Successful Suppression of Endogenous α-1,3-Galactosyltransferase Expression by RNA Interference in Pig Embryos Generated In Vitro

Haiying CHI1), Mariko SHINOHARA1), Takaaki YOKOMINE2), Masahiro SATO3), Sonshin TAKAO2), Mitsutoshi YOSHIDA1) and Kazuchika MIYOSHI1)

1)Laboratory of Animal Reproduction, Faculty of Agriculture, Kagoshima University, Kagoshima 890-0065, Japan
2)Laboratory of Frontier Medicine, Frontier Science Research Center, Kagoshima University, Kagoshima 890-8544, Japan
3)Section of Gene Expression Regulation, Frontier Science Research Center, Kagoshima University, Kagoshima 890-0065, Japan

Abstract. RNA interference (RNAi) technology using small interfering RNAs (siRNA) has been widely used as a powerful tool to knock down gene expression in various organisms. In pig preimplantation embryos, no attempt to suppress the target gene expression with such technology has been made. The purpose of this study is to demonstrate that the RNAi technology is useful for suppression of endogenous target gene expression at an early stage of development in pigs. Alpha-1,3-Galactosyltransferase (α-GalT) is an enzyme that creates the Galα1-3Gal (α-Gal) epitope on the cell surface in some mammalian species, and removal of the epitope is considered to be a prerequisite for pig-to-human xenotransplantation. We decided to suppress the endogenous α-GalT mRNA expression in pig early embryos, since reduction of α-GalT synthesis is easily monitored by cytochemical staining with Bandeiraea simplicifolia isoelectin-B4, a lectin that specifically binds to the α-Gal epitope, and by RT-PCR analysis. Cytoplasmic microinjection of double-stranded RNA and pronuclear injection of an siRNA expression vector into the embryos generated in vitro resulted in a significant reduction in expression of the α-GalT gene and α-Gal epitope in blastocysts, at which stage the α-Gal epitope is abundantly expressed. Somatic cell nuclear transfer of embryonic fibroblasts stably transfected with an siRNA expression vector also led to a significant reduction in the level of α-GalT mRNA synthesis together with decreased amounts of the α-Gal epitope at the blastocyst stage. These results indicate that the RNAi technology is useful for efficient suppression of a target gene expression during embryogenesis in pigs and suggest the possibility of production of siRNA-expressing pigs for use in xenotransplantation.

Key words: Blastocyst, α-1,3-Galactosyltransferase, Pig, RNAi, Somatic cell nuclear transfer

Successful Suppression of Endogenous α-1,3-Galactosyltransferase Expression by RNA Interference in Pig Embryos Generated In Vitro

Haiying CHI1), Mariko SHINOHARA1), Takaaki YOKOMINE2), Masahiro SATO3), Sonshin TAKAO2), Mitsutoshi YOSHIDA1) and Kazuchika MIYOSHI1)

1)Laboratory of Animal Reproduction, Faculty of Agriculture, Kagoshima University, Kagoshima 890-0065, Japan
2)Laboratory of Frontier Medicine, Frontier Science Research Center, Kagoshima University, Kagoshima 890-8544, Japan
3)Section of Gene Expression Regulation, Frontier Science Research Center, Kagoshima University, Kagoshima 890-0065, Japan

Abstract. RNA interference (RNAi) technology using small interfering RNAs (siRNA) has been widely used as a powerful tool to knock down gene expression in various organisms. In pig preimplantation embryos, no attempt to suppress the target gene expression with such technology has been made. The purpose of this study is to demonstrate that the RNAi technology is useful for suppression of endogenous target gene expression at an early stage of development in pigs. Alpha-1,3-Galactosyltransferase (α-GalT) is an enzyme that creates the Galα1-3Gal (α-Gal) epitope on the cell surface in some mammalian species, and removal of the epitope is considered to be a prerequisite for pig-to-human xenotransplantation. We decided to suppress the endogenous α-GalT mRNA expression in pig early embryos, since reduction of α-GalT synthesis is easily monitored by cytochemical staining with Bandeiraea simplicifolia isoelectin-B4, a lectin that specifically binds to the α-Gal epitope, and by RT-PCR analysis. Cytoplasmic microinjection of double-stranded RNA and pronuclear injection of an siRNA expression vector into the embryos generated in vitro resulted in a significant reduction in expression of the α-GalT gene and α-Gal epitope in blastocysts, at which stage the α-Gal epitope is abundantly expressed. Somatic cell nuclear transfer of embryonic fibroblasts stably transfected with an siRNA expression vector also led to a significant reduction in the level of α-GalT mRNA synthesis together with decreased amounts of the α-Gal epitope at the blastocyst stage. These results indicate that the RNAi technology is useful for efficient suppression of a target gene expression during embryogenesis in pigs and suggest the possibility of production of siRNA-expressing pigs for use in xenotransplantation.

