EFFECT OF GREEN FLUORESCENT PROTEIN (GFP) ON THE DEVELOPMENT OF CANINE INTERGENERIC EMBRYO

PENGARUH GREEN FLUORESCENT PROTEIN (GFP) PADA PERKEMBANGAN ANJING DARI EMBRYO INTERGENERIS

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ABSTRACT

The present study investigated the effect of green fluorescent protein on the development of canine intergeneric clone embryo with bovine oocyte recipient. Cumulus oocyte complexes (COCs) were collected from slaughterhouse and matured in TCM-199 supplemented with 10% (v/v) fetal bovine serum (FBS) (Life Technologies), 0.005 U/ml bovine FSH (Antrin®, Denka Kanagawa, Japan) and 1 μg/ml estradiol (Sigma-Aldrich) at 39 °C in a humidified atmosphere of 5% CO_2 in air and donor cell tranfected with enhanced green fluorescent protein. In this experiment GFP have no negative effect in fusion or embryo development but the expression rate were decreased in 2 cell stage and then the expression gradually decreased with progression of embryo development.

Key words: intergeneric, clone embryo, canine

ABSTRAK

Penelitian ini meneliti pengaruh dari protein yang berpendar hijau (GFP) pada perkembangan dari kloning embrio anjing antargenus dengan menggunakan oosit sapi. Kesatuan cumulus-oosit sapi berasal dari rumah potong hewan dan dimasakkan di dalam media TCM-199 yang ditambahi dengan 10% FBS (Life technology), 0,005 U/ml FSH sapi (Antrin®, Denka Kanagawa, Japan) dan 1 μg/ml estradiol (Sigma-Aldrich) pada 39°C dalam kelembaban udara 5% CO_2 dan sel donor ditempeli dengan protein yang berpendar hijau. Dalam penelitian ini, GFP tidak mempunyai pengaruh yang negatif dalam penyatuan atau perkembangan embrio, tetapi laju gambarannya menurun dalam stadium 2 sel dan secara perlahan menurun seiring perkembangan dari embrio.

Kata kunci: intergenerik, klon embrio, anjing

INTRODUCTION

Interspecies somatic cell nuclear transfer method was firstly applied for conservation of endangered animals. The highly publicized that an adult sheep had been cloned from the nucleus of a frozen somatic cell (Wilmut *et al.*, 1997) speculated that cloning technologies might be applied to increase population sizes of endangered species, or even restore them following extinction (Bawa *et al.*, 1997; Cohen, 1997). Interspecies nuclear transfer also provides a possible approach to clone animal species which oocyte were difficult to obtain (Wen *et al.*, 2003; 2005).

Several studies have shown that oocyte cytoplasm from bovine, rabbits and sheep can support early development of embryos produced by nuclear transfer of somatic cells nuclei from various mammalian species (Dominko *et al.*, 1999; White *et al.*, 1999; Cohen et al., 1999; Lanza et al., 1999; Chen *et al.*, 1999; 2002; Wen *et al.*, 2003). Recently, the success of cloning gaur (Lanza et al., 2000) and mouflon (Loi *et al.*, 2001) have demonstrated that the technique of interspecies cloning can be practically applied to save highly endangered species, such as the giant panda, Ovis orientalis musilmon, buffalo, and bos gaurus (White *et al.*, 1999; Lanza *et al.*, 2000; Chen *et al.*, 2002).

The oocyte used for interspecies nuclear transfer should be easy to obtain, able to dedifferentiate other species nuclei and support development the hybrid embryos (Wen *et al.*, 2003). Donor cell marker is required to recognized and make sured the successful of nuclear transfer. Green fluorescence protein (GFP) chromophore is a single peptide composed of 238 amino acid, which adsorbs blue light and emits green light without the need of substrate or any pretreatment, formed during an autocatalytic reaction that's does not require any cofactor, small size and need a simple detection methods (Ikawa *et al.*, 1999). GFP expression has been shown in a broad range of organisms and cells including mammalian cells (Wacker *et al.*, 1997), yeast (Doyle and Botstein, 1996), plant cells (Reichel *et al.*, 1996), C elegans (Chalfie et al., 1994), sea urchin (Arnone *et al.*, 1998), zebrafish (Higashijima *et al.*, 1997), mammalian embryo (Takada *et al.*, 1997), xenopus (Pownal *et al.*, 1998) and drosophila (Hazelrigg *et al.*, 1998).

