Identification and cloning of the porcine ROSA26 promoter and its role in transgenesis

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Abstract

Several promoters from different origins have been used to drive transgene expression in pigs with variable results. In this study, we identified porcine ROSA26 promoter located on chromosome 13. Porcine ROSA26 promoter was cloned and its activity was examined using enhanced green fluorescence protein (EGFP) as a reporter. EGFP expression was detected in adult liver derived cells (LDC) by flow cytometry and confocal microscopy after transient transfection. Stable LDC expressing EGFP were established and used as nuclear donors for somatic cell nuclear transfer (SCNT). Two hundred thirty-three reconstructed embryos were transferred to two recipients. One pregnancy was established. A total of four fetuses were collected at day 30 of gestation, one normal and three resorbed. All fetuses had EGFP gene integration. Confocal microscope analysis showed that EGFP was expressed in fetal liver, heart, and isolated fetal fibroblasts. These results indicated that ROSA26 promoter is a ubiquitous promoter and can be utilized in pig transgenesis.

Keywords: ROSA26 locus, promoter, transgenesis, enhanced green fluorescence protein

Introduction

The current goal of xenotransplantation is to genetically modify pigs to overcome the acute humoral response and thrombocytopenia that limits long term transplantation of pig organs [1]. The success of xenotransplantation relies on the application of molecular biology tools to insert a transgene or silence a xenoantigen gene to prevent platelet consumption or rejection of the xenografts. Recent progress in pig cloning has significantly improved transgenic efficiency, through the selection of transgenic cells prior to somatic cell nuclear transfer (SCNT). To date, pigs transgenic for genes regulating human complement, apoptosis, coagulation, inflammation and platelet activation have been generated [2]. Transgenic pigs expressing graft-protective proteins such as human CD46, CD55, CD59 and CD39 reduced the incidence of hyperacute rejection [3,4]. However, transgenesis has some limitations, such as unpredictability of expression and inheritance of the transgene. This unpredictability is due to transgene random integration (positional effect) as well as promoter silencing. It is important to select a promoter that will drive a significant level of expression of the transgene in all or the desired pig tissue. A variety of promoters originating from human, mouse, chicken, and virus have been used in pig transgenesis with variable outcome, including the promoters for the genes of human β 2m, EF-1a, CD46, mouse H-2K^b, chicken b-actin, cytomegalovirus and respiratory syncytial virus [5].

The ROSA26 locus has become established as the preferred docking site for transgene expression. Mouse, human and rat

ROSA26 locus have been characterized. The mouse ROSA26 promoter was identified by random retroviral gene trapping in embryonic stem cells [6]. Targeting of the ROSA26 site with the reporter b-gal resulted in ubiquitous expression in various tissues and in hematopoietic cells during embryonic development [7]. The human and rat ROSA26 loci have been subsequently identified and showed to accommodate ubiquitous expression of the reporter genes [8,9]. Besides transgenes, short hairpin RNA (shRNA) expression within the mouse ROSA26 locus created a reproducible and inducible knockdown of gene expression [10,11]. The ROSA26 locus is an ideal docking site for transgenes as it supports stable expression and overcomes concerns of gene disruption or alteration through insertional mutagenesis. Yet, the pig ROSA26 promoter and ROSA26 locus have not been characterized.

In this study, we cloned and sequenced the porcine ROSA26 promoter located on chromosome 13. Porcine ROSA26 promoter strength was compared to the mouse H-2K^b and CMV promoters *in vitro*, and *in vivo* activity was examined in 30-day old fetuses.

Materials and methods

Cloning porcine ROSA26 promoter

The porcine ROSA26 promoter sequence was obtained by searching pig genome database (NCBI) using mouse ROSA26 promoter sequence and human ROSA26 promoter sequence as references. Primers ROSA26-F (5'GGCGCGCCAGGATGCAAGAGTCTTAGGC3') and ROSA26-R (5'ACGCGTGTGTTTT CACACACGGCGGGAGG3') were designed to amplifying a 3.9 kb

