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Generation of Venus fluorochrome expressing transgenic handmade cloned buffalo embryos using Sleeping Beauty transposon

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ABSTRACT

The objective of this study was to optimise the electroporation conditions for efficient integration of Venus construct in buffalo fetal fibroblasts using Sleeping Beauty (SB) based transposition and to produce Venus expressing transgenic cloned embryos through handmade cloning (HMC) approach. Primary culture of buffalo fetal fibroblast cells was established and subsequently cultured cells were co-transfected with Venus and helper plasmid at different combinations of electroporation condition. In different combinations of voltage, time and plasmid dose, we observed that 300 V, single pulse for 10 ms in 2 mm cuvette and 1.5–2.0 µg transposons with 200–300 ng transposase dose was optimum for expressing Venus fluorescence in cells via electroporation. After electroporation, the cells were cultured for 2–3 days and then Venus expressing cells were picked with the help of a Pasteur pipette under the fluorescence microscope to enrich them through single cell culture method before using as donor cells for HMC. In vitro matured oocytes were reconstructed with either transfected or non-transfected buffalo somatic cells by electric fusion followed by activation. The reconstructed, activated embryos were cultured in 400 µL of Research Vitro Cleave medium supplemented with 1% fatty acid-free BSA in 4-well dish, covered with mineral oil and incubated in an incubator (5% CO_2 in air) at 38.5 °C for 8 days and the developmental competence was observed. The percentage of cleaved, 4–8 and 8–16 cells stage embryos generated through Venus expressing cells were comparable with control, whereas, the morula (21.0 vs 53.0%) and blastocysts (10.5 vs 30.6%) produced through Venus expressing cells was found low as compared to control. These results indicate that fetal fibroblasts transfected with Venus could be used as donor cells for buffalo cloning and that Venus gene can be safely used as a marker of foreign gene in buffalo transgenesis.

1. Introduction

The desired gene could be transferred into the animal by various methods including microinjection of foreign DNA into the pronuclei of the zygote and nuclear transfer of embryonic stem cells with a foreign gene. Recent introduction of active methods of transgenesis and availability of annotated genome depositories enhance precise genetic engineering to manipulate livestock genomes (Mátés et al., 2009; Garrels et al., 2016; Yum et al., 2016). Now transgenic animals are being produced using established methodologies either of DNA microinjection into zygotes (Mátés et al., 2009; Garrels et al., 2016) or somatic cell nuclear transfer (SCNT) with transgenic somatic cells (Alessio et al., 2016). Recently, SCNT has been considered as a promising approach for generating transgenic animals, as it allows evaluation of the transgene expression in somatic cells by molecular techniques, such as PCR and Southern blot analysis before using these cells in SCNT. Further, in SCNT, offspring sex can be as per choice and is known before birth by choosing the donor cells. Apart from these, transgenic somatic cells could also be propagated almost limitless number of passages and frozen stored for long periods of time. The SCNT also ensures that animals produced are 100% transgenic and that every cell of an animal will have the transgene, thereby saving time and cost, associated with recipient animals. Generation of transgenic animals by SCNT has been achieved in animals including sheep (Wilmut et al., 1997), goat (Zhang et al., 2015), pig (Betthauser et al., 2000; Giraldo et al., 2012) and cattle (Kato et al., 1998). A simplified handmade cloning (HMC) procedure (Vajta et al., 2001), has been applied for the successful generation of genetically engineered pigs (Zhang et al., 2012), and sheep (Zhang et al., 2013).

Stable genetic manipulation of somatic donor cells (Alessio et al., 2016) could be achieved through various mechanisms including random genomic insertion of plasmid DNA (Watanabe et al., 2005) and

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transduction with recombinant retroviral vectors derived from gamma-retroviruses or lenti-viruses (Park et al., 2002). DNA transposons, which can integrate into the chromosomes of the host cells, have been successfully used for transgenesis and insertional mutagenesis in several invertebrate models. The discovery of the Sleeping Beauty (SB) transposon expanded the utility of transposon-based technologies in vertebrate species. During transposition, a single copy of a gene of interest flanked by the inverted terminal repeats (ITR) of a transposon is stably incorporated into the genome by the transposase (enzymatic factor). The drawbacks of classical methods for transgenesis can be overcome by utilizing transposase-catalyzed gene delivery, as it increases the efficiency of chromosomal integration and preferentially favours single-copy (monomeric) insertion events (Garrels et al., 2011a). Porcine primary cells were transduced with SB plasmids expressing human b1 or a2 integrin under the control of a promoter specific for suprabasal keratinocytes were successfully employed to generate cloned Gottingen mini pigs expressing eGFP (Jakobsen et al., 2011) and pig (Staunstrup et al., 2012) by HMC.

To achieve efficient integration of desired exogenous genes into the fetal fibroblasts, various strategies such as lipid based delivery (Hyun et al., 2003), viral delivery (Lai et al., 2002), and electroporation (Ramsoondar et al., 2003; Watanabe et al., 2005) have been used to successfully produce transgenic animals. Among these strategies, electroporation, a physical technique is safe, simple and inexpensive. It applies controlled electrical pulse to cells, inducing a transient destabilization of the cell membrane; and makes short-lived rearrangements in the structure of lipid bilayer membranes that have a lifetime of milliseconds to minutes. Creating pores in cell membranes provide transient access to the cytosol. It is mostly used in vitro to transfect cell types which show low efficiencies of DNA uptake using other techniques, such as lipofection or calcium–phosphate mediated transfection (Neumann et al., 1982). It also provides the constant conditions and as a result, has the highest efficiency (Yorifuji et al., 1989). Hence, optimizing the conditions for electroporation of any particular cell type is primarily empirical. In the present study, we optimised the electroporation condition for efficient integration of Venus construct (derivative of the enhanced yellow fluorescent protein) in buffalo fetal fibroblasts using SB transposition and produced Venus expressing transgenic cloned embryos through HMC approach.