Key words: Blastocyst, α-1,3-Galactosyltransferase, Pig, RNAi, Somatic cell nuclear transfer

Successful Suppression of Endogenous α-1,3-Galactosyltransferase Expression by RNA Interference in Pig Embryos Generated In Vitro

Haiying CHI1), Mariko SHINOHARA1), Takaaki YOKOMINE2), Masahiro SATO3), Sonshin TAKAO2), Mitsutoshi YOSHIDA1) and Kazuchika MIYOSHI1)

1)Laboratory of Animal Reproduction, Faculty of Agriculture, Kagoshima University, Kagoshima 890-0065, Japan
2)Laboratory of Frontier Medicine, Frontier Science Research Center, Kagoshima University, Kagoshima 890-8544, Japan
3)Section of Gene Expression Regulation, Frontier Science Research Center, Kagoshima University, Kagoshima 890-0065, Japan

Abstract. RNA interference (RNAi) technology using small interfering RNAs (siRNA) has been widely used as a powerful tool to knock down gene expression in various organisms. In pig preimplantation embryos, no attempt to suppress the target gene expression with such technology has been made. The purpose of this study is to demonstrate that the RNAi technology is useful for suppression of endogenous target gene expression at an early stage of development in pigs. Alpha-1,3-Galactosyltransferase (α-GalT) is an enzyme that creates the Galα1-3Gal (α-Gal) epitope on the cell surface in some mammalian species, and removal of the epitope is considered to be a prerequisite for pig-to-human xenotransplantation. We decided to suppress the endogenous α-GalT mRNA expression in pig early embryos, since reduction of α-GalT synthesis is easily monitored by cytochemical staining with Bandeiraea simplicifolia isoelectin-B4, a lectin that specifically binds to the α-Gal epitope, and by RT-PCR analysis. Cytoplasmic microinjection of double-stranded RNA and pronuclear injection of an siRNA expression vector into the embryos generated in vitro resulted in a significant reduction in expression of the α-GalT gene and α-Gal epitope in blastocysts, at which stage the α-Gal epitope is abundantly expressed. Somatic cell nuclear transfer of embryonic fibroblasts stably transfected with an siRNA expression vector also led to a significant reduction in the level of α-GalT mRNA synthesis together with decreased amounts of the α-Gal epitope at the blastocyst stage. These results indicate that the RNAi technology is useful for efficient suppression of a target gene expression during embryogenesis in pigs and suggest the possibility of production of siRNA-expressing pigs for use in xenotransplantation.

Key words: Blastocyst, α-1,3-Galactosyltransferase, Pig, RNAi, Somatic cell nuclear transfer

Successful Suppression of Endogenous α-1,3-Galactosyltransferase Expression by RNA Interference in Pig Embryos Generated In Vitro

Haiying CHI1), Mariko SHINOHARA1), Takaaki YOKOMINE2), Masahiro SATO3), Sonshin TAKAO2), Mitsutoshi YOSHIDA1) and Kazuchika MIYOSHI1)

1)Laboratory of Animal Reproduction, Faculty of Agriculture, Kagoshima University, Kagoshima 890-0065, Japan
2)Laboratory of Frontier Medicine, Frontier Science Research Center, Kagoshima University, Kagoshima 890-8544, Japan
3)Section of Gene Expression Regulation, Frontier Science Research Center, Kagoshima University, Kagoshima 890-0065, Japan

Abstract. RNA interference (RNAi) technology using small interfering RNAs (siRNA) has been widely used as a powerful tool to knock down gene expression in various organisms. In pig preimplantation embryos, no attempt to suppress the target gene expression with such technology has been made. The purpose of this study is to demonstrate that the RNAi technology is useful for suppression of endogenous target gene expression at an early stage of development in pigs. Alpha-1,3-Galactosyltransferase (α-GalT) is an enzyme that creates the Galα1-3Gal (α-Gal) epitope on the cell surface in some mammalian species, and removal of the epitope is considered to be a prerequisite for pig-to-human xenotransplantation. We decided to suppress the endogenous α-GalT mRNA expression in pig early embryos, since reduction of α-GalT synthesis is easily monitored by cytochemical staining with Bandeiraea simplicifolia isoelectin-B4, a lectin that specifically binds to the α-Gal epitope, and by RT-PCR analysis. Cytoplasmic microinjection of double-stranded RNA and pronuclear injection of an siRNA expression vector into the embryos generated in vitro resulted in a significant reduction in expression of the α-GalT gene and α-Gal epitope in blastocysts, at which stage the α-Gal epitope is abundantly expressed. Somatic cell nuclear transfer of embryonic fibroblasts stably transfected with an siRNA expression vector also led to a significant reduction in the level of α-GalT mRNA synthesis together with decreased amounts of the α-Gal epitope at the blastocyst stage. These results indicate that the RNAi technology is useful for efficient suppression of a target gene expression during embryogenesis in pigs and suggest the possibility of production of siRNA-expressing pigs for use in xenotransplantation.