DNA fragment harboring potential porcine ROSA26 promoter by PCR using domestic pig genomic DNA as a template. *Ascl* and *MluI* sites were added to the 5' of forward and reverse primers, respectively (underlined). Pwo SuperYield DNA Polymerase (Roche, Indianapolis, IN) was used and PCR conditions were as follows: 94°C, 2 minutes; 94°C, 15 seconds, 55°C, 30 seconds; 72°C 4 minutes for 15 cycles; 94°C, 15 seconds, 55°C, 30 seconds; 72°C 4 minutes and 5 seconds added to each cycle for 25 cycles; and a final extension step of 72°C for 8 minutes. The PCR product was subsequently cloned into pCR-XL-TOPO vector (Invitrogen, Carlsbad, CA) to generate pCR-XL-ROSA26. Porcine ROSA26 promoter was sequenced using primers 7124 (5'ATTACCTCTGTCCTCCAAGCAGCC3'), 7807 (5'CACCATTCACTTCGCATC 3'), 8571(5'TTTCTGTCGCACCCTTACC 3'), 9334 (5'GGAAAAGCCTGGAATACG3'), 9740 (5' GTCCC-AAATGAGCGAAAC 3'), 10241 (5'GTCAGTTTGCTCCTTCTCG 3'), 10415 (5' AATGAGTCCAGGCAACACC3'), 10632 (5' GCATCCTTACGGTCAGATAAC3').

Construction of transgenic vector

CMV promoter and multiple cloning site (MCS) of pEGFP-N1 (Clontech, Palo Alto, CA) was replaced by a linker *Ascl*-*NruI*-*Ascl*-*Sall*-*MluI*-*PvuI*-*BamHI*. A 3.9 kb fragment containing potential porcine ROSA26 promoter was excised from pCR-XL-ROSA26 with *Ascl* and *MluI* digestion and inserted to the promoterless pEGFP-N1 between *Ascl* and *MluI* sites, resulting in plasmid pROSA26-EGFP. The control vector was constructed by cloning of EGFP cDNA to the downstream of murine MHC class I H-2K^b promoter at *EcoRI* site, resulting in plasmid pH-2K^b-EGFP.

Transient transfection

Liver derived cells (LDCs) were isolated as described previously [12]. LDCs were cultured in combination media (α -MEM:EGM-MV 3:1) (Invitrogen/Lonza) supplemented with 10%FBS (Hyclone), 10% horse serum (Invitrogen, Cat # 16050-122), 12 mM Hepes and 1% Pen/Strep (Invitrogen, Carlsbad, CA) on collagen I coated plates and maintained at 37°C with 5% CO₂ and 10% O₂. To compare promoter strength among ROSA26, H-2K^b, and CMV, LDC were transfected with pROSA26-EGFP, pH-2K^b-EGFP and pEGFP-N1 by using NeonTM Transfection System (Invitrogen, Carlsbad, CA) as per the manufacturer instructions. 3X10⁵ cells were mixed with 1.5 μ g of each DNA, respectively, and electroporated at 1300V, 30 ms, 1 pulse. Then cells were cultured at 37°C with 5% CO₂ and 10% O₂. After 48 hours, cells were harvested and EGFP expression was examined by flow cytometry. Untransfected LDC were used as a control. Data was shown as a representative histogram and relative mean fluorescent intensity with standard deviation. Relative mean fluorescent intensity was determined by setting the highest value in each experiment to 1 to allow accurate comparison between experiments. Flow cytometric analysis of EGFP reporter expression is a sensitive way for the detection of promoter activity [13].

Establishment of EGFP stable cell line

LDC at 80-90% confluence were harvested with trypsin and washed with calcium and magnesium free DPBS (Invitrogen, Carlsbad, CA). pROSA26-EGFP was linearized by *AscI* digestion. Transfection was performed by using NeonTM Transfection System (Invitrogen). Briefly, 10⁶ cells were suspended in 120 μ l of R buffer and 2 μ g of linearized DNA was added. Cells were electroporated at 1300V, 30 ms, 1 pulse, and plated onto collagen I coated plates (BD) in the culture media without antibiotics. After 48 hours, the culture media was replaced with selection media containing 100 μ g/ml of G418 (Invitrogen). After 10 days of G418 selection the EGFP positive cells were isolated by flow sorting. The selected cells were expanded and the second flow sorting was performed to purify and enrich the EGFP positive cells.

