 hrs. At 23 hrs after initiation of maturation, spermatozoa were prepared for *in vitro* fertilization according to a method described by Parrish et al. (1988). Cryopreserved semen was thawed in 37℃ water and placed in 10 ml m-PBS for washing and centrifugation, and then capacitated with 400 μl of 50 ng/ml heparin solution for 15 minutes at 39℃. The capacitated sperm were diluted with TL-FERT medium to approximately 1×10^6 sperm/ml in drops containing the oocyte.

3. DNA construct and microinjection

The transgene was constructed with neomycin gene (neo) driven by a promoter known to be active in preimplantation embryos. The transgene contained a 3.2 kb segment of the chicken β actin promoter, β-galactosidase (β-gal) coding regine of the lac Z gene with the nuclear locating signal of large T antigen of SV40, an internal ribosome entry site and the neo coding regine. This construct produces a bi-cistronic mRNA allowing for translation of the lac Z gene product and cap independent translation of the neo gene product. The DNA was diluted in buffer (10 mM Tris+2 μM EDTA, pH 7.6) to 3~4 ng/μl. The larger or the most clearly discernable pronucleus was injected with a volume of DNA solution adequate to effect a visible swelling(1 to 5 pl).

After removal from fertilization drops, ova were washed 3 times in Hepes-TALP, and were then vortexed with approximately 1 ml of the medium to be used for microinjections in 15 ml microcentrifuge tubes for 2 min to remove cumulus cells. Denuded ova were then centrifuged at 14,000×g to aid in the visualization of pronuclei (Wall et al., 1985). Injections were performed under an inverted microscope (Zeiss or Nikon) equiped with Nomarski optics at 400× magnification. Embryos were placed in a drop of 3~5 μl of medium on a glass depression slide. Manipulations were carried out with the aid of Leitz or Narishige micromanipulators. Embryos were held by a fine, polished, holding pipet (outer diameter approximately 150 μm) and the plasma and nuclear membranes were penetrated with an injection pipet (tip diameter approximately 1~2 μm). DNA was injected with the aid of an oil-filled microsyringe (Stoling) until expansion of the pronucleus was visible (25% expansion or less). In the majority of embryos, the most visible pronucleus was injected. If both pronuclei were equally visible, the larger pronucleus was injected.

4. *In vitro* culture system

In vitro culture of treated and control zygotes took place in CR1aa medium supplemented with 10% FCS, 10 mM glycine and 1 mM alanine over neomycin (G418) resistant BRL cells at 50~60% confluency. Culture medium was replaced with new monolayer cells every 48 or 72 hrs. After 48 hrs injection, embryos with greater th-

an 2 cells were assigned to the control (medium alone) or G418 treatments (100 μg/ml of medium), respectively. The development rate of blastocyst at day 8 (insemination : day 0) was evaluated and moved out for use of staining or establishment of ICM colonies.

5. Histochemical staining

A β-actin promoter was chosen because the β-actin gene is widely expressed and activated early. It is expressed from the two cell stage in mice (Davidson, 1986; Bonnerot et al., 1987; Takeda and Toyoda, 1991) and bovine (Gagne et al., 1994; Kubisch et al., 1994). Expression of lacZ gene was detected by β-galactosidase (β-gal) histochemical staining. Whole embryos were rinsed with PBS, pH 7.3, and fixed for 10 min at 4℃ in 0.25% glutaraldehyde in PBS. The embryos were then washed once with PBS and overlaid with a histochemical reaction mixture containing 1 mg/ml of 4-Cl-5-Br-3-indoly-β-gal (X-gal, Sigma), 10 mM potassium ferrocyanide, 10 mM potassium ferricyanide, and 1 mM $MgCl_2$ for 18~24 hrs at 37℃. And the Hoechst 33342 stained of X-gal stained embryos for assessing of unstained nuclei, that is, unexpressed blastomeres.

6. Establishment of ES like-cell and culture conditions

Mouse primary embryonic fibroblasts (PEF) were used as feeder layers. They were established from 14-day old mouse fetuses. The mitotic activity of the fibroblasts was blocked by a 2 hrs incubation in 10 μg/ml mitomycin C. PEF and ES like cells were cultured at 37℃ in a humidified atmosphere of 5% CO_2 and air. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 0.1 mM β-mercaptoethanol, 0.1 mM non-essential amino acids, NEM amino acids, and antibiotics was used for embryonic cell culture. Bovine blastocyst derived cell cultures were mailtained in 1:1 mixture of DMEM (low glucose) and M199 containing 10% FBS, 0.1 mM β-mercaptoethanol, 0.1 mM non-essential amino acids, NEM amino acids, and antibiotics, and nucleotide supplementation as described by Robertson (1987). The medium was changed every 2 days.