The present study aim to know the effect of green fluorescent protein on the development of canine intergeneric embryo.

MATERIAL AND METHOD

Collection of oocytes and in vitro maturation (IVM)

Ovaries from Holstein cows were collected from Garak slaughterhouse in Seoul and transported to the laboratory in Theriogenology and Biotechnology, Seoul National University within 2 h in 0.9% (w/v) NaCl solution at 35 °C. Cumulus-oocyte complexes (COCs) were retrieved from antral follicles 2–8 mm in diameter by aspiration with an 18-gauge hypodermic needle attached to a 10-ml disposable syringe. The COCs with evenly granulated cytoplasm and enclosed by more than three layers of compact cumulus cells were selected, washed three times in HEPES-buffered tissue culture medium (TCM)-199 (Life Technologies, Rockville, MD) supplemented with 0.5% (w/v) BSA (fatty acid free, fraction V, Sigma-Aldrich Corp., St. Louis, MO), 2 mM sodium bicarbonate and 10 mM HEPES (Sigma-Aldrich) and 1% (v/v) solution of penicillin and streptomycin (Sigma-Aldrich). For maturation, COCs were cultured in four-well dishes (30–40 COCs per well, Nunclon, Roskilde, The Netherlands) for 20–22 h in 500 µl bicarbonate-buffered TCM-199 supplemented with 10% (v/v) FBS (Life Technologies), 0.005 U/ml bovine FSH (Antrin®, Denka Kanagawa, Japan) and 1 µg/ml estradiol (Sigma-Aldrich) at 39 °C in a humidified atmosphere of 5% CO_2 in air.

Preparation of recipient oocytes for somatic cell nuclear transfer

At 20–22 h of maturation culture, expanded cumulus cells of COCs were removed by repeated pipetting in 0.1%

hyaluronidase in hCR2aa, and oocytes with first polar bodies were selected. Oocytes were then enucleated in hCR2aa supplemented with 10% FBS and 7 μg/ml cytochalasin B (Sigma-Aldrich) under an inverted microscope equipped with a micromanipulation system (Narishige, Tokyo, Japan). Each oocyte was held with a holding micropipette (120 μm inner diameter) and the zona pellucida was partially dissected with a fine glass needle to create a slit near the first polar body. The first polar body and adjacent cytoplasm presumably containing the metaphase-II chromosomes were extruded by squeezing with the needle. Oocytes were then stained with 5 μg/ml bisbenzimide (Hoechst 33342, Sigma-Aldrich) for 15 min and observed under an inverted microscope equipped with epifluorescence at 200× magnification. Oocytes still containing DNA material were excluded. The enucleated oocytes were placed in TCM-199 supplemented with 10% FBS and used for SCNT.

Preparation of donor canine cells

Canine fibroblast were isolated from ear skin of afghanhound dog. Canine ear skin were recovered and washed three times in D-PBS. After washing, the tissues were minced by a surgical blade on a 100 mm culture dish, followed by dissociation by 0.25% (w/v) trypsin containing 1 mM EDTA for 1 to 2 h at 38°C. Trypsinized cells were washed once by centrifugation (300xg, 2 min) and subsequently seeded into 100 mm culture dishes and cultured for 6-8 day in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1 mM sodium pyruvate, 1 % (v/v) non-essential amino acid and 10 μl/ml penicillin/streptomycin solution in a humidified atmosphere of 95% air, 5% CO_2 at 38°C before removal of unattached clumps of cells or explants. The attached cells were passage by trypsinisation when confluent.

Transfection of EGFP Gene into Ear Fibroblasts

The plasmid pEGFP-N1 encoding a red-shifted variant of wild-type GFP that has been optimized for brighter fluorescence and higher expression in mammalian cells was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). The day before transfection, confluent ear fibroblasts (at passage 3–5) were trypsinized, counted, and plated into 35-mm culture dishes to reach 80% confluency on the day of transfection. One microliter (1 μg) of pEGFP-N1 and 3 μl of FuGENE-6 (Roche Diagnostics, Indianapolis, IN) were diluted with 97 μl of serum-free DMEM. After 15 min of incubation at room temperature, 101 μl DNA-medium mixtures were added into 2 ml of cell culture medium. The cells were cultured for 2–3 days until confluency and passaged once to achieve stable integration of the gene into chromosomes before use for SCNT.