Somatic cell nuclear transfer

Animal use in this study was approved by the Institutional Animal Care and Use Committee (IACUC). SCNT was performed using *in vitro* matured oocytes (DeSoto Biosciences Inc. St Seymour, TN. and Minitub of America, Mount Horeb, WI.). Cumulus cells were removed from the oocytes by pipetting in 0.1% hyaluronidase. Only oocytes with normal morphology and a visible polar body were selected for cloning. Oocytes were incubated in manipulation media (Ca-free NCSU-23 with 5% FBS) containing 5 mg/mL bisbenzimidazole and 7.5 mg/mL cytochalasin B for 15 min. Following this incubation period, oocytes were enucleated by removing the first polar body and metaphase II plate, and one single cell was injected into each enucleated oocyte. Electrical fusion was induced with a BTX cell electroporator (Harvard Apparatus, Hol-liston, MA). Couples were exposed to two DC pulses of 140 V for 50 ms in 280 mM Mannitol, 0.001 mM CaCl₂, and 0.05 mM MgCl₂. One hour later, reconstructed oocytes were activated by two DC pulses of 120 V for 60 ms in 280 mM Mannitol, 0.1 mM CaCl₂, and 0.05 mM MgCl₂ [14]. After activation, oocytes were placed back in NCSU-23 medium with 0.4% bovine serum albumin BSA and cultured at 38.5°C, 5% CO₂ in a humidified atmosphere, for less than 1 h before being transferred into the recipient. Recipients were synchronized occidental pigs on their first day of estrus.

Genotyping of EGFP transgenic fetuses

The pregnancy was terminated at 30 day and fetuses were harvested. Genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen). The EGFP sequence in pEGFP-N1 was used to design the forward primer (5'-CTGGTCGAGCTGGACGGCGACG-3') and reverse primer (5'-CACGAAGTCCAGCAGGACCATG-3') to amplify 630 bp PCR product. The 20 μ l of reaction mixture contained 10 μ l of 2 x Go-Taq Green Master Mix (Promega, Madison, WI), 5 pmol of each primer, and 50 ng of genomic DNA. The initial denaturation was performed at 94°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 45 sec,

and a final extension step of 72°C for 5 min. Genomic DNA extracted from domestic cells and EGFP positive LDC were used as negative and positive control, respectively.

Confocal microscope analysis of EGFP expression in LDC and fetal tissues

The presence of EGFP expression driven by the ROSA26 promoter was examined in LDC, fetal tissue and fetal fibroblasts isolated from genetically modified pigs using confocal microscopy. Cells or frozen sections were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) and blocked with 50% Odyssey buffer (LI-COR Biosciences, Lincoln, NE) in PBS. Liver derived cells were labeled with anti-porcine CD31 antibody detected with donkey anti-goat secondary antibody conjugated to Dylight 549 in PBS. Cell nuclei were stained with DAPI (Invitrogen) and mounted with Prolong Gold (Invitrogen). Fetal tissue and fibroblasts were labeled with anti-EGFP antibody conjugated to alexa fluor 647 and stained with DAPI. Images were collected with an Olympus FV1000 confocal microscope.

Results

Cloning of pig ROSA26 promoter

The porcine ROSA26 promoter region was identified by blast searching the NCBI pig genome database using the human and mouse ROSA26 promoter sequences, respectively. A sequence with 86% identity to human ROSA26 promoter and 79% identity to mouse ROSA26 promoter was located on porcine chromosome 13. A DNA fragment containing the potential porcine ROSA26 promoter region was amplified by PCR, cloned, and sequenced, and compared to mouse and human (Figure 1). The cloned DNA fragment shared 99% identities to the sequence on *sus scrofa* chromosome 13 (NCBI).

Porcine ROSA26 promoter activity *in vitro*

EGFP expression driven by the porcine ROSA26 promoter in LDC was detected by confocal microscopy after transient transfection (Figure 2). To further assess the promoter strength we used flow cytometry to compare the porcine ROSA26, murine MHC class I H-2K^b and CMV promoters. The H-2K^b promoter, suggested to be moderate and ubiquitous, was used to drive human a 1,2-FT, CD55, CD59 and TRAIL on endothelial cells in transgenic pigs [3,15]. The CMV promoter is commonly known to be a strong promoter *in vitro*. Flow cytometry analysis showed that some LDC transfected with pH-2K^b-EGFP were only slightly brighter than the LDC transfected with pROSA26-EGFP, suggesting that the strength of the murine H-2K^b promoter is similar to the porcine ROSA26 promoter. The CMV promoter was much stronger than murine H-2K^b and porcine ROSA26 promoters *in vitro* (Figures 3a and 3b).

Somatic cell nuclear transfer

Liver derived cells positive for EGFP enriched by G418 selection and flow sorting were used as nuclear donors for SCNT. Two

hundred thirty-three cloned embryos were transferred to two gilts. One became pregnant. A total of four fetuses were collected at day 30. One fetus was normal and three fetuses were resorbed.