7. Alkaline phosphate staining

To confirm of ES like-cell colony on the STO monolayers, cell cultures were fixed in 4% formaldehyde in PBS for 10~15 min. After washing with distilled water, the fixed cultures were incubated for 15~30 min in distilled water containing 240 μg/ml fast blue RR salt and 40 μl/ml AS-MX phosphate at pH 8.6. The reaction was stopped by rinsing with PBS or water.

8. Statistical analysis

Statistical analysis (Chi-square test) was carried out for the proportions of development and staining of embryos for each class of development.

Ⅲ. RESULTS AND DISCUSSION

1. Development competence of injected or non-injected embryos

As shown Table 1 and 2, non-microinjected zygotes were cultured in the presence of 0, 50, 100, and 200 μg/ml of G418 to identify the appropriate concentration of G418 for selection. After 8 days of culture in these treatments, 44/145 (30.3%), 13/150 (8.7%), 1/151 (0.7%), and 0/134 (0.0%) developed to the blastocyst stage in 0, 50, 100, and 200 μg/ml of G418, respectively. A total of 1,127 zygotes were microinjected and placed into culture (without G418) and subsequently 710 (63.0%) cleaved. At 48 hrs post-injection, embryos (≥2-cell) were ran-

Table 1. Development competence of non-injected bovine embryos in various G418 concentration

Concentration of G418 (μg/ml)	No. of embryos used	No. of blastocyst developed at day 8 (%)
0	145	44(30.3)[a]
50	150	13(8.7)[b]
100	151	1(0.7)[c]
200	134	0(0.0)[c]

* Values with different superscripts were significantly ($P<0.05$) different.

domly assigned to control (medium alone) or G418 (100 μg/ml) treatments. A control culture of 343 non-microinjected embryos from the same replicates of embryos were also placed into control medium. After 8 days in culture, 54/343 (15.7%) and 22/367 (6.0%) of the microinjected embryos developed to the blastocyst stage in control and G418 media, respectively. A total of 151/740 (27.2%) of the non-microinjected embryos placed in the control medium developed to the blastocyst stage.

The pronuclei of cow eggs are more elastic and less firmly anchored in the cytoplasm than pronuclei of other species and consequently are the most difficult to inject. Cow pronuclei will either invaginate and/or move out of the way, unless the injection needle is very sharp. The cytoplasmic material (thought to consist primarily of lipid droplets) that obscures the pronuclei can be displaced by centrifugation without compromising embryo viability (Wall et al., 1985; Wall and Hawk, 1988). Before the progression of transgene expression in developing bovine embryos was studied, a number of preliminary experiments were conducted to establish the system at various laboratory. In the course of this work, that the best time to view pronuclei in bovine embryos produced by IVM-IVF is between 19~22 hrs after culturing the gametes, were confirmed. That the microinjection procedures reduces initial cleavage of the zygote and subsequent progression to blastocyst was also verified. Essentially similar observations have been made for mouse (Gorden et al., 1980; Petters et al., 1987), pig (French et al., 1993) and ovine (Hammer et al., 1985; Wright et al., 1991), as well as bovine embryos (Hawk et al., 1989; Krimpenfort et al., 1991). Whether the developmental failure stems from mechanical or genetic damage or a combination of both remains unclear.

2. Expression pattern of injected embryos and ICM colonies

As shown Table 3, 4 or Fig. 1, blastocysts in the control treatment had a mean of 70.7±4.7 cells of which 23.1±2.6 (32.7%) stained for β-Gal activity while blastocysts in the G418 treatment had a mean of 48.8±7.5 cells of which 40.3±4.1 (82.6%) stained for β-Gal activity ($P<0.05$). In the control treatment 26 of 30 (87.0%) blastocysts had some cells with β-Gal activity

Table 2. Development competence of injected bovine embryos in 100μg/ml G418

Treatments		Results	
		Injected (%)	Control (%)
No. of embryos injected		1,127	740
No. of embryos cleaved		710(63.0)	555(75.0)
No. of embryos treated with	G418	Non-G418	
	367	343	
No. of blastocyst developed	22(6.0)	54(15.7)	151(27.2)

Table 3. Expression pattern of injected exogeneous gene in blastocyst stage

Type of injection	G418 treatment	No. (%) of blastocyst expressed /stained	No. (%) of nuclei expressed		
			Total	Expressed	Unexpressed
Injected	Yes	12 /12(100)	48.8±7.5[a]	40.3±4.1(82.6%)	8.6±5.1
Injected	No	26 /30(87)	70.7±4.7[b]	23.1±2.6(32.7%)	47.7±3.2
Non-injected	No	51	86.3±3.6[c]	–	–

* Mean±S.E., Values with different superscripts in the column were significantly ($P<0.05$) different.

while all of the blastocysts in the G418 treatment had some cell with β-Gal activity ($P<0.05$). ICM colonies in either control or G418 treatments were expressed in the endoderm, ectoderm and trophetoderm cells except of epiblast cells. The β-actin promoter /lacZ gene is not expression and silence expression in epiblast cells.

