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Leading Opinion

Successful genetic modification of porcine spermatogonial stem cells via an electrically responsive Au nanowire injector

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ABSTRACT

Transgenic pigs are quite useful in many biomedical fields, such as xenotransplantation research and the production of biopharmaceutical materials. The genetic transformation of porcine spermatogonial stem cells (pSSCs) followed by differentiation into mature spermatozoa enables the effective production of transgenic pigs. Improving the transfection efficiency of pSSCs, however, has been much desired. Herein, we report the efficient genetic modification of pSSCs by using an electrically responsive Au nanowire injector (E-R Au NWI). This is the first study that shows an exogenous gene is directly delivered into the nucleus of a pSSC by using a 1-dimensional nanomaterial and then successfully expressed to produce a protein. The E-R Au NWI interfaced noninvasively with the nucleus of the pSSC, and the pEGFP-N1 plasmid was delivered by the application of an electrical stimulus without cell damage. Compared to the results of conventional nonviral vector-based gene delivery methods such as jetPEI, Lipofectamine, and electroporation, the E-R Au NWI-based method improved the pSSC transfection efficiency by at least 6.7-fold and even up to 46.7-fold. Furthermore, we successfully obtained transgenic pSSCs containing the human bone morphogenetic protein 2 gene by using E-R Au NWIs. This result suggests that the E-R Au NWI enables the efficient genetic modification of pSSCs and can be employed to produce diverse kinds of transgenic pigs.

1. Introduction

Since the emergence of transgenic techniques with the first successful cloning of eukaryotic DNA in 1974, the production of transgenic animals has been attempted in a variety of species by using diverse transgenic technologies such as microinjection, sperm mediated gene transfer method, somatic cell nuclear transfer, and so on [1–7]. These transgenic animal production methods, however, suffer from low efficiency, cumbersome operation, and high cost [8–11]. To overcome these limitations, various kinds of nanomaterials have recently emerged as effective carriers for the delivery of genes. For example, Thomas et al. reported PEI2 conjugated Au nanoparticles highly increased transfection efficiency of monkey kidney cells and Rosi et al. developed Au NP-oligonucleotide complexes exhibited 99% cellular uptake without cytotoxicity. Feng et al. demonstrated that injectable nanofibrous spongy microspheres are able to transfect intervertebral disc cells with high efficiency. Our group also investigated the electro-triggered, spatioselective, and quantitative gene delivery into a single cell nucleus by using an Au nanowire (NW) nanoinjector [12–15].

Among the various species of animals, the pig has been considered as a useful transgenic model for biomedical research due to the following several advantages: anatomical and physiological similarity to humans, a relatively short gestation period and generation interval, and a large litter size [16–18]. Typically, transgenic pigs have been generated by somatic cell nuclear transfer methods via direct microinjection, pluripotent cells, transposons, viruses, and biopolymers [19–24].

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However, these techniques have rarely showed genomic integration of the transgene [23–26], resulting in extremely low efficiency for the production of full-term transgenic offspring [27,28].

Recently, spermatogonial stem cells (SSCs) have attracted as one of highly useful gene carrier for producing transgenic pigs instead of somatic cells [29–31]. The SSCs can self-renew infinitely and differentiate into sperm cells through spermatogenesis in the seminiferous tubule of the testis [32–34]. Moreover, the chromosome modification of SSCs can be easily induced by a gene delivery system, since the SSC chromosome is characterized by histone-dependent condensation [35]. Although the use of SSCs offers significant advantages for generating genetically modified pigs, transgenic studies employing SSCs rarely reported due to their small number in the testis (0.03% of germ cells in rodent) and complexity of culture environment [36–39]. Up to date, the genetic modification of porcine SSCs (pSSCs) has accomplished by utilizing viral vector systems [31,40]. The genetic modifications derived from viral vector systems, however, can induce carcinogenesis, broad tropism, and immunogenicity, hindering the production of transgenic animals [41–43]. Accordingly, it is promptly necessary to develop a technology capable of improving the transfection efficiency of pSSCs without using viral vectors. The electrically responsive Au NW injector (E-R Au NWI) is a promising technique that can overcome the limitations of typical gene delivery systems. A single-crystalline Au NW can penetrate effectively into a single cell owing to its sharp tip, uniform width along the whole NW, and high mechanical strength. In addition, the superelasticity of the Au NW can minimize the physical damage to the cell [15,44–46]. We previously verified that an E-R Au NWI could introduce an exogenous gene into the nucleus of a cell without cell damage and that the exogenous gene expressed without cell proliferation [15]. This result prompted us to investigate the transfection of pSSCs, the first step in transgenic pig production, by employing an E-R Au NWI.

