

Non-invasive evaluation of embryo morphological plasticity by designing of new transgenic gene cassette

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Abstract

Background: Determination of transgenic embryos from non transgenic embryos sibling is an important step in producing homozygous transgenic mice. These steps need by PCR or southern blotting followed extraction of DNA, but both techniques require skill and consume time.

Objective: The aim of this study was simulation of high accuracy method using novel enhanced green fluorescent protein (*EGFP*) gene cassette to eliminate some consume time in livestock industry to assay high quality embryos and morphological plasticity.

Materials and Methods: We modified pQE-Tri systemic vector with *EGFP* and *IRE5* sequence to trace out coming planning of molecular farming transgene using co-injection method.

Results: The combination of these sequences successfully showed the faint and normal expression of transgene in mouse pre-implantation stage embryos. The low rate of surviving green positive embryos as compare as only medium and physical treatments could be partly from the physical damage caused by microinjection and gene integration. Furthermore, application of the enhanced *GFP* marker facilitated subtle detection of asymmetrical division appeared in some of transgenic embryos.

Conclusion: The results of current mouse simulation model imply that an efficient production and propagation of transgenic livestock can be done by co-injection of every economical gene with this novel transgene and also it can be suitable gene cassette for numerous experiments and study of protein behaviors in living cells.

Key words: *Enhanced green fluorescent protein (EGFP), Transgenic animals, Embryo.*

Introduction

Since the first report of the green fluorescent protein (GFP) expression in *Caenorhabditis elegans* (1), this novel and simple reporter has attracted much interest as an in vivo marker for gene expression. Other gene reporters, which encode the specific enzymes firstly require substrates to detect the gene expression, but the detection of GFP does not require the extraction, substrate loading steps and following toxicity, so we can monitor the presence of GFP by

illuminatin living cells. Already, several substrates have been developed that can be permeable to the plasma membrane and are processed in the cytoplasm for use in living cells (2). However, it is still necessary to load the substrate with these markers (3-5).

On the other hand, the discernment of transgenic embryos from non transgenic embryos sibling is an important step in producing homozygous transgenic mice. These steps need by PCR or southern blotting followed extraction of DNA (4, 6) but both techniques require skill and consume time. Thus, to circumvent these problems, it seems that selection of embryos before implantation with an integrated transgene for embryo transfer will have high efficacy in transgenic technology regarding the long gestation period, limited number of offspring and low

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percentage of implantation if applied to a large number of transgenic animals.

After the production of the founder transgenic animals, it would be necessary to propagate the transgenic animals. Therefore, for the production of large transgenic animals such as cow and pig, the detection of the transgenes at the preimplantation stage would be desirable. Therefore, the method for quick separation of transgenic embryos with high quality will be important for the rate of implantation after embryo transfer. In addition, blastocyst biopsy and subsequent PCR analysis enable us to separate X and Y chromosome bearing embryos (1, 5) which will be important in animal breeding and the production of milk and meat. Several reports have suggested the possibility of using marker genes for this purpose (7, 8). In this context, we describe the designing of novel transgene, which enables us to separate quickly and non-invasively high quality transgenic embryos and also parallel monitoring of the effect of exogenous gene on mammalian cells. We demonstrate this applicability by designing the novel GFP transgene. Moreover, we try to assay morphological plasticity of the produced transgenic embryos after microinjection system to evaluate this new transgenic gene cassette and its usefulness probably in livestock industry.

Materials and methods

Animals

The outbred six-week old CXB RI females were resulted by C57BL/6 × BALB/c mating from the breeding farm of Pasture Institute of Iran. Pregnant female mice were sacrificed humanely for embryo biopsy by cervical dislocation method (9).

