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Targeted Toxin as a Useful Reagent for Enrichment of α-Gal Epitope-Negative Cells Used for Somatic Cell Nuclear Transfer in Pigs

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1. Introduction

α-1,3-Galactosyltransferase (α-*GalT*) is a key enzyme in mediating the synthesis of the Galα1-3Gal (α-Gal) epitope on the cell surface of some mammalian species. Removal of the epitope is considered a prerequisite for xenotransplantation (Cooper et al., 1994). Thus, the production of genetically modified (GM) pigs that lack the expression of the α-Gal epitope has been the main target for pig-to-human xenotransplantation, and this can be performed by somatic cell nuclear transfer (SCNT) of porcine cells lacking α-*GalT* messenger RNA (mRNA) synthesis obtained by gene targeting (Lai et al., 2002; Dai et al., 2002; Phelps et al., 2003; Ramsoondar et al., 2003; Harrison et al., 2004; Kolber-Simonds et al., 2004; Takahagi et al., 2005). However, this approach is labor-intensive and time-consuming, and sometimes it is difficult to obtain knockout (KO) pigs. Other methods of genetically modifying pigs to obtain reduced levels of α-Gal epitope have been reported. One involves the production of cells/piglets overexpressing *Clostridium perfringens*-derived endo-β-galacosidase C (EndoGalC), which is capable of digesting α-Gal epitope (Yazaki et al., 2009; Himaki et al., 2010), and the other involves the production of cells/blastocysts overexpressing small interference RNA (siRNA) targeted to α-*GalT* mRNA (Yu et al., 2005; Zhu et al., 2005; Chi et al., 2010). Recently, zinc finger nuclease-mediated destruction of the endogenous α-*GalT* gene was reported by Hauschilda et al. (2011). In any case, however, for SCNT-mediated transgenesis, acquisition of a population of pure GM cells is a prerequisite because contamination of nontransfected cells or cells with low transgene expression would decrease the SCNT efficiency. Historically, cytotoxicity-based selection of α-Gal epitope-negative cells was first used for selection of cells completely lacking expression of *α-GalT* mRNA (Sharma et al., 2003). This can be performed by using specific antibodies (monoclonal antibodies) recognizing the α-Gal epitope and complement. Kolber-Simonds et al. (2004) used anti-α-Gal epitope antibodies from naive baboon plasma (natural antibodies) and baby rabbit complement. Baumann et al. (2004) also used a method similar to that of Kolber-Simonds et al. (2004). Although these cytotoxicity-based methods appear to be simple and convenient, it

is often difficult to obtain α-Gal epitope-specific antibodies using these methods, and the strength of complement is sometimes variable, depending on the quality used. Fujimura et al. (2008) used magnet-activated cell sorting in the selection of α-Gal epitope-negative cells. This selection process successfully eliminates α-Gal epitope-positive cells by using biotin-labeled *Bandeiraea simplicifolia* isolectin-B$_4$ (IB4), a lectin that specifically binds to α-Gal epitope (Vaughan et al., 1994). However, in this case, contamination by a small number of α-Gal epitope-positive cells often occurs in the eluent after passage through the streptoavidin-conjugated column. Thus, Phelps et al. (2003) used a bacterial toxin, *Clostridium difficile* toxin A, which binds with high affinity to α-Gal epitope, to obtain *α-GalT*-deficient pig cells. This method appears very simple and easy to perform, except that the reagent itself is not always available, limiting its use.

We have recently proposed another approach for the elimination of α-Gal epitope-positive cells. This approach is called targeted toxin technology (http://www.ATSbio.com), with which α-Gal epitope-expressing cells can be efficiently eliminated by incubation of target cells in the presence of toxin-labeled IB4. This selection procedure is rapid and simple because α-Gal epitope-expressing porcine cells are rapidly removed after incubation with toxin-labeled IB4 for 1–2 h at 37°C and subsequent cultivation in normal medium for more than 10 days, as will be described later in more detail.