Herein, we report the efficient production of transgenic pSSCs by using an E-R Au NWI. An E-R Au NWI can approach the nucleus of pSSCs without causing serious damage and can deliver cargo selectively through the application of an electrical stimulus. The E-R Au NWI-based genetic modification of pSSCs showed from 6.7 times to 46.7 times higher efficiency than conventional nonviral-mediated transfection methods. Furthermore, we successfully produced transfected pSSCs by delivering a functionally designed plasmid containing the human bone morphogenetic protein 2 (hBMP2) gene which is marketed by the Food and Drug Administration as a biosimilar. To the best of our knowledge, this work is the first case of gene delivery into the pSSC nucleus and successful expression of the protein by using a 1-dimensional nanomaterial as a nonviral gene delivery system. We anticipate that the E-R Au NWI-based genetic modification of pSSCs will lead to the efficient production of various kinds of transgenic pigs.

2. Experimental section

2.1. Preparation of E-R Au NWIs

Au NWs were synthesized in the vapor phase as reported in our previous study [47]. To detach a single Au NW, a W tip loaded on a piezoelectric stage (Sigma Koki) was used. To prevent undesired electrochemical reactions, the entire W tip was insulated with UV-curing optical adhesive (Norland) and nail varnish. After E-R Au NWIs preparation, the scanning electron microscopy (SEM) images of E-R Au NWIs were taken using a FEI Nova 230 (FEI, USA).

2.2. Attachment of plasmids to E-R Au NWIs

Before loading plasmids on E-R Au NWIs, they were electrochemically cleaned in 0.1 M phosphate buffer solution (pH = 7.4) by cyclic voltammetry measurement. As-cleaned E-R Au NWIs were then incubated in 20 mM cysteamine (CA, $HSCH_2CH_2NH_2$) in ultrapure water for 30 min, and excess CA was washed away with ultrapure water. The CA-modified E-R Au NWIs were then incubated in a solution of 100 nM plasmid dissolved in ultrapure water overnight at room temperature. The successful loading of plasmids onto the E-R Au NWI was confirmed by using a DAPI (4',6-diamidino-2-phenylindolem dihydrocholoride) (D1306, Invitrogen, USA) as a DNA intercalator. For the comparison, one E-R Au NWI was incubated with CA and plasmids sequentially, and another E-R Au NWI was incubated with CA only. These two E-R Au NWIs were then incubated in 500 nM of DAPI for 5 min at the dark room. After washing, the fluorescence images of two E-R Au NWIs were obtained by a confocal laser scanning microscope (LSM780, Carl Zeiss, Jena, Germany).

2.3. Animals

Crossbred (Landrace × Yorkshire) or purebred (Yorkshire × Yorkshire) piglets were generously provided by Gumbo Inc. (Wonju, Korea), and the testes were isolated from 1- to 4-day-old piglets through routine castration surgery performed at a local farm. All of the animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Kangwon National University (Approval No. KW-131106-1) and performed in accordance with the Animal Care and Use Guidelines of Kangwon National University (Gangwon-do, South Korea).

2.4. Isolation of SSCs from porcine testes

Male neonatal testes were transported from a local farm (Gumbo) to our laboratory in ice-cold Dulbecco's phosphate-buffered saline (DPBS; Welgene Inc., Daegu, Korea) supplemented with 1% (v/v) antibiotic-antimycotic solution (Welgene) within 1 h. To isolate the testicular cells from the testes, the tunica albuginea and the epididymis were removed, and the seminiferous tubules were digested with 0.1% (w/v) type IV collagenase (Worthington Biochemical, Lakewood, CA, USA) in high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Welgene) at 37 °C for 15 min. The fragmented seminiferous tubules were dissociated sequentially and separately by 0.1% (w/v) hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) in high-glucose DMEM and 0.25% trypsin-EDTA (Welgene) at 37 °C for 10 min. The dissociated testicular cells were washed twice with DMEM containing 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY), and myoid and sertoli cells in the dissociated testicular cells were eliminated by filtration using a 70 μm nylon strainer (SPL, Pocheon, Korea). Red blood cell lysis buffer (Sigma-Aldrich) was used to eliminate erythrocytes in the collection of dispersed cells. Then, the pSSCs were sorted from the isolated testicular cells by Petri dish plating using the postdifferential plating method previously described [69]. Briefly, 5×10^6 testicular cells were resuspended in high-glucose DMEM supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) antibiotic-antimycotic solution, then plated on a 100 mm SPL Petri dish coated with 0.1% (w/v) gelatin (Sigma-Aldrich). After a 16 h incubation at 37 °C, 1×10^6 cells suspended in medium were plated on a 35 mm Petri dish in high-glucose DMEM supplemented with 15% (v/v) heat-inactivated FBS, 0.1 mM β-mercaptoethanol (Gibco), 1% (v/v) nonessential amino acid (NEAA; Gibco), 2 mM L-glutamine (Invitrogen, Carlsbad, CA, USA), 1% (v/v) antibiotic-antimycotic solution, 10^3 units/ml mouse leukemia inhibitory factor (LIF; Chemicon International, Inc., Temecula, CA, USA), and 10 ng/ml glial cell-derived neurotrophic factor (GDNF; R&D Systems, Inc., Minneapolis, MN, USA) and incubated at 37 °C overnight. Then, the suspended pSSC populations were collected, and only SSC populations showing greater than 95% positivity against pSSC-specific proteins (OCT4, NANOG, promyelocytic leukemia zinc finger [PLZF] and GDNF family receptor alpha-1 [GFRα1]) and less than 5% positivity against a leydig cell-specific protein (luteinizing hormone receptor [LHR]) and a sertoli cell-specific protein (GATA4) were assigned to the following experiments.