Key words: Blastocyst, α-1,3-Galactosyltransferase, Pig, RNAi, Somatic cell nuclear transfer
Construction of dsRNA

Construction of dsRNA was performed according to the published nucleotide (nt) sequence of pig α-GalT mRNA (GenBank Accession No. L36535 [26]) using sense RNA (5′-ACGCUAUAGGCAACGAAAAGGAACA-AG-3′) and antisense RNA (3′-UA-UGCGAUUACCGUUCUUCUUCGUU-5′). The synthetic dsRNA produced by iGENE (Tokyo, Japan) was diluted with distilled water that had been treated with 0.5 µg/ml of diethylpyrocarbonate (DEPC), an inhibitor of RNase, according to the manual described by Sambrook [27], and then stored at –80°C prior to use. This DEPC-treated water is hereafter referred to as DEPC-water. Of dsRNA, pronuclear injection of an siRNA expression vector DNA and SCNT of somatic cells stably transfected with an siRNA expression vector) to examine whether they can confer efficient suppression of endogenous α-GalT gene expression in pig pre-plantation embryos generated in vitro.

Materials and Methods

Construction of dsRNA

Construction of dsRNA was performed according to the published nucleotide (nt) sequence of pig α-GalT mRNA (GenBank Accession No. L36535 [26]) using sense RNA (5′-ACGCUAUAGGCAACGAAAAGGAACA-AG-3′) and antisense RNA (3′-UA-UGCGAUUACCGUUCUUCUUCGUU-5′). The synthetic dsRNA produced by iGENE (Tokyo, Japan) was diluted with distilled water that had been treated with 0.5 µg/ml of diethylpyrocarbonate (DEPC), an inhibitor of RNase, according to the manual described by Sambrook et al. [27], and then stored at –80°C prior to use. This DEPC-treated water is hereafter referred to as DEPC-water.

Construction of siRNA expression vectors

Two siRNA expression vectors, pPR4 and pPR5 (Fig. 1A), were constructed by inserting chemically synthesized siRNA oligonucleotides (siR4 and siR5) into a human U6 promoter-based vector, pGEME PUR hU6 (iGENE), which contains a selection marker unit comprising the SV40 early promoter, puromycin resistance gene; SV40 early, SV40 enhancer and early promoter; puro, puromycin resistance gene.

Fig. 1. A: Two siRNA expression vectors, pPR4 and pPR5, constructed by inserting siR4 and siR5 (shown as a hatched box) downstream of the human U6 promoter (hU6p) in the pGEME PUR hU6 plasmid, respectively. B: Plasmid pPNERS5 constructed by inserting an siRS expression unit (comprising hU6p and siR5) upstream of the cytomegalovirus promoter (CMVp) in the pEGFP-N1 plasmid, neo, neomycin resistance gene; SV40 early, SV40 enhancer and early promoter; puro, puromycin resistance gene.

In vitro maturation (IVM) of oocytes

IVM was performed as previously described [28]. Briefly, ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory within 2 h in 0.9% (w/v) NaCl containing 100 mg/l of kanamycin sulfate (Meiji Seika, Tokyo, Japan) at 32–34°C. The follicular contents were recovered by aspiration using an 18-gauge needle (Terumo, Tokyo, Japan) and 5-ml disposable syringe (Nipro, Osaka, Japan) from the follicles (2–5 mm in diameter). The cumulus-oocyte complexes (COCs) were gathered from the follicular contents and washed twice with TL-HEPES-polypolyvinyl alcohol (PVA) [29] and the maturation medium, respectively. The maturation medium consisted of 90% (v/v) TC-199 with Earle’s salts (Gibco, Grand Island, NY, USA) supplemented with 0.91 mM sodium pyruvate (Sigma-Aldrich Chemical, St. Louis, MO, USA), 3.05 mM glucose (Wako Pure Chemical Industries, Osaka, Japan), 0.57 mM cytosine hydrochloride hydrate (Sigma-Aldrich Chemical), 10 ng/ml epidermal growth factor (Sigma-Aldrich Chemical), 10 IU/ml eCG (ASUKA Pharmaceutical, Tokyo, Japan), 10 IU/ml hCG (ASUKA Pharmaceutical), 100 µg/ml amikacin sulfate (Meiji Seika), 0.1% (w/v) PVA and 10% (v/v) pig follicular fluid prepared according to the procedure described by Yoshida et al. [30]. Only COCs possessing a compact cumulus mass and evenly granulated ooplasm were selected and used for the experiments. COCs (20 to 50) were transferred to a droplet of maturation medium (200 µl) under paraffin oil (Nacalai Tesque, Kyoto, Japan) in a 35-mm polystyrene dish (Becton Dickinson Labware, Oxford, CA, USA) and cultured at 38.5°C in an atmosphere of 5% CO₂ in air.