Targeted toxins consist of the ribosome-inactivating protein saporin (Stirpe et al., 1992), which is conjugated to a target molecule that recognizes a cell-specific marker. When the conjugate is administered to cells of interest, it binds to the target cells and is absorbed, releasing saporin, which inactivates ribosomes. Cells not expressing the target molecule do not bind or absorb the conjugate, and are not affected. This can be performed simply by co-incubating target cells with the targeted toxins for a short period before culture in normal conditions, as described previously. It does not require additional treatment, such as that with a complement to kill target cells, and the targeted toxins themselves are commercially available from Advanced Targeting Systems Inc. (San Diego, CA, USA). When the targeted toxin is not available, it is possible to form a complex between saporin and the molecule of interest (which must be expressed on the cell surface) by hand. In this context, targeted toxins are useful as a powerful tool for removing unwanted cells from a pool of GM cells. In fact, negative selection using targeted toxins has already been proven useful in vivo (Wiley and Kline, 2000; Vulchanova et al., 2001; Tarpley et al., 2004) and in vitro (Akasaka et al., 2010).

In our previous experiments (Akasaka et al., 2010), we demonstrated that porcine embryonic fibroblasts (PEFs) expressing the EndoGalC gene strongly can survive after treatment with IB4 conjugated with saporin (hereafter referred to as IB4-SAP), but those that do so weakly or not at all died within 3 or 4 days after the treatment. When the surviving cells were inspected for possible expression of α-Gal epitope on their cell surface using fluorescence-labeled IB4, no distinct fluorescence was noted, indicating the success of the targeted toxin technology.

2. Results and perspective

In this review, we demonstrate another successful attempt performed in our laboratory of enriching porcine cells with highly reduced amounts of α-Gal epitope. First, the PEFs were transfected with the siRNA expression vector pPNER5 (Fig. 1A), which carries enhanced

green fluorescent protein (*EGFP*) cDNA and neomycin resistance gene (*neo*) expression units, together with an siRNA fragment targeted to the middle region of α-*GalT* mRNA (Chi et al., in press). We expected that the PEFs stably transfected with the pPNER5 plasmid would exhibit EGFP-derived fluorescence but decreased the expression of α-Gal epitope on their cell surface. Staining of transfected cells cultured for 10 days without drug selection with Alexa Fluor 594 (red fluorescence)-labeled IB4 revealed that approximately 64% of fluorescent cells (132 cells counted in total) were less distinctly stained with lectin (arrows in Fig. 1D-a–c). The image analysis of these cells demonstrated 60–95% reduction in the level of α-Gal epitope expressed in the normal cells. However, the remaining fluorescent cells were distinctly stained with lectin (arrowheads in Fig. 1D-d–f), suggesting silencing or low levels of siRNA expression from the integrated pPNER5 plasmid. To eliminate these fluorescent cells (but still expressing the α-Gal epitope) and nonfluorescent cells (probably nontransfected cells), the cells were treated with IB4-SAP (1×10^6 cells; 10 days after transfection). Inspection of the cells 1 day after the treatment revealed massive cell death in the IB4-SAP-treated group (Fig. 1C-a) but not in the control group (SAP alone; Fig. 1C-b), indicating the effectiveness of IB4-SAP in killing α-Gal epitope-expressing cells. Four days after the IB4-SAP treatment, the cells were passaged from a 30-mm dish onto a 60-mm dish and cultured in the absence of a selection drug for approximately 2 weeks. The number of colonies generated ranged 1–5. These colonies were next picked up by a paper method (Nakayama et al., 2007) and propagated systematically. Of the 3 colonies tested, all exhibited bright green fluorescence but reduced levels of α-Gal epitope expression on their cell surface, as evidenced by the staining with Alexa Fluor 594-labeled IB4 (Fig. 1D-g–i). We next performed SCNT using the IB4-SAP-treated pPNER5 transfectants. Out of 154 enucleated oocytes reconstituted with pPNER5-PEFs, the developing blastocysts exhibited bright green fluorescence around an embryo (Fig. 2A-b, e), suggesting success of the SCNT. Staining the blastocyst derived from the SCNT of the pPNER5-PEFs with Alexa Fluor 594-labeled IB4 demonstrated a great decrease in fluorescence on its cell surface (Fig. 2A-c). This was in contrast with the blastocyst derived from the SCNT of the pEGFP-N1-PEFs (Nakayama et al., 2007), which exhibited extensive staining with lectin (Fig. 2A-f). Reverse transcription-polymerase chain reaction (RT-PCR) analysis demonstrated that all of the tested samples (5/5 tested) exhibited complete loss of the target 586-base pair (bp) band corresponding to the endogenous α-*GalT* mRNA (lanes 1–5 in Fig. 2B). In contrast, all (2/2 tested) of the SCNT blastocysts derived from eggs reconstituted with pEGFP-N1-PEF nuclei exhibited a clear band of 586 bp (lanes 8 and 9 in Fig. 2B). These data indicate the effectiveness of RNA interference (RNAi) in the SCNT-derived porcine embryos and suggest the usefulness of IB4-SAP for enrichment of porcine cells with highly reduced levels of α-Gal epitope prior to SCNT-mediated production of GM piglets suitable for pig-to-human xenotransplantation.