2.5. Plasmid delivery into pSSCs by E-R Au NWIs

The pSSCs (1×10^2) were adhered onto a 35-mm cell culture dish coated with vitronectin, and the dish was placed on the stage of an optical microscope (CKX-41; Olympus, Tokyo, Japan). For gene delivery into the pSSCs, an E-R Au NWI mounted on a micromanipulator was operated under the optical microscope. The E-R Au NWI was connected to an electrochemical workstation (CHI 660D) and was slowly immersed in the pSSC culture medium (pSSCCM) until it was positioned in the same focal plane as the pSSCs. The pSSCCM consisted of Stempro-34 medium (Invitrogen) supplemented with insulin-transferrin-selenium (Invitrogen), 60 μM putrescine dihydrochloride (Sigma-Aldrich), 6 mg/ml D-(+)-glucose (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 0.11 mg/ml sodium pyruvate (Sigma-Aldrich), 1 μl/ml DL-lactic acid (Sigma-Aldrich), 5 mg/ml bovine serum albumin (BSA, Sigma-Aldrich), 2 mM L-glutamine, 0.05 mM β-mercaptoethanol, 1% (v/v) FBS, 1% (v/v) NEAA, MEM vitamin solution (Sigma-Aldrich), 1% (v/v) antibiotic–antimycotic solution, 0.1 mM ascorbic acid (Sigma-Aldrich), 10^3 units/ml mouse LIF, 10 ng/ml GDNF, 30 ng/ml β-estradiol (Sigma-Aldrich), 60 ng/ml progesterone (Sigma-Aldrich), 20 ng/ml human epidermal growth factor (EGF; Peprotech, Inc., Rocky Hill, NJ, USA), and 10 ng/ml human basic fibroblast growth factor (bFGF; Peprotech, Inc.). For the delivery of plasmids by an electrical stimulus from the E-R Au NWI, the reference electrode and counter electrode were also immersed in the pSSCCM. After insertion of the E-R Au NWI into the nucleus, an electrical stimulus of −0.8 V was immediately applied for 2 min to detach the plasmids from the E-R Au NWI. The transfected pSSCs were stabilized for 24 h at 31 °C on a culture dish coated with testicular stromal cells as feeder cells in pSSCCM.

2.6. Transfection of pSSCs by electroporation

For this purpose, pSSCs (5×10^5) diluted in 200 μl Hanks' balanced salt solution (HBSS; Welgene) were transferred into a Gene Pulser cuvette (Bio-Rad, Richmond, CA, USA) with a distance of 0.4 cm between electrodes. Subsequently, 1 μg of pEGFP-N1 plasmid (4.7 kb) was delivered into their cytoplasm using a single electric pulse derived from the Gene Pulser Apparatus (Bio-Rad) at a voltage of 275 and a capacitor setting of 500 μF. The transfected pSSCs were stabilized for 24 h at 31 °C on a culture dish coated with testicular stromal cells as feeder cells in pSSCCM.