Parthenogenetic activation (PA) of oocytes

After 40–42 h of culture, the cumulus mass was removed by pipetting with 0.1% (w/v) hyaluronidase (Sigma-Aldrich Chemical). Only oocytes with clearly extruded polar bodies were selected for the PA experiment. Matured oocytes were placed between two wire electrodes (1 mm apart) of the chamber slide filled with activation medium (comprising 250.3 mM sorbitol, 0.1 mM Ca(CH₃COO)₂, 0.5 mM Mg(CH₃COO)₂ and 1 mg/ml bovine serum albumin (BSA; Nacalai Tesque)) at 32–34°C. The follicular contents were recovered by aspiration using an 18-gauge needle (Terumo, Tokyo, Japan) and 5-ml disposable syringe (Nipro, Osaka, Japan) from the follicles (2–5 mm in diameter). The cumulus-oocyte complexes (COCs) were gathered from prepubertal gilts at a local slaughterhouse and transported to the laboratory within 2 h in 0.9% (w/v) NaCl containing 100 mg/l of kanamycin sulfate (Meiji Seika, Tokyo, Japan) at 32–34°C. The follicular contents were recovered by aspiration using an 18-gauge needle (Terumo, Tokyo, Japan) and 5-ml disposable syringe (Nipro, Osaka, Japan) from the follicles (2–5 mm in diameter). The cumulus-oocyte complexes (COCs) were gathered from the follicular contents and washed twice with TL-HEPES-polypolyvinyl alcohol (PVA) [29] and the maturation medium, respectively. The maturation medium consisted of 90% (v/v) TC-199 with Earle’s salts (Gibco, Grand Island, NY, USA) supplemented with 0.91 mM sodium pyruvate (Sigma-Aldrich Chemical, St. Louis, MO, USA), 3.05 mM glucose (Wako Pure Chemical Industries, Osaka, Japan), 0.57 mM cytosine hydrochloride hydrate (Sigma-Aldrich Chemical), 10 ng/ml epidermal growth factor (Sigma-Aldrich Chemical), 10 IU/ml eCG (ASUKA Pharmaceutical, Tokyo, Japan), 10 IU/ml hCG (ASUKA Pharmaceutical), 100 µg/ml amikacin sulfate (Meiji Seika), 0.1% (w/v) PVA and 10% (v/v) pig follicular fluid prepared according to the procedure described by Yoshida et al. [30]. Only COCs possessing a compact cumulus mass and evenly granulated ooplasm were selected and used for the experiments. COCs (20 to 50) were transferred to a droplet of maturation medium (200 µl) under paraffin oil (Nacalai Tesque, Kyoto, Japan) in a 35-mm polystyrene dish (Becton Dickinson Labware, Oxford, CA, USA) and cultured at 38.5°C in an atmosphere of 5% CO₂ in air.

In vitro fertilization (IVF)

The sperm-rich fraction of ejaculates was obtained from male Claw miniature pigs [32] using the gloved-hand method. Semen samples were washed three times by suspending them in 0.9% (w/v) NaCl containing 10 ng/ml BSA and 100 µg/ml kanamycin.
sulfate and subsequently subjecting them to brief centrifugation (1,000 × g, 5 min). Washed spermatozoa were subsequently diluted to 2 × 10^7 cells/ml in modified IVF-TALP [30] without caffeine and then subjected to IVF. After 40–42 h of culture, the cumulus mass of the oocytes was partially removed by vortexing with 0.1% (w/v) hyaluronidase. After the oocytes (50 to 55) were washed and transferred to modified IVF-TALP supplemented with 0.27 mM CaCl_2·2H_2O, 5.07 mM NaHCO_3, 0.1 mM Na pyruvate, 2 mM caffeine-sodium benzoate (Sigma-Aldrich Chemical) and 0.3% BSA, spermatozoa were introduced with a final concentration of 2.5 to 5 × 10^4 cells/ml. After incubation for 6–7 h, the oocytes were removed from the modified IVF-TALP, washed 3 to 6 times and cultured in DM under 5% CO_2.

Microinjection and culture

Microinjection was carried out using an inverted microscope (Olympus, Tokyo, Japan) equipped with a PC controlled micromanipulation system (Suruga Seiki, Shizuoka, Japan) and an IM-300 motorized injector (Narishige, Tokyo, Japan). After electrical pulse stimulation of oocytes for 10 to 12 h, the pronuclei were visualized after brief centrifugation of the oocytes. About 5 pl of 0.5 µg/ml dsRNA was injected into the cytoplasm of PA-derived pronuclear (PN) stage embryos. As a control, about 5 pl of DEPC-water was also injected. About 10 pl of 2.5 or 5 µg/ml siRNA expression vector was injected into the pronuclei of the IVF-derived PN stage embryos. Concomitantly, a similar amount of distilled water was injected as a control. The injected embryos (12 to 23) were washed with DM three times and cultured at 38.5 C in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 37 C. After 4 days of culture in vitro, embryos were transferred to a droplet (50 µl) of DM supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) for an additional 3 days of culture. The cleavage rate and blastocyst formation efficiency of these cultured embryos were evaluated at 2 and 7 days in culture, respectively.