Our major concern is how long this RNAi-mediated suppression of endogenous α-*GalT* mRNA will continue beyond the blastocyst stage. Therefore, it is required to transfer the SCNT blastocysts into recipients for obtaining cloned GM fetuses or animals that have been carried to term in which reduced levels of α-Gal epitope expression are expected to be maintained.

In conclusion, we demonstrate the usefulness of targeted toxin technology here, using IB4-SAP for enriching GM porcine cells in which α-Gal epitope synthesis is almost suppressed. These enriched cells are useful for cell transplantation or for SCNT-mediated generation of GM pigs that are optimized for xenotransplantation.
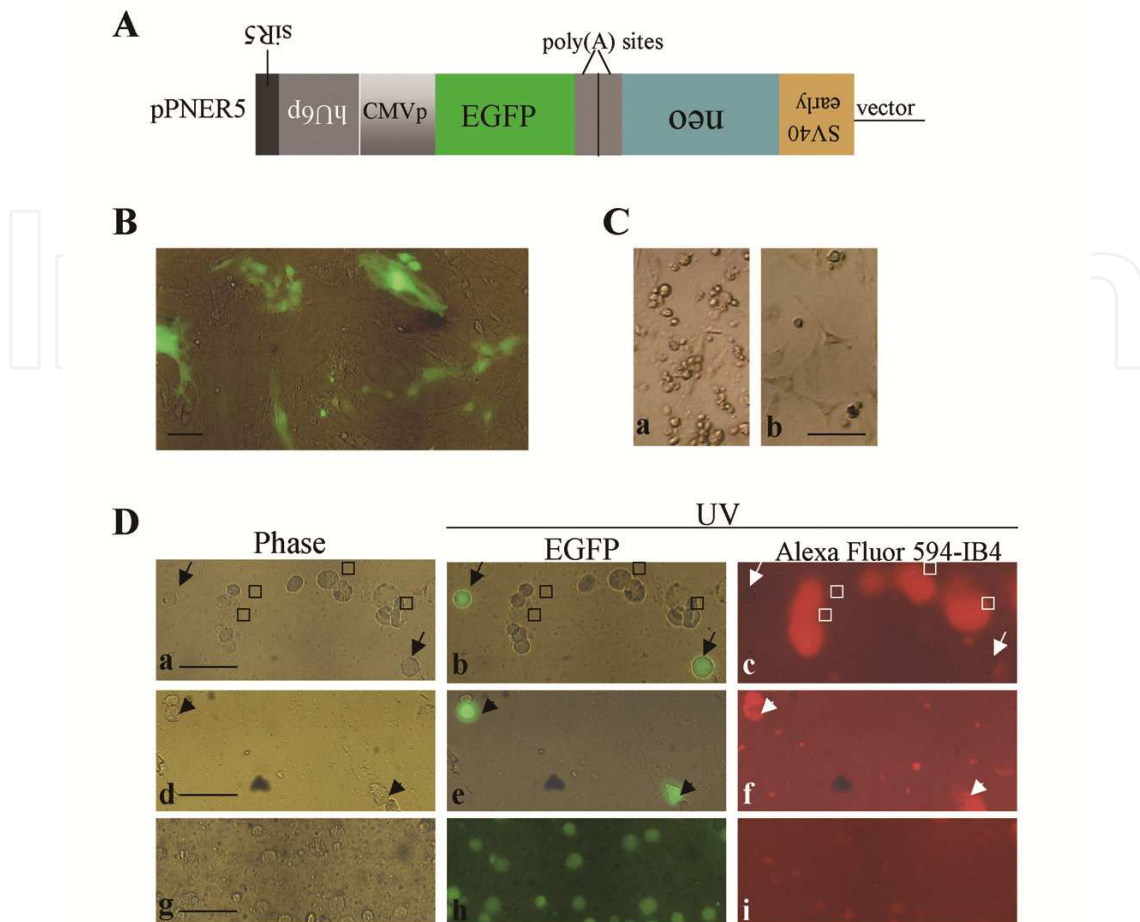
Fig. 1. A. Plasmid pPNER5 constructed by inserting a siRNA expression unit (comprising hU6p and siR5) upstream of the cytomegalovirus promoter (CMVp) in pEGFP-N1 plasmid. Abbreviations: EGFP, enhanced green fluorescent protein cDNA; neo, neomycin resistance gene; siR5, siRNA targeted to *α-GalT* mRNA; SV40 early, SV40 enhancer and early promoter; hU6p, human U6 promoter. B. EGFP expression in pPNER5-transfected PEFs cultured in G418-free PEF medium for 10 days after transfection. Approximately 10% of the cells were observed to express EGFP-derived green fluorescence. Bar = 25 μm. C. Cells 1 day after treatment with pPNER5-PEFs with IB4-SAP (a) or control SAP alone (b). Before dish-washing, a number of floating cells are visible in the IB4-SAP-treated dish (a) but almost none in the control dish (b). Bar = 