2.7. Transfection of pSSCs by nonviral transfection reagents

According to the manufacturer's instructions, 1 μg of pEGFP-N1 plasmids (4.7 kb) was dissolved in 50 μl of 150 mM NaCl in distilled water or 50 μl of Opti-MEM medium (Invitrogen), and 2 μl of jetPEI (22 kDa, linear polyethylenimine derivative; Polyplus, Graffenstaden, France) or 2 μl of Lipofectamine 2000 (Invitrogen) stock solution was diluted in 48 μl of 150 mM NaCl in distilled water or 48 μl Opti-MEM medium. Subsequently, jetPEI-DNA or Lipofectamine-DNA complexes were prepared by gently mixing the plasmid DNA solution and the diluted jetPEI or Lipofectamine 2000 solution and incubating the mixture for approximately 30 min at room temperature. Before transfection, pSSCs (1×10^5) were plated in 24-well culture dishes coated with 5 μg/ml vitronectin in 500 μl of pSSCCM. After washing with DPBS the next day, 500 μl of Opti-MEM medium was added to the 24-well culture dishes containing pSSCs. Then, the pSSCs were treated with the prepared transfection reagent-DNA complexes for 6 h at 31 °C, washed with DPBS, and stabilized for 24 h in pSSCCM.

2.8. Immunocytochemistry

The transfected or non-transfected pSSCs were plated on confocal dishes (SPL) coated with 5 μg/ml vitronectin (Gibco) and stabilized at 31 °C for 24 h in pSSCCM. After rinsing twice with DPBS, the cells in pSSCCM were stained with anti-rabbit GFRα1 antibody (SC-10716; Santa Cruz Biotechnology, Dallas, TX, USA) at 31 °C for 2 h and the detection of GFRα1 primary antibody was performed by incubating Alexa Fluor 546-conjugated donkey anti-rabbit IgG antibody (A10040; Invitrogen) diluted in pSSCCM at 31 °C for 2 h. Subsequently, the stained cells were counterstained at room temperature for 15 min in DPBS containing 5 μg/ml Hoechst33342 (Sigma-Aldrich) and the counterstained cells were rinsed twice with DPBS. Finally, after adding pSSCCM to the rinsed cells, the localization of GFRα1, EGFP, and Hoechst33342 in pSSCs were monitored under a confocal laser scanning microscope (LSM880, Germany).

2.9. Flow cytometry

Electroporated or transfected pSSCs were transferred to a flow cytometry tube, and the EGFP-positive pSSCs were detected using a FACS Calibur (Becton, Dickinson and Co. Franklin Lakes, NJ, USA). The data were analyzed using BD CellQuest Pro software (Becton, Dickinson and Co.).

2.10. PCR analysis

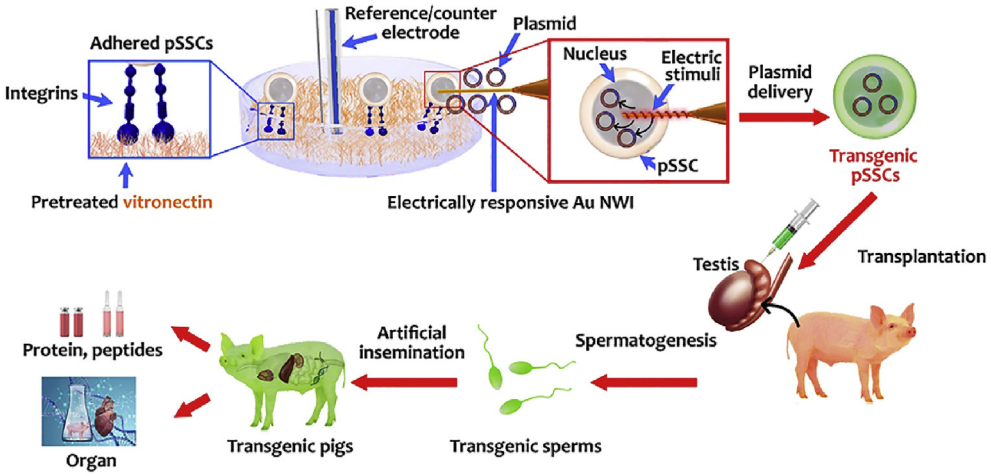
Total mRNA was extracted from pSSCs using the Dynabeads® mRNA Direct™ Kit (Ambion, Austin, TX, USA) followed by cDNA synthesis using ReverTra Ace® PCR RT Master Mix with gDNA remover (Toyobo, Osaka, Japan) according to the manufacturers' instructions. Moreover, the gDNA of pSSCs was extracted using a FavorPrep Plasmid Extraction Mini Kit (Favorgen) according to the manufacturer's instructions. Subsequently, TProfessional Trio Thermocycler (Biometra, Goettingen, Germany) was used for PCR amplification with the following conditions: 9 min at 95 °C for the initial denaturation followed by 45 cycles of 30 s at 92 °C, 30 s at 60 °C for annealing of the GAPDH and human BMP2 primers and at 58 °C for annealing of the EGFP primer, and 30 s at 72 °C. The final extension was performed at 72 °C for 5 min. The PCR products were fractionated by electrophoresis in 1% (w/v) agarose gels (Sigma-Aldrich), stained with RedSafe (Intron Biotechnology Inc., Seongnam, Korea) and visualized under the Gel Doc XR + imaging system (Bio-Rad). All primers were designed by Primer3 software (Whitehead Institute/MIT Center for Genome Research) with pig gDNA and cDNA sequences obtained from GenBank. The general information and sequences of primers are shown in Table S1 (Supporting Information).