Construction of EGFP expression vectors

In brief, the CAG promoter, which was composed of the CMV enhancer, a fragment of the chicken β -actin promoter and rabbit β -globin intron was used. The IRES-EGFP fragment covering the coding sequence from plasmid pIRES2-EGFP (Clontech, Palo Alto, CA) was digested with *Bgl*II and *Not*I and inserted into the pQE-Tri system expression vector (Qiagen, Hilden, Germany) containing the CAG promoter and rabbit β globin termination region. Transgenes were excised immediately downstream of the polyadenylation signal and upstream of the CMV-chicken β actin promoter with the *Pae*I and *Esp*3I enzymes and the entire

insert was gel purified (QE-IRES2-EGFP; 3.1 kb). For construction of another GFP expression vector used as control, in brief the chicken β actin promoter was amplified by PCR with 5' primer; TACGTATCGAGGTGAGCCCCACGTTCT and 3' primer; GCTAGCCGGTACACGCCAGAAG from the genomic DNA of Iranian poultry (Research Center of Animal Science, Karaj, Iran) as a template. *Sna*BI and *Nhe*I sites included in the PCR primers were used to introduce this amplification product into pIRES2-EGFP instead of the short chicken β actin promoter sequence that there was in the first designed construct. The entire insert with the promoter and coding sequence was excised with *Ase*I and *Afl*III and gel-purified (Qiagen, CA) (Figure 1).

Microinjection and production of transgenic embryos

The outbred six-week old CXB RI females were superovulated by injecting 5IU of PMSG and hCG, respectively, at 48 h intervals and then fertilized one-cell embryos of CXB RI females mated with CXB RI males were collected from the oviduct. Transgenic green embryos were produced by microinjecting the purified *Pae*I and *Esp*3I fragment with QIAEX (QIAGEN, CA) into male pronuclei of recovered fertilized eggs. This procedure was also performed using *Ase*I and *Afl*III fragment resulted from the second construct. After microinjection (Nikon 300, Hoffman) of EGFP transgenes, embryos were cultured.

Preparation of embryos and in vitro fertilization (IVF)

On the day following hCG injection and for sperm mediated gene transfer using IVF technique, mature oocytes (MII) were recovered by opening the oviducts in pre-warmed M16 medium supplemented with 10% bovine serum albumin. At the same time, mature male CXB RI was killed to remove the tail of epididymis. The extracted spermatozoa were then cultured in M16 droplets for 2 h at 37°C, 5%CO₂. The IVF took place approximately 5 h post sperm incubation.

Observation and selection of embryos

For quality prediction of preimplantation embryos after injection using our novel transgene, three major standard criteria were used; 1- the rate of development, 2- the degree of fragmentation, 3- the intensity of EGFP expression (10). Also to evaluate the ability of development to blastocyst (4 dpc) and viability of transgenic embryos,

EGFP-transgene injected (EGFP treatment), T₁₀E_{0.1} buffer injected (physical treatment) and non-injected IVF eggs (medium treatment) were cultured. The incorporation and separation of green and non green embryos was examined by placing step by step developing embryos under UV light using a Zeiss (Germany/Axiocvert) inverted fluorescence microscope with filters BP

450-490, FT 510, LP 520. EGFP was distinguishable by shedding the different excitation light. The eggs expressing EGFP emit green when excited with 488 nm and detected at 500-to 530 nm band pass filter. Separated embryos were washed and cultured in M16 medium for development and further assay using PCR analysis.

