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Development of porcine transgenic nuclear-transferred embryos derived from fibroblast cells transfected by the novel technique of nucleofection or standard lipofection

M. Skrzyszowska [a,*], M. Samiec [a], R. Słomski [b,c], D. Lipiński [b,c], E. Mały [c]

[a] *Department of Animal Reproduction Biotechnology, National Research Institute of Animal Production, Krakowska 1, 32-083 Balice/Kraków, Poland*
[b] *Institute of Human Genetics, Polish Academy of Sciences, Strzeszyńska 32, 60-479 Poznań, Poland*
[c] *Department of Biochemistry and Biotechnology, Agricultural University, Wołyńska 32, 60-637 Poznań, Poland*

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Abstract

The aim of our study was to determine the in vitro developmental potential of porcine nuclear-transferred (NT) embryos that had been reconstructed with *Tg*(*pWAPhGH-GFPBsd*) transgene-expressing fibroblast cells. The gene construct was introduced into fibroblast cells by the novel method of nucleofection or standard lipofection. NT oocytes derived from foetal and adult dermal fibroblast cells were stimulated by either simultaneous fusion and electrical activation (Groups IA and IB) or sequential electrical and chemical activation (Groups IIA and IIB). The percentages of cloned embryos that reached the morula and blastocyst stages were 152/254 (59.8%) and 77/254 (30.3%) or 139/276 (50.4%) and 45/276 (16.3%) in Groups IA or IB, respectively. The rates of NT embryos that developed to the morula and blastocyst stages were 103/179 (57.5%) and 41/179 (22.9%) or 84/193 (43.5%) and 27/193 (14.0%) in Groups IIA and IIB, respectively. In conclusion, the in vitro developmental competences of porcine transgenic NT embryos that had been reconstructed with the *Tg*(*pWAPhGH-GFPBsd*) gene-transfected fibroblast cells were relatively high. Further, the nucleofection efficiency of all the porcine fibroblast cell lines as estimated by intra-vitam fluorescent evaluation based on the index of reporter eGFP transgene expression was nearly 100%. However, PCR analysis for transgene screening confirmed the absence of *Tg*(*pWAPhGH-GFPBsd*) fusion gene in some of the nucleofected cell lines. To our knowledge, the novel method of nucleofection is the first to transfect nuclear donor cells in the production of transgenic cloned embryos.

Keywords: Porcine; Fibroblast cell nucleofection; Nuclear transfer; Oocyte activation; Transgenic embryo

1. Introduction

Swine embryo engineering (somatic cell cloning combined with transgenesis) is a particularly important research field within assisted reproduction technologies, and may relate to both xenotransplantology [1,2] and the creation of animal bioreactors for the production of biopharmaceuticals [3,4]. Nonetheless, the overall efficiency involving the generation of viable transgenic embryos and/or offspring with the aid of somatic cell cloning remains relatively low.

A significant aspect of applying somatic cell nuclear transfer (SCNT) technology to generate transgenic embryos and/or offspring is the origin/type of nuclear donor cells and the in vitro techniques utilized to obtain

* Corresponding author. Tel.: +48 12 25 88 162; fax: +48 12 28 56 162.
E-mail address: mskrzysz@izoo.krakow.pl (M. Skrzyszowska).