2.11. Statistical analysis

Statistical analyses were performed with the Statistical Analysis System program. Analysis of variance was performed to identify significance, and significant differences among the different treatments were determined with the least-squares test or Duncan's test. *P* values less than 0.05 were indicative of significant differences.

3. Result and discussion

Sperm, the male germ cell, plays a crucial role in transferring genetic heritability and can be a mediator in the production of transgenic pigs [48–51]. Although some studies have reported the genetic modification of sperm, they have lacked a comprehensive approach to the successful generation of transgenic sperm because of the difficulty of controlling the various factors that affect the interaction between sperm and exogenous DNA [50]. In particular, the nuclei of sperm are densely integrated with specific structural proteins, and thus, it is very difficult for foreign genes to enter the sperm nucleus [52–54]. Moreover, the introduced foreign DNA may be degraded by sperm nucleases, lowering the efficiency of genomic integration [55,56]. Since genetically modified SSCs have the ability to differentiate into sperms cells bearing their transformed genetic traits, pSSCs are considered as highly useful



Scheme 1. Delivery of plasmid into a pSSC nucleus via E-R Au NWI and prospective applications of the transgenic pSSCs.

gene carriers for producing transgenic pigs.

3.1. Development of an E-R Au NWI-based gene delivery system for generating transgenic pSSCs

Scheme 1 shows the process of delivering a plasmid into the pSSC nucleus via an E-R Au NWI and the prospective application of the transgenic pSSCs. To transfer a plasmid into a pSSC nucleus via an E-R Au NWI, pSSCs isolated from piglet testes should be immobilized firstly rather than allowed to float in a medium. We adopted vitronectin to immobilize pSSCs in a cell culture dish. Victronectin can bind to the heterodimeric integrins $\alpha_V\beta_1$ existing in the membrane of pSSCs [57]. We pretreated a cell culture dish with 5 μg/ml vitronectin and immobilized pSSCs on the dish by 3 h of incubation. After the adhesion of the pSSCs, the plasmid was delivered into the pSSC nucleus by using a 3-electrode system that consists of an E-R Au NWI (working electrode), a saturated calomel electrode (reference electrode), and a Pt wire (counter electrode). The E-R Au NWI was fabricated from single-crystalline Au NW as previously reported [47]. In brief, single-crystalline Au NWs were synthesized on a sapphire substrate by chemical vapor transport. The vertically grown Au NW was then attached to a W tip under optical monitoring. The Au NW-attached W tip was insulated with a nail varnish except for the Au NW, completing the fabrication of the E-R Au NWI. Plasmids were then electrostatically attached to the surface of an E-R Au NWI.

The successful attachment of plasmids onto the E-R Au NWI was confirmed by using a DAPI as a DNA intercalator. To investigate the successful plasmid loading, two E-R Au NWIs were prepared. One was incubated with CA and plasmids sequentially, and another was incubated with CA only. These two E-R Au NWIs were then incubated in DAPI at the dark room. After washing, the fluorescence images of two E-R Au NWIs were obtained (Fig. S1). The plasmid-loaded E-R Au NWI only showed the blue fluorescence of DAPI, indicating the successful loading of plasmids onto the E-R Au NWI.

To deliver the plasmid into pSSCs, the plasmid-attached E-R Au NWI was injected into the nucleus of a pSSC, and an electrical stimulus was applied. If genetic modification of the pSSCs was successfully achieved by an E-R Au NWI, the transgenic pSSCs were transplanted into the recipient testis and differentiated into mature sperm cells in vivo. In addition, the transgenic pSSCs could differentiate into mature sperm cells in a well-controlled in vitro culture system. Ultimately, transgenic pigs may be obtained through in vitro fertilization or intrauterine insemination with transgenic sperm and they will be used to acquire information on human diseases, to produce specific organs that are not subject to immune rejection, and to produce high-value products for pharmaceutical use such as proteins, hormones, therapeutic antibodies, and vaccines [58–64].