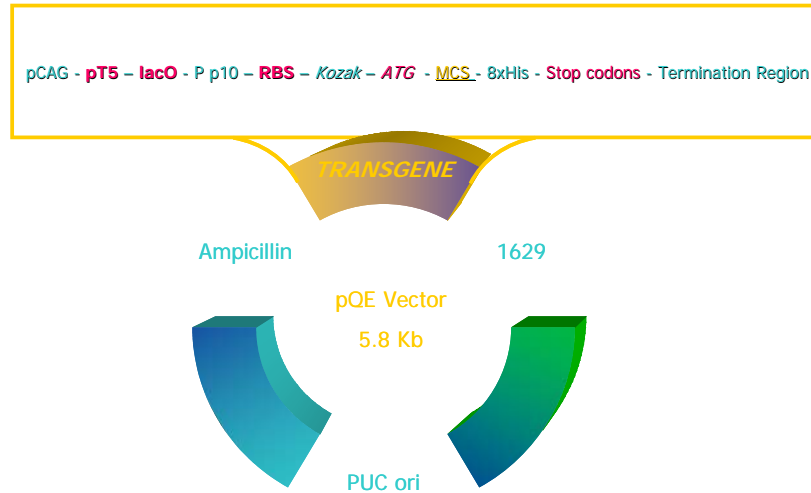


Figure 1. Structure of novel transgene. The linearized QE-IRES2-EGFP transgene (3130 bp) from the pQE vector contains the CMV-IE and chicken beta actin globin (CAG), T5 promoter, lac operator (Lac O), ribosome binding site (RBS); kozak consensus sequence, start codon (ATG), His tag sequence (8xHis), MCS; containing of inserted desired IRES-EGFP sequence (1367 bp) for bicistronic expression, stop codon and termination region.

PCR amplification of embryo samples

One blastocyst was transferred into an Eppendorf PCR tube with 10 µl of dH₂O, overlaid with 30 µl of light mineral oil. The samples were heated for 10 min at 97°C. After cooling down, 1 µl of proteinase K stock solution (10 mg/ml) was added to each tube, and then treated for 1 h at 56°C. Proteinase inactivation was done for 10 min at 97°C. Then 3.5 µl of dH₂O, 2 µl of 10X PCR buffer (100 mM Tris-HCl, pH 9.0, 500 mM KCl, 1% Triton X-100), 1.5 µl of MgCl₂ stock solution (25 mM), 1 µl of dNTP mixture (each 5 mM of dATP, dCTP, dGTP, dTTP) and 0.5 µl of each primer (50 µM) were added to individual tubes. The primers used for the detection of the GFP were: 5' TGGAGAGGGTGAAGGTGATGC and 3' TGTGTGGACAGGTAATGGTTG. Finally, 5 µl of polymerase solution (2.5 units of Taq polymerase in 1×PCR conditions) were added. Therefore PCR reaction was performed by: 40 cycles of denaturation at 94°C for 30 s, annealing at 54 °C for 30 s and extension at 72 °C for 40 s. After the completion of 40 cycles, 5 µl of each

sample was analyzed immediately by 1% agarose gel electrophoresis.

Protein analysis

The DNA construct was transformed into *E. coli* strain JM109. An overnight culture of bacteria (5 ml) was diluted into 300 ml of LB medium. At OD₆₀₀ = 0.4, the culture was induced by IPTG (1 mM) and incubation was continued for 4 hr at 37°C.

Cells were then pelleted and resuspended in 10 ml of Sonication buffer (SB 50 mM Na-phosphate PH 7.8, 300 mM NaCl) and after washing with PBS were lysed by sonication (6 times for 30 s in an ice bath). The lysate was clarified by centrifugation at 14000 rpm for 20 min and applied to 1 ml Ni-NTA (Qiagen) column equilibrated with SB (pH 8).

The column was washed with 50 ml of SB (PH 6) and the protein eluted with 8 ml of SB (pH 4.5). SDS-PAGE method was performed under non-reducing conditions using a 12% gel. Gels were stained with 0.1% Coomassie brilliant blue R250 (Biorad, Richmond, CA) and destained in

isopropanol:acetic acid:water (1:1:8) by gently shaking at RT.

Results

Expression of EGFP in preimplantation mouse embryos

Of 72 embryos microinjected with novel EGFP transgene, 69 survived and were cultured for further development. After 4 days in vitro culture followed by microinjection, 44 embryos developed to blastocysts. In this period, EGFP was easily

detected by fluorescence microscopy at two-cell, to morula and blastocyst stages in 17 embryos (EGFP-positive) while embryos 27 were EGFP-negative. The percentage of survived embryos to developed blastocyst in EGFP expressing embryos was less than physical and medium treatment (IVF) groups. Also in our transgenic embryos the percentage of EGFP positive embryos from surviving embryos after microinjection was remarkably higher than most published data which means creating of good morphological plasticity (Table I, Figure 2).