Donor cells for SCNT

Pig embryonic fibroblasts (PEFs) were collected from a male fetus of a Clawn miniature pig on Day 30 of pregnancy. Body tissues were cut into small pieces and cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium (11320–033; Gibco) supplemented with 10% FBS at 37 C. After reaching confluence, cells were passaged several times. The cells used as donors for gene transfer between culture passages 4 and 8. Cells (1 × 10^7) were transfected with a nucleofection system (AMAXA Biosystem, Amaxa GmbH) according to the method of Nakayama et al. [33]. Briefly, 85 µl of nucleofector solution (for primary cells) containing 100 µl of supplement was mixed with 15 µl (10 µg) of linearized pPNER5 plasmid DNA and then added to the cell pellet. After suspending them, the cell suspension was subjected to nucleofection with program B16 (which is one of the programs recommended by the manufacturer). These cells were then plated onto a 100-mm plastic dish (Becton, Dickinson and Company) with 10 ml of DMEM/Ham’s F-12 medium containing 10% FBS at 37 C in a humidified atmosphere of 5% CO_2 in air. After 5 days of culture, G418 stock solution (Geneticin; Invitrogen) at a final concentration of 500 µg/ml was added to the medium to select potential donor cells. Colonies exhibiting uniform expression of EGFP were picked up, according to the method of Nakayama et al. [33]. A single cell suspension was prepared by standard trypsinization prior to SCNT.

SCNT, culture and counting of cell number

SCNT was performed according to the method of Miyoshi et al. [31]. Briefly, nuclei from the G418-selected transfectants or normal PEFs were each introduced into a single enucleated oocyte by cell fusion. SCNT was performed on 199 and 133 enucleated oocytes using nuclei from the G418-selected transfectants or from wild-type cells, respectively. Development of SCNT-treated embryos was evaluated in view of cleavage rate and blastocyst formation efficiency at 2 and 7 days in culture, respectively. At the end of culture, blastocysts were placed onto slides with a drop of mounting medium consisting of glycerol and Dulbecco’s phosphate buffered saline (PBS; Nissui Pharmaceutical, Tokyo, Japan) (9:1) containing 100 µg/ml Hoechst 33342 (Sigma-Aldrich Chemical). A cover slip was then placed onto the drop, and the edge of the cover slip was sealed with nail polish to prevent evaporation. Cell number of blastocysts was recorded by counting the number of Hoechst 33342-stained nuclei under ultraviolet light.

Assessment of embryos in vitro

At the end of culture, blastocysts were washed with PBS and then incubated in 100 µg/ml FITC-labeled BS-I-B4 (Sigma-Aldrich Chemical) in PBS for 1 h at room temperature. After washing, fluorescence (the presence of the α-Gal epitope) in these blastocysts was assessed under an inverted fluorescence microscope (Olympus). The specificity of BS-I-B_4 for α-Gal epitope was confirmed by incubation of embryos in PBS containing 50 mM D-galactose (WAKO, Osaka, Japan) and 100 µg/ml FITC-labeled BS-I-B_4 for 1 h at room temperature. After observation for fluorescence, these embryos were subjected to the RT-PCR analysis.

Expression of EGFP in the PEF transfectants and SCNT blastocysts (that were unstained with BS-I-B_4) was examined under a fluorescence microscope (Nikon, Tokyo, Japan) with DM filters (Nikon).

RT-PCR analysis

According to the method described by Klebe et al. [34], total RNA was isolated from embryos and stored at −80 C immediately before use. The RT reaction was carried out using a GeneAmp® RNA PCR Kit (Applied Biosystems, Foster City, CA, USA). For the RT reaction, 6 µl of RNA sample was mixed with a solution containing 3.2 µl of 25 mM MgCl_2, 2 µl of 10× PCR Buffer II, 1 µl of RNase inhibitor (20 U/µl), 1 µl of murine leukemia virus reverse transcriptase (50 U/µl; Applied Biosystems) and 1 µl of 50 µM Oligo d(T)_{18} (Applied Biosystems) in a total volume of 20 µl. The reaction was performed at 42 C for 60 min, at 99 C for 5 min and then at 4 C for 5 min using a thermal cycler (PC-800; Astec, Japan). After RT, the mixture was stored at −20 C prior to use.

For PCR, 5 µl of RT mixture was mixed with a solution containing 1.2 µl of 25 mM MgCl_2, 2 µl of 10× PCR buffer, 0.125 µl of GeneAmp® AmpliTaq Gold® DNA polymerase (5 U/µl;
ments were pooled. The statistical significance of percentage data was determined by ANOVA. Statistical analysis was performed by using a StatView 5.0 program (SAS Institute, Cary, NC, USA).

Experimental design

In Experiment 1, the effects of dsRNA microinjected into the cytoplasm of PA-derived embryos on their subsequent in vitro development and the expression of an endogenous α-GalT mRNA were assessed. We used PA-derived embryos because they have developmental competence that is almost equivalent to that of IVF-derived embryos, as suggested by Kurihara et al. [36], and because PA-derived embryos can be more easily prepared than IVF-derived embryos. For testing the expression of an endogenous α-GalT mRNA in dsRNA-injected embryos, we evaluated the intensity of the 586-bp band corresponding to α-GalT mRNA in relation to that of the 233-bp band corresponding to β-actin transcripts as an internal control using the PA-derived blastocysts. As shown in Fig. 2, the majority of the samples (as exemplified by lanes 4–11) had both bands with relatively equal degrees of band intensity. Notably, 20% (2/10; lanes 3 and 12 in Fig. 2) of the samples tested exhibited a reduction in the level of the 586-bp band, while the 233-bp band was clearly visible. The reason why such event occurred remains unclear. In any case, we have to consider that about 20% of the control blastocysts did not exhibit a 586-bp band (Fig. 4A, lanes 6 and 7). However, the remaining 28% (4/14) of the control blastocysts did not exhibit a 586-bp band (Fig. 4A, lanes 4 and 5). In contrast, all of the dsRNA-injected embryos (9/9 tested) exhibited complete loss or decreased intensity of a 586-bp band in the ethidium bromide-stained gels (Fig. 4A, lanes 8 to 12).