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viable donor nuclei for cloning procedures. Genetically transformed cells can be selected in vitro, and only the cells with stable, integrated transgenes are used as a source of nuclear donor cells to reconstruct the enucleated oocytes through somatic cell cloning. For the establishment of transgenic cell lines, various selection markers with different actions and advantages have been employed, including either antibiotics such as neomycin/geneticin G418 [5–7], puromycin [8] and blasticidin S [9], or enhanced green fluorescent protein (eGFP) [10–12]. Although antibiotics have been used successfully in generating genetically modified cells, antibiotics induce cellular damage, a shortening of the life-span of cultured somatic cells, a diminishment of the frequency of cell population doublings, replicative senescence and chromosomal abnormalities after the long-term selection of somatic cells ranging from 8 to 14 days of in vitro culture [5,8,13,14]. Since it was first introduced by Chalfie et al. [15], the eGFP-mediated genetic reporter system, which was derived from the biochemiluminescent jellyfish *Aequorea victoria*, is now emerging and provides us with a valuable xenogeneic selection marker because of its expression in a broad range of organisms and its lack of reported obvious adverse biological (i.e., cytotoxic) effects [12,16–18]. So far, the 238 amino acid residue eGFP protein has been applied for various purposes as a useful marker among others for the vital monitoring of the efficiency of somatic cell transfection and selection of in vitro cultured transgenic cells [19–21]. It has already been used for visual transgene screening and the controlling/assessment of its expression in situ in the preimplantation embryos of different mammalian species [22–25]. Although it has been shown in several studies that eGFP transgene overexpression determined by the brightest eGFP protein-derived fluorescence emission may reduce to a very limited degree the viability of transfected cells, no deleterious effects of eGFP at faint-moderate expression levels on the development of SCNT-derived transgenic embryos to the blastocyst stage have been reported [10,16,26,27].

In mice and cattle, eGFP reporter vectors have been effectively used to select transgenic embryos produced by intrapronuclear microinjection [16,18,24,28]. Furthermore, intra-vitam eGFP-mediated selection of nuclear donor cells has been applied to generate transgenic embryos and/or offspring by SCNT in mice [21], cattle [17,19,29,30], sheep [31,32] and goats [14]. Finally, porcine eGFP transgene-expressing blastocysts and/or offspring have been created from cultured fibroblast cells that were most often transfected with gene constructs (fusion genes) by replication-defective viral vectors or liposome carriers [4,25,33,34]. But to our knowledge, the production of porcine cloned embryos using somatic cells undergoing the novel method of nucleofection has not yet been reported. The nucleofection strategy – a combination of lipofection and electroporation – enables targeted transduction of the gene construct directly into the nucleus of the somatic cell, mediated through liposome carriers. Moreover, compared to the standard transfection methods for somatic cells, such as lipofection or electroporation, the nucleofection technique allows a considerable shortening of the time needed to verify transgenesis efficiency. This verification is performed through live-eGFP reporter gene expression controlling, from 24 to 48 h and up to even 4–6 h after the transfection procedure [35,36].

This is also the first report in which the effect of different protocols of oocyte artificial activation on both the preimplantation development of porcine transgenic cloned embryos and eGFP expression rates and patterns in the blastocysts generated were determined. This determination was depended on the type of lipofected or nucleofected nuclear donor fibroblast cells. To date, there are few studies focused on applying eGFP transgenic cells to porcine SCNT technology. Among them, foetal fibroblast cells have been used for the generation of genetically transformed embryos/offspring because of their rapid growth rate and potential for multiple mitotic cell divisions before replicative senescence under in vitro culture conditions [4,37–39]. In turn, among adult somatic cells, only ear skin-derived fibroblast cells have been utilized in the production of porcine eGFP gene-expressing cloned embryos and/or offspring [3,23,39,40]. However, none of the studies on swine somatic cell cloning have compared the preimplantation developmental capability and intra-vitam eGFP expression profiles between nuclear-transferred (NT) embryos derived from oocytes reconstituted with different types of transgenic fibroblast cells and stimulated by using different activation treatments. Therefore, in this study we investigated the effect of oocyte activation protocols on the in vitro developmental abilities of porcine cloned embryos that had been reconstituted with *Tg(pWAPhGH-GFPBsd)* gene-transfected foetal and adult dermal fibroblast cell nuclei.

2. Materials and methods

2.1. Preparation of the Tg(pWAPhGH-GFPBsd) gene construct

The PCR primers (TibMolBiol) were designed on the basis of human growth hormone gene (Accession