Porcine ROSA26 promoter activity *in vivo*

All four fetuses showed EGFP transgene integration (Figure 4a). Variable expression of EGFP in fetal tissues was observed by confocal microscope analysis. Moderate EGFP expression was found in fetal liver and weak EGFP expression was observed in fetal heart as compared to control fetal liver tissue. Control fetal heart tissue was unavailable. EGFP expression was also observed in isolated fetal fibroblasts and tissues (Figure 4b). These results suggest that porcine ROSA26 is a ubiquitous promoter *in vivo*.

Discussion

In this study, we successfully cloned, sequenced and evaluated the porcine ROSA26 promoter to control the expression of a reporter gene. EGFP driven by the porcine ROSA26 promoter was expressed in LDC *in vitro* and liver, heart and fibroblasts from 30-day old fetus.

Several promoters from different origins have been previously used to drive transgene expression in pigs with variable results. The CMV promoter has been widely used for transgene expression but is prone to silencing by methylation [16] and unpredictable transgene expression ranging from low to strong in different tissues [17]. The Chicken b-actin promoter coupled with enhancer elements from the CMV promoter and rabbit b-globin drives EGFP expression in transgenic pigs in a stable and uniform manner [18]. However, the same promoter was unpredictable when driving the human A20 gene [19]. A study demonstrated that human intercellular adhesion molecule 2 (ICAM-2) promoter was much weaker in pig than in mice, suggesting that there may be a need for additional regulatory elements to achieve species-specific gene expression in pig [20]. Mouse major histocompatibility complex class I gene H-2K^b promoter was used to express human TRAIL (tumor necrosis factor- α -related apoptosis-inducing ligand) in transgenic pigs. TRAIL expression was detected in all the tissues yet with variable expression levels [15]. Expression strength of the ROSA26 promoter was comparable to the H-2K^b promoter and similar to previous reports in the human and mouse, demonstrating moderate activity *in vitro* and in range of tissues *in vivo*.

Development of α 1,3-galactosyltransferase knockout (GGTA1-KO) pigs overcame a major hurdle in xenotransplantation by reducing antibody mediated rejection. Recently, we have successfully generated GGTA1/CMAH double knockout pig using ZFN technology [21]. This double knockout pig deficient in N-glycolylneuraminic acid (Neu5Gc) and a-Gal exhibits much less human antibody binding compared to GGTA1-KO pig. Generation of transgenic pigs expressing human complement regulatory proteins, human anti-apoptosis,

continuation of **Figure 1**.

Figure 1. Alignment of cloned porcine ROSA26 promoter with human and mouse ROSA26 promoters.

anti-inflammatory and anti-coagulation genes on GGTA1/ CMAH double KO background may be crucial to the success of xenotransplantation.

Reliable and consistent expression of transgenes depends on the selection of suitable promoters as well as the transgene integration site. Endogenous porcine ROSA26 and the other ubiquitous exogenous promoters such as PGK, EF1a, UBC, CAG could be used to achieve a desired expression level of transgene at the ROSA26 locus. Integration of exogenous promoters at ROSA26 in both sense and antisense orientation had a large effect on transgene expression [22]. Targeting transgenes to the ROSA26 locus by homologous recombination is inefficient and time consuming in porcine somatic cells used for SCNT to generate transgenic pigs. Currently, several methods have been developed to accelerate the process of

genomic modification by generation of a DNA double-strand break (DSB) at a specified locus in various species, such as zinc finger nucleases (ZFN), transcription activator-like effector nucleases (Talens) and clustered regularly interspaced short palindromic repeat (CRISPR)/Cas system [23-25]. Combined with these genetic engineering tools, transgenes could be efficiently delivered to the porcine ROSA26 locus where they can be driven by the endogenous ROSA26 promoter.

In conclusion, our study demonstrated that the porcine ROSA26 promoter is ubiquitous and can be utilized in pig transgenesis. Targeting transgenes to ROSA26 locus along with further genetic modification of the pig genome may provide suitable organs for xenotransplantation.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	PL	CB	JE	PJC	AJT
Research concept and design	✓	✓	--	--	✓
Collection and/or assembly of data	✓	✓	✓	--	--
Data analysis and interpretation	✓	✓	✓	--	--
Writing the article	✓	✓	--	--	--
Critical revision of the article	✓	✓	✓	✓	✓
Final approval of article	--	--	--	--	✓
Statistical analysis	✓	✓	--	--	--

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