Table I. Microinjection experiment with novel transgene and CXB FI superovulated eggs. a) Positive samples; *EGFP* gene treatment embryos, b) T₁₀E_{0.1} buffer injected embryos; physical treatment and c) non-injected embryos are for evaluation of medium effect.

	Recovered eggs	Embryos injected	Embryos survived	Blastocyst developed embryos	EGFP-positive embryos
Positive	121	72	69	44	17
TE buffer injected	152	89	81	73	-
Non-injected (IVF Control)	173	-	-	156	-

Transgenesis identification from EGFP expressing blastocyst

To verify the amplified sequence and increase sensitivity, we performed two rounds of PCR analysis firstly using EGFP-positive blastocyst genomic DNA and secondly EGFP transgene-specific primers. On the nested PCR analysis, transgene specific 696 bp bands were amplified from first round PCR amplification proved that all the selected green embryos inherited EGFP transgene. No amplification was detected with genomic DNA from EGFP-negative embryos (Figure 3, 4, 5).

Protein analysis

After sub-cloning of IRES-EGFP sequence into pQE Tri systemic vector, we didn't see the evidence of EGFP protein expression into bacterial cells in which it was detected before ligation of IRES segment with EGFP gene. The SDS-PAGE experiment confirmed this phenomenon. This was additional confirmation to check the accuracy of EGFP expression pattern following of sub-cloning and changing of expression shift into mammalian cells. The reason was the existence of IRES sequence, which had made a long distance disruption between RBS and start codon of EGFP gene in bacterial cells (Figure 6).

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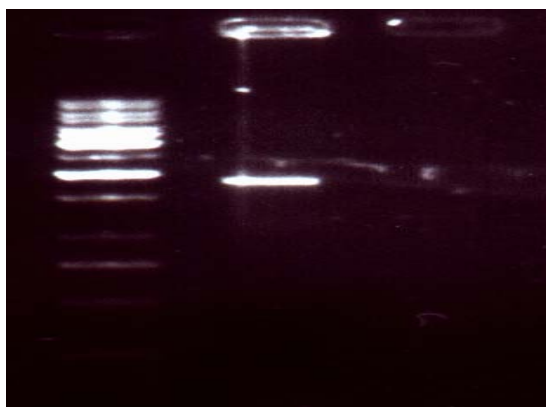


Figure 2. The cloning of chicken beta actin promoter from Iranian poultry Lane 1: 1kb DNA ladder (Fermentas), Lane 2: Amplification product (1700 bp), Lane 3: Negative control.

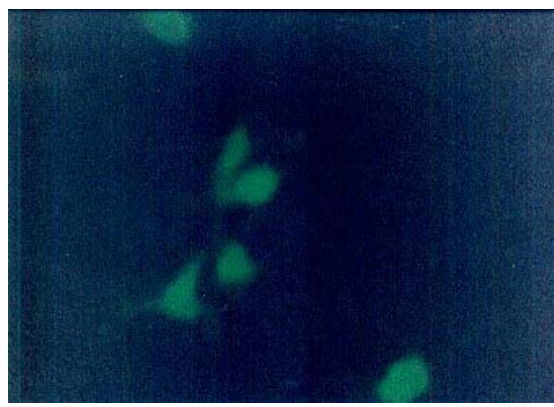


Figure 3. In vitro transfection of novel designed transgene into eukaryotic COS 7 cell lines before microinjection experiment using lipofection method (QIAGEN effectene transfection kit).