The most of bovine embryos injected with β -actin lacZ proved to be mosaics in the control treatment group. Kubisch et al. (1995) reported that almost all putative transgenic embryos injected with either construct showed a mosaic pattern of lacZ expression, with an average of only 2~3 cells staining at the eight-cell stage and the majority of cells in positive the blastocysts showing no evidence of experiments. Almost 100% mosaicism has been observed after microinjection of an SV40-lacZ gene into mouse embryos, although a far higher proportion of blastomeres at the four- and eight-cell stage stain blue than was observed here in bovine embryos (Takeda and Toyoda, 1991). It is possible that such mosaicism results from regulatory events controlling transcription or translation that can silence expression selectively in some blastomeres and not in others. The most likely explantation for the observed mosaicism is that expression stems largely from nonintegrated DNA, which is transiently transcribed. Powell et al. (1992) showed that bovine zygotes have a high ability to generate large ligation products from cytoplasmically injected DNA. Such concatamerization might ultimately lead to circularization, making the exogenous DNA more resistant to exonucleolytic degradation and more likely to be asymmetrically distributed at cell division. Moreover, circularization could facilitate and enhance transcription from a microinjected gene (Mertz, 1982; Harland et al., 1983; Bevilacqua et al., 1992).

These experiments do not provide much insight into why production of transgenic offspring is so much less efficient in cattle than in mice. One possibility, which might provide an alternative explantation to the one above for the extreme mosaicism noted in bovine embryos, is that the timing of transgene integration depends upon when the embryonic genome is activated.

Table 4. Expression pattern of exogeneous gene in ICM colonies established

Type of injection	Type of treatment	No. of blastocyst used	No. (%) of ICM colonies established	Expression pattern in ES cells	
				Epiblast	Trophoblast
Injected	G418	10	2(20.0)[a]	Negative	Positive
Injected	Non-G418	24	7(29.2)[a]	Negative	Positive
Non-injected	Non-G418	93	32(34.4)[a]	Negative	Positive

* Values with same superscripts in the column were not significantly($P<0.05$) different.