Results

Experiment 1

When dsRNAs were microinjected into the cytoplasm of PN-stage PA-derived embryos, the developmental rate of the injected embryos to the blastocyst stage was similar to that of embryos injected with DEPC-water (41% vs. 55%; Table 1). Staining with FITC-labeled BS-I-B4 demonstrated that all of the developing blastocysts (5/5 tested) derived from the dsRNA-injected embryos exhibited a great reduction in fluorescence (Fig. 3a, b). In contrast, the control blastocysts derived from the DEPC-water-injected embryos were extensively stained with lectin (Fig. 3c, d). A single embryo-based RT-PCR demonstrated that 72% (10/14 tested) of the control blastocysts derived from the DEPC-water-injected embryos exhibited a clear band of 586 bp corresponding to an endogenous α-GalT mRNA (Fig. 4A, lanes 6 and 7). However, the remaining 28% (4/14) of the control blastocysts did not exhibit α-GalT mRNA (Fig. 4B, lanes 1 to 3; Table 3). In contrast, almost all (75–92%) of the siRNA expression vector-injected embryos exhibited complete loss or decreased intensity of a 586-bp band in the ethidium bromide-stained gels (Fig. 4A, lanes 8 to 12).

Experiment 2

When two types of siRNA expression vector DNA (pPR4 and pPR5) were microinjected into the pronuclei of PN-stage IVF-derived embryos, the blastocyst formation rates of the injected embryos were similar to those of embryos injected with distilled water (Table 2). A single embryo-based RT-PCR demonstrated that about half (43%, 9/21 tested) of the embryos injected with distilled water exhibited reduced levels of α-GalT mRNA (Fig. 4B, lanes 1 to 3; Table 3). In contrast, almost all (75–92%) of the siRNA expression vector-injected embryos exhibited complete loss or decreased intensity of the 586-bp band (Fig. 4B, lanes 5 to 12; Table 3). In particular, α-GalT mRNA synthesis was significantly depressed (P<0.05) suppressed when pPR5 was injected at 2.5 µg/ml (Table 3).

Experiment 3

We next performed SCNT using the PEF clone stably transfected with linearized pPNER5 plasmid (Fig. 1B) as a donor nuclei. Prior to SCNT, we confirmed that one of these pPNER5 transfectants in fact exhibited EGFP-derived fluorescence homogeneously (Fig.
SUPPRESSION OF α-1,3-GALT EXPRESSION

5a, b). The control untransfected PEF cells were never fluorescent (data not shown). Staining of the pPNER5 transfectant with Alexa Fluor 587 (red fluorescence)-labeled BS-I-B4 demonstrated a great decrease in fluorescence on the cell surface (data not shown).

Table 4 summarizes the results in regard to the developmental

Fig. 3. a, b: Reduced expression of the α-Gal epitope in a blastocyst developed from a dsRNA-injected PN-stage embryo. Note the marked reduction in fluorescence on the cell surface of a blastocyst stained with FITC-labeled BS-I-B4. c, d: Control blastocyst developed from a water-injected PN-stage embryo showing strong fluorescence throughout the embryo. a, c: bright field; b, d: dark field. Scale bars = 50 μm.

Table 1. Developmental ability of PA-derived embryos microinjected with dsRNA

<table>
<thead>
<tr>
<th>Reagents injected</th>
<th>No. embryos microinjected</th>
<th>No. (%) of embryos developed to 2-cell stageb</th>
<th>Blastocyst stagec</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEPEC-water (control)</td>
<td>121</td>
<td>75 (62)</td>
<td>41 (55)</td>
</tr>
<tr>
<td>dsRNA</td>
<td>134</td>
<td>66 (49)</td>
<td>27 (41)</td>
</tr>
</tbody>
</table>

* Experiments in each treatment group were replicated 3 times. b % = no. 2-cell embryos/no. embryos microinjected. c % = no. blastocysts/no. 2-cell embryos.