Reconstruction of enucleated oocytes, fusion and activation

Trypsinized, a single cells with a smooth surface were selected under an inverted microscope equipped with a FITC filter (wavelength: excitation 489 nm and emission 508 nm) and were transferred into the perivitelline space of enucleated oocytes through the same slit that was made during enucleation. The couplets were subsequently placed in a fusion medium comprising 0.26 M mannitol, 0.1 mM $MgSO_4$, 0.5 mM HEPES and 0.05% (w/v) BSA, and transferred into a cell fusion chamber with a stainless steel wire electrode (3.2 mm gap; BTX, San Diego, CA) after equilibration for 3 min. Fusion was induced by two DC pulses of 1.75 kV/cm for 15 μs using an Electro-cell Manipulator (BTX 2001). The fusion of the donor cell and the ooplast was determined 1 h after electric stimulation under a stereomicroscope (Nikon Corp., Tokyo, Japan). Only fused embryos were selected and subjected to chemical activation. For transgenic embryos, fusion was confirmed by observing GFP expression in recipient cytoplasm under DIC microscopy equipped with FITC filter (Nikon Corp.). Chemical activation was induced by incubating embryos in HEPES-buffered TCM-199 (hTCM) containing 5 μM ionomycin (Sigma-Aldrich) for 4 min at 39 °C. Reconstructed embryos were then washed thoroughly in

ionomycin-free hTCM and further incubated for 4 h in mSOF medium supplemented with 1.9 mM 6-dimethylaminopurine (Sigma-Aldrich).

In vitro culture (IVC)

A group of 5–7 reconstructed embryos were cultured in a 25 µl microdrops of mSOF supplemented with 0.8% BSA (Choi et al., 2002) under embryo-tested mineral oil at 39 °C with 5% CO_2, 5% O_2 and 90% N_2 in a humidified atmosphere. The embryos were evaluated at 48, 96, 144 and 192 h after activation for in vitro development. The GFP expression in embryo was determined under a FITC filter.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) and protected least significant difference (LSD) test using general linear models (PROC-GLM) in a statistical analysis system (SAS) program to determine differences among experimental groups. When a significant treatment effect was found in each experimental parameter, data were compared by the least squares method. Statistical significance was considered where the P value was less than 0.05.

RESULT AND DISCUSSION

In this study we want to learn about GFP effect on the development of intergeneric canine clone embryo and results from this experiment demonstrated that EGFP have no negative effect in fusion or embryo development (Table) but the expression rate