25 μm. D. Cytochemical staining with IB4 lectin. a–f, Cells 10 days after transfection with linearized pPNER5 plasmid stained with Alexa Fluor 594-labeled IB4 lectin. Note that the fluorescent cells exhibit a mosaic pattern for lectin staining, indicating a mixture of cells expressing (indicated by arrowheads in d–f), not expressing, or weakly expressing α-Gal epitope (indicated by arrows in a–c). In contrast, untransfected nonfluorescent PEFs exhibit strong red fluorescence on the cell surface (denoted by quadrants in a–c). g–i, Cells surviving for 1 month after IB4-SAP treatment were examined for the presence of α-Gal epitope on their surfaces by Alexa Fluor 594-labeled IB4. As expected, almost all the cells treated with IB4-SAP were slightly or not at all stained with lectin. Phase, microphotographs taken under light; UV, microphotographs taken under UV + light. Bar = 25 μm.
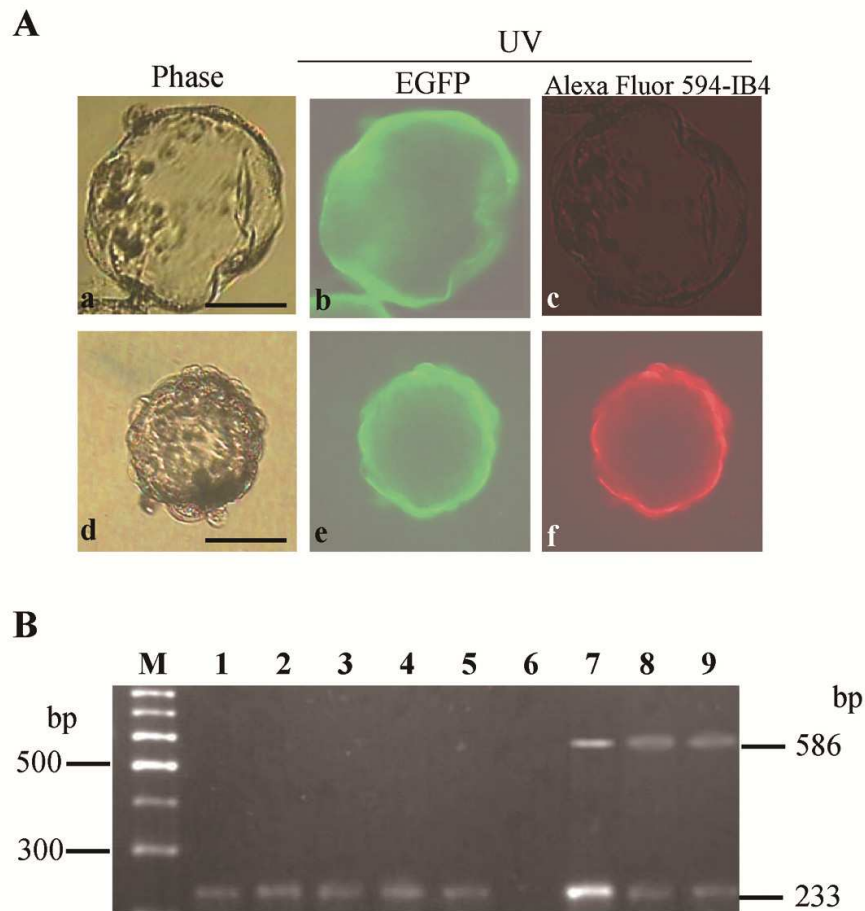
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Fig. 2. A. a–c, Reduced expression of α-Gal epitope in blastocysts derived from oocytes reconstituted with the pPNER5-PEFs. Developing SCNT blastocysts were stained with Alexa Fluor 594-labeled IB4 before observation using a fluorescence microscope. Note the bright green fluorescence around the whole embryo (b) but marked reduction in red fluorescence on its cell surface (c). d–f, Control blastocyst derived from the oocytes reconstituted with the control pEGFP-N1-PEFs. Note the bright green (e) and red (f) fluorescence around the embryo. Phase, microphotographs