Table 2. Developmental ability of IVF-derived embryos microinjected with siRNA expression vectors

<table>
<thead>
<tr>
<th>siRNA expression vectors</th>
<th>Concentration (µg/ml)</th>
<th>No. embryos microinjected</th>
<th>No. (%) of embryos developed to 2-cell stageb</th>
<th>Blastocyst stagec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled-water (control)</td>
<td>2.5</td>
<td>93</td>
<td>56 (60)d</td>
<td>21 (38)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>72</td>
<td>36 (50)e</td>
<td>8 (22)</td>
</tr>
<tr>
<td>pPR4</td>
<td>5</td>
<td>84</td>
<td>38 (45)e</td>
<td>9 (24)</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>99</td>
<td>49 (49)e</td>
<td>12 (24)</td>
</tr>
<tr>
<td>pPR5</td>
<td>5</td>
<td>108</td>
<td>45 (42)e</td>
<td>9 (20)</td>
</tr>
</tbody>
</table>

* Experiments in each treatment group were replicated 3 times. b % = no. 2-cell embryos/no. embryos microinjected. c % = no. blastocysts/no. 2-cell embryos. d,e Numbers with different superscripts differ significantly (P<0.05).
data obtained through SCNT experiments. The cell number at the blastocyst stage was significantly (P<0.05) different between the SCNT blastocysts derived from pPNER5-transfected PEFs and those from untransfected PEFs. Fluorescence microscopic observation confirmed that all of the SCNT blastocysts (8/8 tested) derived from embryos reconstituted with pPNER5 transfectant nuclei expressed EGFP in their trophectoderm layers (Fig. 5c, d). RT-PCR analysis demonstrated that all of the tested samples (8/8 tested) exhibited complete loss of a target 586-bp band corresponding to the endogenous \(\alpha\)-GalT mRNA. In Fig. 4C, a portion of the data is shown (lanes 1 to 5). In contrast, about half (57%, 4/7 tested) of the SCNT blastocysts derived from embryos reconstituted with untransfected PEF nuclei exhibited a clear band of 586 bp (Fig. 4C, lanes 8 to 12).

**Table 3.** Summary regarding detection of a 586-bp band corresponding to endogenous \(\alpha\)-GalT mRNA in blastocysts obtained after pronuclear injection of siRNA expression vectors

<table>
<thead>
<tr>
<th>siRNA expression vectors</th>
<th>Concentration (µg/ml)</th>
<th>Percentage of samples identified with reduced expression of endogenous (\alpha)-GalT mRNA after RT-PCR analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled-water (control)</td>
<td></td>
<td>43 (9/21)(^b)</td>
</tr>
<tr>
<td>pPR4</td>
<td>2.5</td>
<td>75 (6/8)(^b,c)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>89 (8/9)(^b,c)</td>
</tr>
<tr>
<td>pPR5</td>
<td>2.5</td>
<td>92 (11/12)(^b)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>89 (8/9)(^b,c)</td>
</tr>
</tbody>
</table>

\(^a\) Experiments in each treatment group were replicated 3 times. RT-PCR products were separated in 2% agarose gels and stained with EtBr. Samples exhibiting reduced intensity of the 586-bp band relative to the amounts of \(\beta\)-actin transcripts are defined as those with reduced expression of \(\alpha\)-GalT mRNA. Values in parentheses indicate no. of samples exhibiting reduced expression of \(\alpha\)-GalT mRNA/no. of samples tested. \(^b\) Numbers with different superscripts differ significantly (P<0.05).

**Table 4.** Developmental ability of SCNT embryos after reconstitution with a stable PEF clone carrying pPNER5 plasmid

<table>
<thead>
<tr>
<th>Donor nuclei</th>
<th>No. enucleated oocytes</th>
<th>No. (%) of oocytes fused</th>
<th>No. (%) of 2-cell embryos(^b)</th>
<th>No. (%) of blastocysts(^c)</th>
<th>Mean no. ± SEM of cells in blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untransfected PEF</td>
<td>133</td>
<td>79 (59)</td>
<td>61 (77)</td>
<td>9 (15)</td>
<td>51.9 ± 3.8(^d)</td>
</tr>
<tr>
<td>PEF clone carrying pPNR5</td>
<td>199</td>
<td>138 (69)</td>
<td>109 (79)</td>
<td>8 (7)</td>
<td>30.1 ± 2.9(^e)</td>
</tr>
</tbody>
</table>

\(^a\) Experiments in each treatment group were replicated 3 times. \(^b\) % = no. 2-cell embryos/no. oocytes fused. \(^c\) % = no. blastocysts/no. 2-cell embryos. \(^d\)-\(^e\) Numbers with different superscripts differ significantly (P<0.05).

**Fig. 5.** EGFP expression in the pPNER5-transfected PEFs (a, b) and the blastocysts (c, d) derived from embryos reconstituted with those cells. Note that one clone carrying pPNR5 obtained after selection with G418 exhibits strong and uniform expression of EGFP-derived fluorescence. Furthermore, the SCNT blastocysts exhibit uniform and bright fluorescence in their trophectoderm layers. a, c: bright field; b, d: dark field. Scale bars = 50 µm.

**Discussion**

Attempts to suppress the expression of \(\alpha\)-GalT mRNA by RNAi technology in pig cells have been made by a few laboratories. For example, Yu et al. [37] demonstrated that expression of the \(\alpha\)-Gal epitope was markedly suppressed by transfection with siRNA targeted to the \(\alpha\)-GalT mRNA sequence. Interestingly, these siRNA-incorporating cells are resistant to cytotoxicity after treatment with normal human serum and complements. Zhu et al. [38–40] also obtained similar results achieved by Yu et al. [37] using pig endothelium. These results suggest feasibility of RNAi technology in genetic manipulation of pig cells. Unfortunately, there is no report on successful suppression of a target gene expression in pig preimplantation embryos with this RNAi technology.