3.2. Generation of transgenic pSSCs using an E-R Au NWI-based gene delivery system

To verify pSSC transfection, the first step in transgenic pig production, we delivered pEGFP-N1 plasmid (4.7 kb) containing the CMV promoter into a pSSC nucleus using an E-R Au NWI. To attach the plasmid to the E-R Au NWI, the surface of the Au NW was modified with cysteamine (CA). The plasmid can bind electrostatically to the amine group because the plasmid has a (−) charge originating from the phosphorous backbone, whereas the amine group exhibits a (+) charge in neutral solution. The attached plasmid can be released selectively by applying a −0.8 V electrical stimulus to the E-R Au NWI for 2 min. This electrical stimulation induces an electrochemical reaction, the reductive desorption of thiol ($Au\text{-}S\text{-}R + H^+ + e^- \rightarrow Au + HS\text{-}R$) [65–70], enabling the detachment of the plasmid from the surface of the E-R Au NWI. During the application of the electrical stimulus to an E-R Au NWI interfacing with a pSSC, no electrolysis of the surrounding water was observed and only a minute current (−0.9 ~ −0.4 nA) was detected between the E-R Au NWI and the counter electrode (Fig. S2) [71].

Fig. 1a depicts an optical image of an E-R Au NWI inserted into a single pSSC. The E-R Au NWI interfaced well with the pSSC. We clearly confirmed that the Au NW had entered the pSSC nucleus through the change in focal plane (Fig. 1b). Moreover, the morphology and membrane integrity of the pSSC were shown to be preserved after the insertion of the E-R Au NWI (Fig. 1c,d and Fig. S3). We further investigated the cell damage in the pSSCs by varying the diameter of the E-R Au NWI. When the diameter of the E-R Au NWI was 278 nm, the cell was viable, and no damage was induced after injection and the application of an electrical stimulus for 2 min (Fig. S4a-c). When thicker E-R Au NWI (325 nm) was used, a cytosolic leak occurred after injection and the application of an electrical stimulus for 1 min (Fig. S4d-f). An E-R Au NWI of 594 nm resulted in severe cell damage after injection and the application of an electrical stimulus for 30 s (Fig. S4g-i). Furthermore, Table S2 and S3 demonstrate that the usage of E-R Au NWI with a diameter of less than 300 nm in the transfection of a pSSC induced the lowest morphological abnormality and the highest transfection efficiency compared to those with a diameter of 300 nm or more. Based on this result, we employed E-R Au NWIs with diameter thinner than 300 nm to transfer plasmids into pSSCs without causing detrimental damage.

Fig. 2a–d shows fluorescence images of pSSCs 1 day after plasmid delivery using an E-R Au NWI. Blue fluorescence derived from Hoechst33342 was observed in the nucleus of the pSSC with red

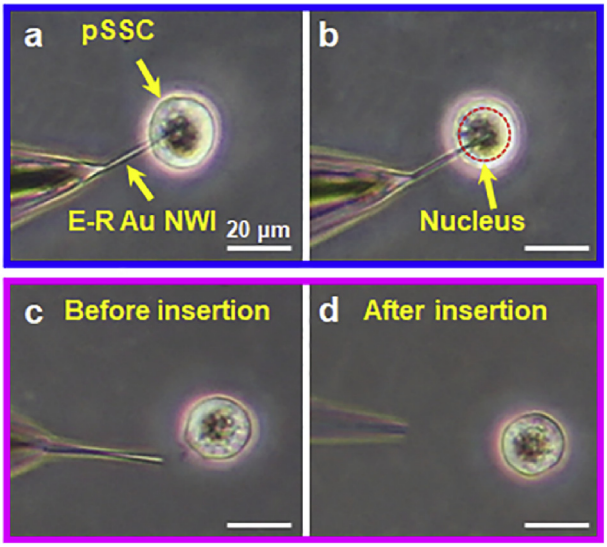


Fig. 1. (a) Optical image of E-R Au NWI inserted into a single pSSC. (b) Optical image of E-R Au NWI inserted into the nucleus of a pSSC. (c,d) Optical images of pSSC (c) before and (d) after insertion of an E-R Au NWI. The morphology and membrane integrity of the pSSC were preserved after insertion of the E-R Au NWI. The scale bars indicate 20 μm.