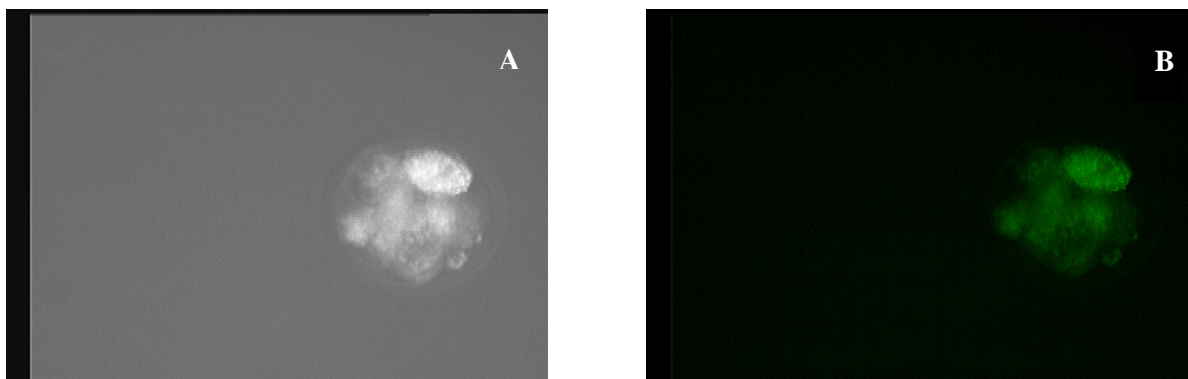


Figure 4. Photomicrographs of EGFP expression in preimplantation mouse embryos. EGFP expression was monitored at 8 cell (2.5 dpc) using fluorescent microscope. After microinjection of transgenes, embryos exhibited uniform EGFP patterns that they were clearly and faintly visualized in %25 preimplantation stage embryos. Also this photo clearly shows the asymmetrical division of embryo. The picture was photographed with 20× objectives. A) Light microscope, B) fluorescent microscope

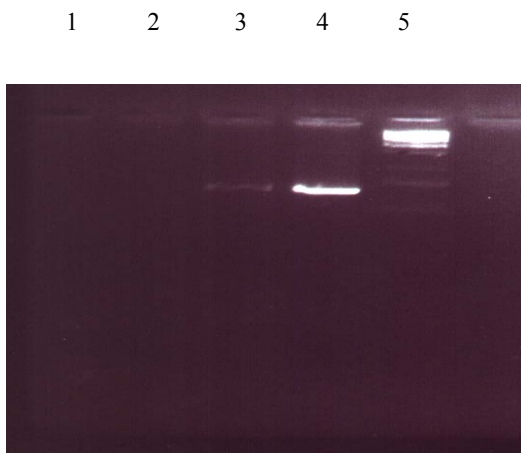


Figure 5. PCR analysis of selected embryos. Lane 1: Negative control without DNA. Lane 2: EGFP negative embryo, Lane 3 and 4: Nested 696 bp amplification products using EGFP DNA positive embryos. Lane 5: 100 bp DNA ladder (Fermentas).

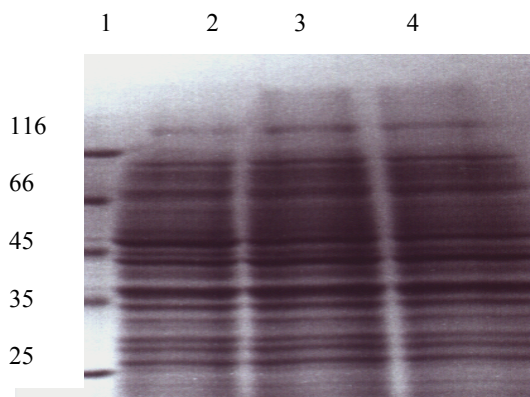


Figure 6. SDS-PAGE analysis of EGFP product from novel pQE-IRES2-EGFP gene construct after transformation into *E. coli*. Lane 1: Molecular weight markers in kDa. Lane 2: Bacterial lysate before induction by IPTG, Lane 3-4: Bacterial lysate after induction, 1 and 5 h later, respectively. EGFP expression in combination with pQE Tri-systemic vector was not detected in prokaryotic system following of inserting IRES2-EGFP segment. It was done to be sure unique protein expression in mammalian cells after modifying the vector from prokaryotic expression system.