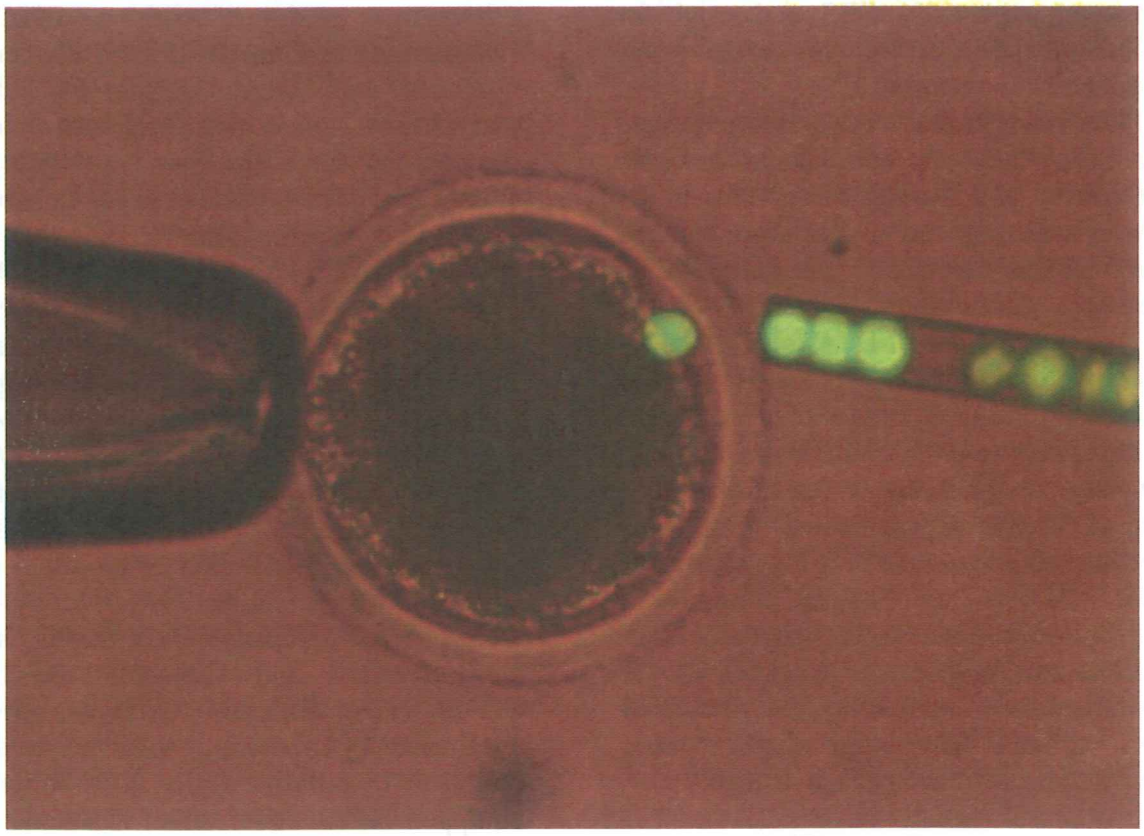

Figure 1. A single transfected donor cell was transfer into the perivitelline space of an enucleated oocyte. Donor cells were examined by fluorescent microscopy using a standard fluorescein isothiocyanate (FITC) filter set (exitation wavelength; 450-490).

were decreased in 2 cell stage and then the expression gradually decreased with progression of embryo development. We used EGFP as a marker to determined gene expression into adult cell and nuclear transfer embryo derived from that cell. GFP is though to be a non toxic biological marker since introduced first time in 1994 by Chalfie and colleagues (Chalfie *et al.* 1994). Hanazono *et al.* (1997) reported that high expressing cells (the brightest cells) died within a matter of days after transfection while low level expressing cell grew faster and dominated the culture. They hypothesized that this was due to a deleterious effect of the gene product. Hadjantonakis *et al.* (1995) reported a variation of GFP expression on transfected mouse ES cells and interpreted this phenomenon as an effect of gene copy number or the site of integration, while they did not report any deleterious effect of GFP on ES cell viability.

In several species, including pigs, enhanced green fluorescent protein (EGFP) gene was successfully used as an indicator without any adverse biological effects on in vitro development of transfected embryos (Park *et al.*, 2001; Takada *et al.*, 1997; Perry *et al.*, 1999; Chan *et al.*, 2000) and transgenic offspring carrying the GFP gene have been successfully produced in mice, monkeys, and pigs (Park *et al.*, 2001; Takada *et al.*, 1997; Chan *et al.*, 2000). That result also support our result in this study, the expression of GFP did not affect the development of NT unit with no difference result of embryo development (Table)

After fusion GFP was expressed all fused embryo, however expression rate were decreased in 2 cell stage and then the expression gradually decreased with progression of embryo development. This phenomenon suggested that constructed gene was not inserted stably into donor cell but transient. Theoretically, GFP expression of 1-cell stage should result from cytoplasm of the donor cell. Some protein and mRNA (Parry and Prather, 1995) from the donor cells were introduced with injection of the nuclei. After injection of the donor nucleus, GFP protein would be dispersed into the cytoplasm of oocytes and be detected and GFP mRNA also could produce GFP in the cytoplasm. The timing of the transtition from maternal to zygote control of embryonic development (maternal to zygotic transcript transition; MZTT) is the 8~16 cell in cattle (Barnes *et al.*, 1993). Therefore, if the donor nucleus remodeled and reprogrammed, detectable GFP in early embryo stage (until 8~cell) should result from the donor cell, but detection of GFP at later stage (especially in more 8~cell) should be result of net transcript from the NT embryo. With cell fusion by transfer the donor cell into perivitelline space, most of the cytoplasmic material (some protein, mRNA, etc.) of donor cell was introduced into the oocyte. Thus, both GFP message and protein might be detected in the early embryo (before the onset of transcription). In this study, no expression of GFP observed in early stage (before MZTT) was might be that inserted gene transcription or translation in the donor cell was not complete and no expression of GFP observed in late stage (after MZTT) was might be that transfection of gene is not stable but transcient by incomplete integration. Additionally,