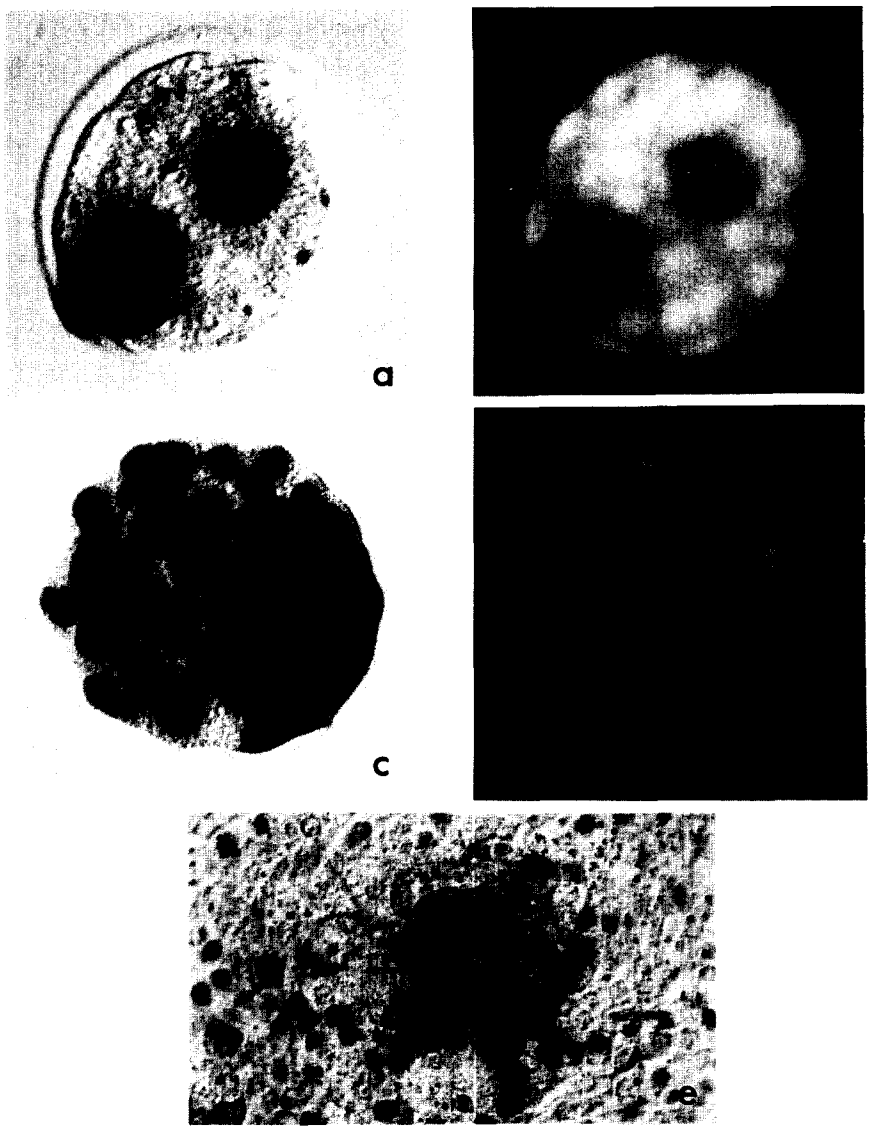

Fig. 1. Preimplantation embryos were fixed and stained with X-gal at different developmental stages. A blastocyst expressing lacZ with mosaic pattern under fluorescence and non-fluorescence (a and a'), whole expressing pattern under fluorescence and non-fluorescence(b and b') and ICM colony stained with X-gal/AP stained (c).

Recombination rate are markedly higher in transcriptionally active DNA (Blackwell et al., 1986; Rohdewohld et al., 1987; Schulz et al., 1987; Thomas and Rothatein, 1989; Nickoloff and Reynolds, 1990). Therefore, integration into the mouse genome might occur earlier than in bovine embryos. Integration at the four- or eight-cell stage, being a relatively rare event, wo-

uld probably create only a single transgenic blastomere in a multicellular embryo and a patchy distribution of expression at later developmental stages.

These results clearly show an enrichment of blastocysts expressing the transgene in the majority of their cells after culture in the presence of G418. The exogeneous gene was not express in the epiblast cells and silence in ICM colonies except of another cells. Eventhough the higher rate cell number expressed of exogeneous gene, a total cell number was decrease significantly ($P<0.05$) of which might be also drop of the production of transgenic animals. But futher studies need anticipated to determine the viability of these selected embryos and production of transgenic offspring.

Ⅳ. SUMMARY

This study was carried out to evaluate the potential for preselection of transgenic embryos prior to transfer into recipient animals. In these experiments, I used a 3.2 kb transgene which contained the neomycin resistance gene (neo) and lac Z gene driven by the β actin promoter (iacZ/neo). Oocytes were aspirated from abattoir ovaries, matured in TCM-199 supplemented with 10% fetal bovine serum (FBS), 5 μg/ml LH, 0.5 μg/ml FSH, 100 unit/ml penicillin, and 100 μg/ml streptomycin for 22 to 24 hrs then inseminated with a sperm suspension of 1×10^6 sperm/ml containing 5 μg/ml of heparin. At 18~20 hrs after insemination, cumulus cells were removed by vortexing and pronuclei of centrifuged zygotes microinjected with the lacZ/neo construct (3 ng/μl). All cultures were carried out in CR1aa with transfected BRL monolayers containing 3 mg/ml BSA, 20 μl/ml NEM amino acids and 40 μl/ml BME amino acids. To identify the appropriate concentration of G418 for selection, non-microinjected zygotes were cultured in the presence of 0, 50, 100 and 200 μg/ml of G418. After 8 days of culture in these treatments, 44/145 (30.3%), 13/150 (8.7%), 1/151 (0.7%) and 0/134 (0.0%) developed to the blastocyst stage in 0, 50, 100 and 200 μg/ml of G418, respectively. A total of 1,127 zygotes were microinjected and placed into culture (without G418) and subsequently 710 (63.0%) cleaved. At 48 hrs post-injection, embryos ($\geq$2-cell) were randomly assigned to control (medium alone) or G418 (100 μg/ml) treatments. A control culture of 740 non-microinjected embryos from the same replicates of embryos were also placed into control medium. After 8 days in culture, 54/343 (15.7%) and 22/367 (6.0%) of the microinjected embryos developed to the blastocyst stage in control and G418 media, respectively. A total of 151/740 (27.2%) of the non-microinjected embryos placed in the control medium developed to the blastocyst stage. The blastocysts in the control treatment had a mean of 70.7 ± 4.7 cells of which 23.1 ± 2.6 (32.7%) stained for β-Gal activity. Blastocysts in the G418 treatment had a mean of 48.8 ± 7.5 cells of which 40.3 ± 4.1 (82.6%) stained for β-Gal activity ($P<0.05$). In the control treatment 26 of 30 (87.0%) blastocysts had some cells with β-Gal activity while all of the blastocysts in the G418 treatment had some cell with β-Gal activity ($P<0.05$). However, ICM colonies in either control or G418 treatments were not expressed any epiblast cell except of trophetoderm cells. The β-actin promoter/lacZ gene may not be expression or silence expression in epiblast cells.

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