Discussion

The aim of this study was simulation of high accuracy method using novel *EGFP* gene cassette to eliminate some consume time in livestock industry and high quality embryo morphological selection. Southern blotting procedure requires skill and it consumes time if applied to detect a transgene between large numbers of transgenic animals. Also the low efficiency of transgenic animal production by microinjection has been a serious problem, especially in large mammals because of long gestation period and limited number of offsprings. Already the enhanced green fluorescent protein (EGFP) has been known the best marker whose codon usage has optimized for human cells (11). The preliminary usage of substrate is not imperative in *EGFP* gene reporter. Otherwise using of *EGFP* mutant that has an excitation peak at 490 nm, minimized damage to embryos by ultraviolet light (12). Thus non-invasive selection of transgenic embryos was performed successfully at the pre-implantation stage of embryos using *EGFP* gene marker under our novel pQE-IRES-EGFP-Tri systemic gene cassette (Figure 4).

We modified pQE-Tri systemic vector (Qiagen) with EGFP and IRES sequence to trace out coming molecular-farming gene by co-injection method. It means by available GFP variants, which are different in spectra, we are able to study the characters of another co-injected and expressed gene in developmental research area. This novel transgene with restricted role to play bicistronic expression for both desired genes using IRES, also will be suitable for pharmaceutical planning when be co-injected with second gene. It is able to assay every gene after a little modification using three specific promoters located in pQE vector, into three separated prokaryotic, baculovirus and eukaryotic systems. Furthermore, we replaced the chicken beta actin promoter colonized from our poultry in Iran instead of short promoter in pQE vector to evaluate size effect between both promoters. We found no difference at EGFP intensity run by two different size promoters under the fluorescence microscope. Beyond the morphological plasticity, the EGFP is believed to be a non-toxic biological molecule (11, 13), but it's over expression in a few instances is shown to be cytotoxic in transgenic mice even to cause dilated cardiomyopathy (14).

Whereas the combination of these sequences in both our designed constructs successfully showed the faint and normal expression of EGFP in mouse

pre-implantation stage embryos. It was regarded to specific data in which the faint-moderate expression of EGFP in transgenic embryos is compatible with normal embryogenesis in mice (15). The expression of EGFP protein into the cytosol and it's solubility were not seemed to be toxic because of normal and high quality production of embryos. The green fluorescence observed in the morulae stage grew faint in all of the blastocyst stage embryos.

We performed to select embryos at the blastocyst stage. This prevent firstly the effect of transient expression, result of the epichromosomal, and non-integrated transgene and secondly mosaic expression that may be due to the integration of the transgene after the first round of the DNA replication and thirdly for observation of GFP intensity in embryos (16, 17). In both mice and human, 15-50 % of embryos die during the preimplantation period from mechanisms that are largely unknown thus the low rate of surviving EGFP positive embryos comparing to only medium and physical treatments could be partly from the physical damage caused by microinjection and gene integration (Table I). We however decrease this matter because $T_{10}E_{0.1}$ buffer injected eggs showed clearly higher percentage of blastocyst development comparing to EGFP injected embryos. On the other hand, application of IVF was done firstly because of controlling both transgenic and non-transgenic embryos affected by medium and secondly out-coming sperm mediated gene transfer to provide some study about sex selection in livestock industry.

It is related to accelerate genetically additive value of elite animals using blastomer biopsy and followed embryo sexing by PCR analysis (18, 19). It is interesting referring the data from Devgan et al (2004) that the developed blastocyst expressing EGFP can develop in post-implantation stage as well as reproductive out come of transgenic animals (14).

As shown in the experiment by novel EGFP transgene, it is easy to classify transgenic eggs with high morphological plasticity before implantation stage with 100% of accuracy. Many mixed transgenic and non-transgenic embryos were separated under fluorescence microscope and were subjected to PCR analysis and the effect of new transgene was confirmed by standard grading method. Furthermore it was shown briefly the result of physical and transgene injected embryos that it needs to be large analysis on statistical basis.

Conclusion

The results of current mouse simulation model imply that an efficient production and propagation of transgenic livestock can be achieved by co-injection of every economical gene with this novel EGFP transgene and also it can be suitable gene cassette for numerous experiments and behaviors of proteins in living cells.

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