Table. Effect of reconstructed cell with GFP on development of canine intergeneric embryo with bovine oocyte recipient

Treatment	Total oocytes	Fusion (%)	Total cleavage	No. (%) of embryo				
				2 cell	4 cell	8 cell	16 cell	32 cell
Normal	274	189(68)	112 (59)	20 (18)[a]	38 (34)	31 (28)[a]	22 (20)[a]	1 (1)
GFP	264	138 (52)	87 (63)	9 (10)[a]	40 (46)	28 (32)[a]	8 (9)[a]	1 (1)

[a-d]) Values with different superscripts are significantly different ($p<0.05$)

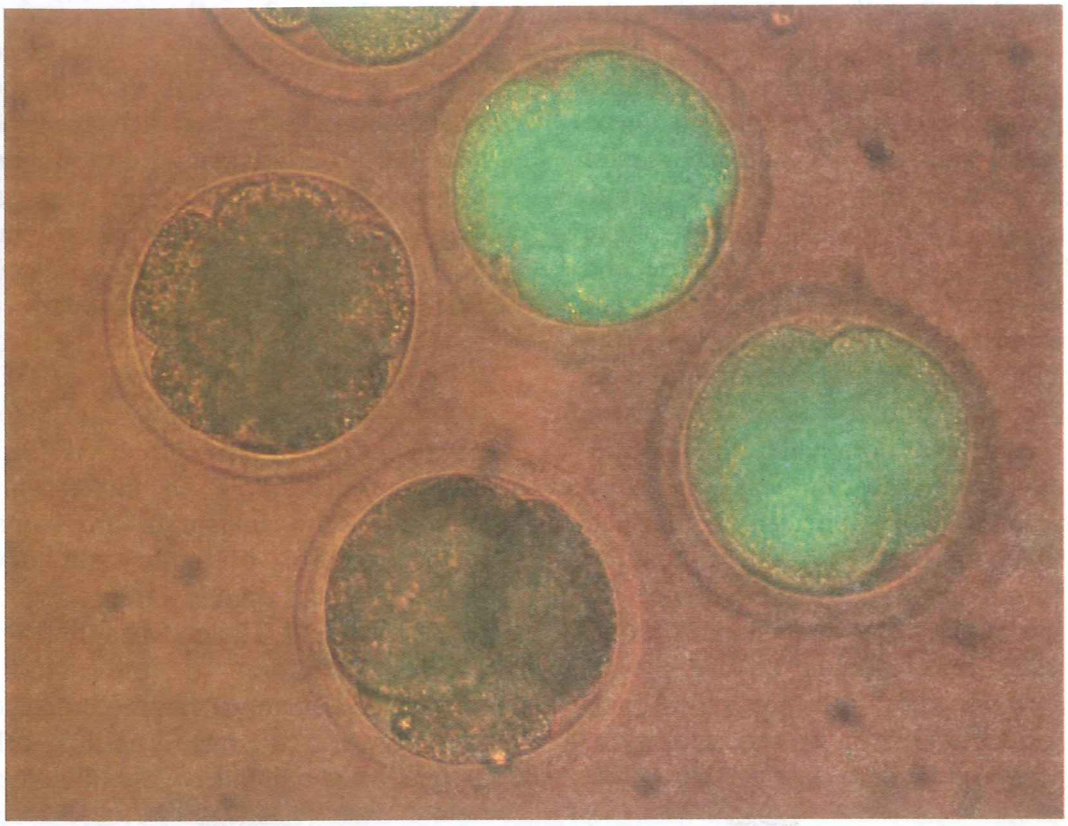

Figure 2. Expression of green fluorescent protein (GFP) in 4 cell intergeneric embryos.

promoters and enhancers regions can also affect expression levels, thus a stronger expression vector containing CMV enhancer may promote more efficient GFP expression in transgenic embryos (Kato *et al.*, 1999).

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