As a first step toward the applicability of this technology to pig
embryos, we microinjected dsRNA into cytoplasm of pig zygotes to test whether these RNAi systems can effectively suppress endogenous α-GalT mRNA expression in them. As expected, all of the developing blastocysts tested exhibited a great reduction in fluorescence when they were stained with FITC-labeled BS-1-B4 (see Fig. 3a, b). These results suggest a decrease in the level of endogenous α-GalT mRNA expression. However, in the control in which DEPC-water alone was injected, only 72% of the developing blastocysts exhibited a target band of 586 bp (see Fig. 4a, lanes 6 and 7). The failure to detect endogenous α-GalT mRNA expression in the remaining 28% of the control blastocysts (see Fig. 4a, lanes 4 and 5) may be due to the inability of the embryos to produce α-GalT mRNA at a detectable level, but not to RNAi-mediated suppression, as mentioned previously.

Suppression of endogenous α-GalT mRNA synthesis was also achieved in the blastocysts derived from embryos receiving pronuclear injection of an siRNA expression vector (see Table 3). This type of RNAi is called “transgenic RNAi” and is now recognized as a useful tool for continuous knocking down of a target gene expression in live animals [41, 42]. However, this technology still has drawbacks. Gene silencing like that in the transgene-based conventional transgenic system often occurs in the resulting animals. From this point of view, SCNT-mediated transgenesis may be more feasible than pronuclear injection-based transgenesis, because, in the former case, we can easily select the transfectants with high transgene expression. In this context, insertion of fluorescent marker gene (i.e., EGFP cDNA) into the siRNA expression vector appears to be of importance, since the strength of transgene expression in transfectants can be monitored under visual inspection for fluorescence. For example, when one PEF transfectant carrying pPNERS (Fig. 1B) was inspected for EGFP-derived fluorescence, it exhibited bright uniform fluorescence, as expected (Fig. 5a, b). Blastocysts developing from the embryos reconstituted with the above PEF transfectant exhibited bright fluorescence throughout the embryo, as expected (Fig. 5c, d). RT-PCR analysis demonstrated that all of these SCNT blastocysts exhibited complete loss of the target band corresponding to endogenous α-GalT mRNA (see Fig. 4C, lanes 1 to 5). Notably, about half (57%) of the SCNT blastocysts derived from embryos reconstituted with untransfected PEFs exhibited a clear band of 586 bp (see Fig. 4C, lanes 8 and 12). The decreased levels of α-GalT mRNA synthesis exhibited by the remaining 43% of the embryos (see Fig. 4C, lane 8) may be ascribed to failure to express their α-GalT mRNA at a level detectable by RT-PCR, as previously pointed out. This phenomenon may be related to the failure of proper reprogramming of donor nuclei after SCNT, since gene activation of embryonic and pluripotency-related genes is sometimes suppressed [43, 44]. In our preliminary data, expression of α-GalT mRNA increased gradually with progression towards the blastocyst stage (unpublished results). Although the α-GalT gene does not belong to the pluripotency-related genes, its expression may be affected by the reprogramming state of donor nuclei. Presently, no reports on successful production of gene-engineered cloned pigs, in which expression of an endogenous target gene is suppressed by expression of siRNA, have been provided. It would be thus worthwhile to create such cloned animals carrying pPNERS transgenes in their genome to test whether the siRNA technology is still effective even in living individuals after birth.

In conclusion, we show here for the first time that RNAi targeted to the endogenous α-GalT mRNA expression is effective in preimplantation pig embryos. We believe that this RNAi-based technique will represent a new approach for overcoming hyperacute rejection of transplants upon pig-to-human xenotransplantation.

Acknowledgments

The authors express their gratitude to the staff of the Kagoshima Meat Inspection Office and Meat Center Kagoshima, Inc. (Kagoshima) for supplying pig ovaries. This study was partly supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (No. 17100007 to ST and 20580311 to MY).

References

4. Ge Q, McManus MT, Nguyen T, Shen CH, Sharp PA, Eisen HN, Chen J. RNA interference of influenza virus protein by directly targeting mRNA for degradation and indirectly inhibiting all viral RNA transcription. Proc Natl Acad Sci USA 2003; 100: 2718–2723. [Medline] [CrossRef]
Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science 2002; 295: 1089–1092. [Medline] [CrossRef]


Strahan KM, Gu F, Preece AF, Gustavsson L, Andersson L, Gustafsson K. CDNA sequence and chromosome localization of pig alpha-1,3-galactosyltransferase. Immunogenetics 1995; 41: 101–105. [Medline] [CrossRef]


Mendus WJ. A semi-quantitative RT-PCR method to measure the in vivo effect of dietary conjugated linoleic acid on porcine muscle PPAR gene expression. Biol Proced Online 2005; 5: 20–28. [Medline] [CrossRef]