fluorescence of GFRα1 (pSSC-specific marker) on the cell surfaces after the immunocytochemistry was performed, and green fluorescence from enhanced green fluorescence protein (EGFP) was observed in the cytoplasm of the pSSC, indicating expression of the delivered gene. Fig. 2e–h shows fluorescence images of pSSCs that did not undergo plasmid delivery. The blue fluorescence of Hoechst33342 and red fluorescence of GFRα1 were observed with no green fluorescence. These results indicate successful E-R Au NWI-based gene delivery into a pSSC. In addition to confocal microscopy analysis, we performed polymerase chain reaction (PCR) and agarose gel electrophoresis analysis after the extraction of total RNA from transfected pSSCs and the synthesis of complementary DNA (cDNA) (Fig. 2i). Transcripts of the EGFP gene (736 bp) were observed in all 6 pSSCs transfected by E-R Au NWIs. If the EGFP gene-encoded plasmids were not transfected into the pSSC genome, the DNA-based plasmids would be fragmented by the DNase used in the cDNA synthesis process, resulting in no detection of EGFP transcripts in the PCR analysis [72]. The fluorescence and PCR results clearly verified that the efficient genetic modification of pSSCs using E-R Au NWIs is possible. Meanwhile, we found that the EGFP fragment could be observed even in pSSCs with a slight cytosol leak caused by plasmid delivery via a rather thick (> 300 nm) E-R Au NWI (Fig. S5). This result suggests that pSSCs can spontaneously recover from a slight cytosol leak induced by an E-R Au NWI. If the physical impacts resulting from thicker E-R Au NWI diameters stimulated the active destruction of pSSC microstructures during the transfection process, necrosis or apoptosis would be observed in the transfected pSSCs, and no EGFP transcripts would be detected by PCR analysis [73–75].

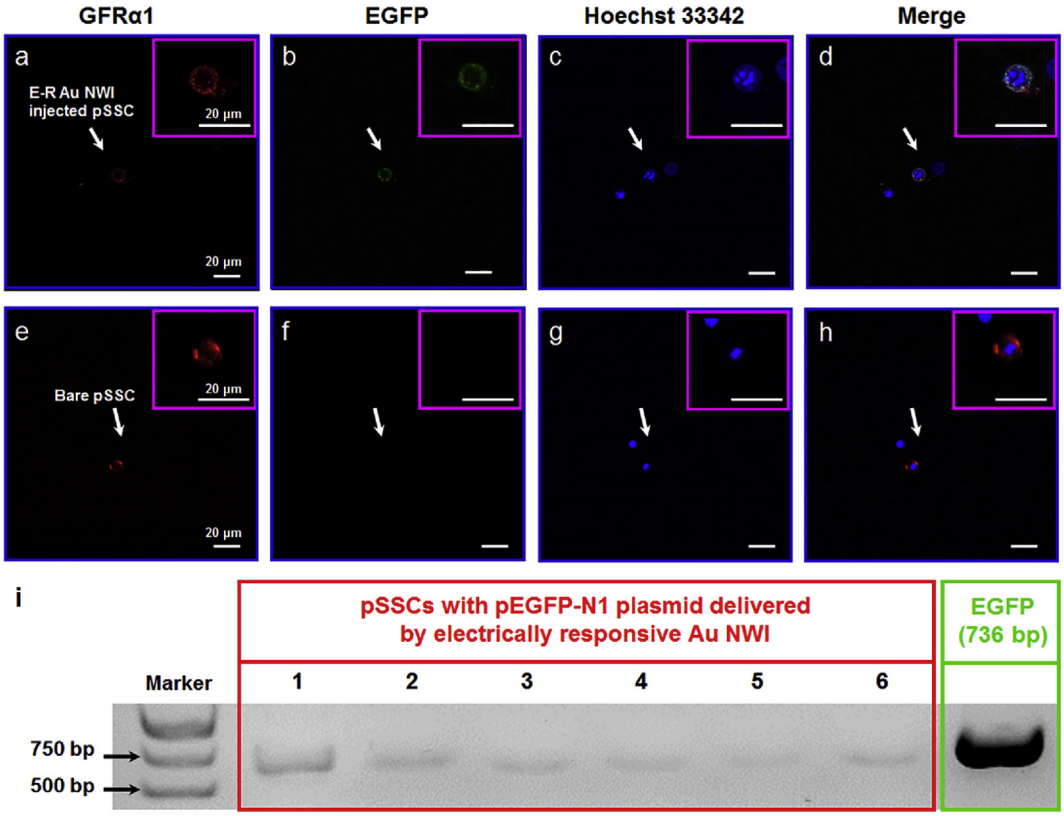


Fig 2. (a–d) Fluorescence image of pSSCs (arrow) obtained 1 day after plasmid delivery using E-R Au NWIs. Insets are magnified images. Blue fluorescence from DNA staining with Hoechst33342 was observed in the nucleus of pSSCs with red fluorescence of GFRα1 (pSSC-specific marker) on the cell surfaces after the immunocytochemistry was performed, and green fluorescence from EGFP was observed in the cytoplasm of pSSCs. The scale bars indicate 20 μm. (e–h) Fluorescence images of pSSCs without plasmid delivery. Insets are magnified images. Only the expression of GFRα1 was detected in pSSCs without expression of EGFP. The scale bars indicate 20 μm. (i) PCR and gel electrophoresis analysis of 6 pSSCs after plasmid delivery using E-R Au NWIs. The transcripts of EGFP gene (736 bp) were observed in all 6 pSSCs transfected by E-R Au NWIs.