taken under light; UV, microphotographs taken under UV + light. Scale bar = 50 μm. B. RT-PCR analysis of endogenous *α-GalT* and *β-actin* mRNA in blastocysts derived from oocytes reconstituted with pPNER5-PEFs. Lanes 1–5, Blastocysts derived from oocytes reconstituted with pPNER5-transfected PEFs; lane 6, water subjected to RT-PCR (negative control); lane 7, PEFs (positive control); lanes 8 and 9, blastocysts derived from oocytes reconstituted with control pEGFP-N1-PEFs.

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Xenotransplantation

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Accompanied by the advent of animal cloning, the technique of nuclear transfer produced alpha1,3-galactosyltransferase-knockout (Gal-KO) pigs in many institutes, including the ones in Japan, at the beginning of 21st Century. In addition, the controversy of the risks of PERV has gradually minimized, because of the fact that there are no cases of PERV infections reported in humans. Furthermore, a large clinical wave for islet allotransplantation resumed the interest of xenotransplantation, especially porcine islet transplantation and some exceptions. Clinical trials were done in many countries so far, such as Sweden, China, Mexico, USA (Inventory of Human Xenotransplantation Practices - IXA and HUG in collaboration with WHO). In addition, a new clinical trial was approved by the government, and resumed the porcine islet transplantation research in New Zealand two years ago.

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