2. Materials and methods

The chemicals and culture media were obtained from the Sigma Aldrich (St. Louis, MO, USA), serum from Gibco (Grand Island, NY, USA), and disposable plasticware from Nunc (Roskilde, Denmark), unless otherwise stated. Cell culture was done at 38.5 °C with 5% CO_2 in air and maximum humidity.

2.1. Ethics statement

Experiments were conducted after following the guidelines laid down by Institute Bio-Safety Committee, ICAR-CIRB, Hisar, India.

2.2. Isolation and culture of buffalo fetal fibroblasts

Isolation, culture and cryopreservation of buffalo fetal fibroblast were performed as per Yadav et al. (2012). Briefly, buffalo fetuses (approximately 50–60 days) from slaughtered animals were separated from uteri and washed twice with sterile phosphate buffered saline. Skin biopsies were taken, minced by scissors into small pieces and washed 6–8 times with DPBS fortified with antibiotic. The tissue pieces were transferred to 25 cm^2 cell culture flask and were cultured in fibroblast cells culture media containing DMEM supplemented with 20% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, 1% multivitamin, 100 U/mL penicillin, and 100 µg/mL streptomycin in a CO_2 incubator (5% CO_2 in air) at 38.5 °C. The explants were removed after achieving confluency. Confluent cultures were treated with trypsin-EDTA (0.25%), and sub-cultured for cell multiplication. At passage-2, the harvested cells were either used for electroporation or frozen in small aliquots in LN_2 for future use.

2.3. Preparation of plasmid and transfection of cells

The plasmids pCMV-SB100X and pT2CAGGS-Venus containing the SB100X transposase (pCMV-SB100X) and a transposon carrying a Venus fluorochrome driven by the ubiquitous CAGGS promoter (pT2CAGGS-Venus) were kindly provided by Dr. Wilfried A Kues, Institut für Nutztiergenetik, Friedrich-Loeffler-Institut, Mariensee, Germany and the same were earlier used (Mátés et al., 2009; Garrels et al., 2011a). This plasmid was transformed into DH5α strain of *E. coli* and purified by commercial anion exchange columns (Endo-Free Plasmid Maxi Kit, Qiagen) and re-suspended in ultrapure water and checked for the absence of bacterial genomic DNA through restriction enzyme digestion method. The concentration of DNA was determined by a Nanodrop photometer; purity and supercoiled conformation were verified by gel electrophoresis. The actively growing buffalo fetal fibroblasts were co-transfected with pT2CAGGS-Venus and pCMV-SB100X at different combination of electroporation condition in 2 mm cuvette with square wave (ECM 2001, BTX, San Diego, CA). The cells were cultured for 2–3 days and then Venus expressing cells were picked with the help of a Pasteur pipette under the fluorescence microscope and further enriched through single cell culture method before using as donor cells for HMC.

2.4. Handmade cloning using Venus expressing somatic cells

HMC was performed as described (Selokar et al., 2012). Briefly, cumulus cells were removed from matured cumulus-oocyte complexes (COCs) by treatment with hyaluronidase (0.5 mg/mL). Oocytes, with completely digested zona pellucida were manually bisected using a microblade to remove nuclei. Each enucleated cytoplast was attached to single transgenic fibroblast and then transferred to fusion medium (0.3 M D-mannitol, 0.1 mM $MgCl_2$, 0.05 mM $CaCl_2$, and 1 mg/mL polyvinyl alcohol [w/v]) for equilibration. The couplets and the remaining demi-cytoplasts were then transferred away from the positive and negative poles, respectively, of the fusion chamber (BTX microslide 0.5 mm gap, model 450; BTX, San Diego, CA, USA). Initially, the triplets were aligned with an AC pulse (4 V) followed by a single DC pulse (160 V for 4 ms) for electrofusion using BTX Electrocell Manipulator 2001 (BTX, San Diego, CA, USA). The triplets were then incubated in T20 medium for 4 h at 38.5 °C. The reconstructed embryos were then activated (~29 h after the start of maturation) by incubating in T20 medium containing 5 µM calcimycin A23187 for 5 min at 38.5 °C. After washing thrice with T20 medium, fused embryos were incubated in 4-well plate containing 400 µL of T20 medium containing 2 mM 6-dimethylamino purine (6-DMAP) covered with mineral oil and incubated in CO_2 incubator at 38.5 °C for 6 h.

2.5. Embryo culture

The reconstructed, activated embryos were cultured in 400 µL of Research Vitro Cleave medium (K-RVCL-50, Cook1 Australia, Queensland, Australia) supplemented with 1% fatty acid-free BSA in 4-well dish (10–15 embryos per well), covered with mineral oil and incubated in a CO_2 incubator (5% CO_2 in air) at 38.5 °C for 8 days. The reconstructed embryos were cultured for 8 days after activation. The cleavage, 4–8, 8–16, morula and blastocyst formation rates were monitored under a stereomicroscope at 2nd and 8th day of culture.

2.6. Fluorescent microscopy excitation of Venus fluorescence

Venus expression was observed under a fluorescence inverted zoom