3.3. Comparison of an E-R Au NWI-based gene delivery system with conventional nonviral gene delivery systems in the pSSCs transfection efficiency

Next, we compared the pSSC transfection efficiency of the E-R Au NWI with that of conventional nonviral transfection methods. First, we tried to transfect pSSCs by using an electroporation method. For this purpose, pSSCs in Hanks' balanced salt solution were transferred to a Gene Pulser cuvette, and the pEGFP-N1 plasmids were delivered to the cytoplasm of the pSSCs by the application of a single electric pulse (275 V and at a capacitor setting of 500 μF). After plasmid delivery, the pSSCs were cultured in a culture dish coated with testicular stromal cells and containing pSSC culture medium (pSSCCM) for 24 h at 31 °C. Second, pSSCs were transfected by using Lipofectamine 2000. Lipofectamine-DNA complexes were prepared by dissolving pEGFP-N1 plasmids and Lipofectamine 2000 in Opti-MEM medium and gently mixing for 30 min at room temperature. Then, pSSCs were treated with the prepared Lipofectamine-DNA complexes at 31 °C for 6 h, and the transfected pSSCs were stabilized in pSSCCM for 24 h. Third, pSSCs were transfected with jetPEI. The jetPEI-DNA complexes were prepared by gently mixing pEGFP-N1 plasmids with jetPEI for 30 min at room temperature. Similar to the Lipofectamine-based transfection method, pSSCs were treated with jetPEI-DNA complexes at 31 °C for 6 h and stabilized in pSSCCM for 24 h. Lastly, plasmids were delivered into the nuclei of pSSCs by using E-R Au NWIs, as depicted in Scheme 1. Each method was independently repeated at least three times. The pSSC transfection efficiencies of the electroporation, Lipofectamine, and jetPEI methods were calculated by counting the EGFP-expressing pSSCs 24 h after plasmid delivery. The pSSC transfection efficiency of E-R Au NWI was determined by the PCR analysis of intracellular EGFP messenger RNA (mRNA) after 24 h of gene delivery.

The EGFP expression of pSSCs transfected by conventional nonviral gene-delivery techniques: electroporation, and cationic lipid- (Lipofectamine) and polyethylenimine- (jetPEI) mediated chemical transfection methods was observed with a confocal microscope (Fig. S6). Unlike the obvious EGFP expression in the pSSCs transfected by E-R Au NWI, the expression of EGFP in pSSCs transfected by Lipofecamine- and jetPEI-mediated transfection was not absolutely observed under the confocal microscope. Justly, the extremely low level of EGFP from their cytoplasm was detected through the flow cytometer (Fig. S7). Even though the electroporation for pSSC transfection showed higher transfection efficiency than pSSCs transfected by other chemical transfection methods, it was still difficult to observe the pSSCs expressing EGFP on their cytoplasm under the confocal microscope because of extremely low EGFP intensity.

Fig. 3 presents the quantitative transfection efficiencies of pSSCs for each gene delivery method. Surprisingly, E-R Au NWI showed 46.7 times higher transfection efficiency than Lipofectamine reagent (56% vs 1.2%), 31 times higher efficiency than the jetPEI reagent (56% vs 1.8%), and 6.7 times higher efficiency than the electroporation method (56% vs 8.3%). Lipofectamine- and polymeric carrier-mediated gene delivery methods are difficult to use in pSSCs [76–78] because they require cell division after gene delivery [78] but no culture system for the effective proliferation of pSSCs has yet been established. Electroporation can modify the genome of pSSCs without proliferation process. However, the weak cell membrane of pSSCs is readily disrupted by a strong electrical stimulus [79], resulting in the induction of cell apoptosis immediately after transfection [80]. The E-R Au NWI is a promising technique that can overcome the limitations of typical chemical/physical gene delivery systems. The E-R Au NWI can approach the nucleus of pSSCs without damages and can deliver plasmids selectively, improving the transfection efficiency of pSSCs. The successful achievement of highly efficient pSSCs transfection by E-R Au NWI could be a new breakthrough in the genetic transformation of pSSCs and the future production of transgenic pigs.

Notably, the pSSC transfection efficiencies of conventional nonviral transfection methods are significantly lower than the previously reported transfection efficiency of chicken SSCs [81]. This difference between chicken SSCs and pSSCs may be attributed to the high susceptibility of pSSCs to chemical and physical stimuli, so that this result suggests that the efficiency of plasmid delivery can vary by species even for the same cell type.

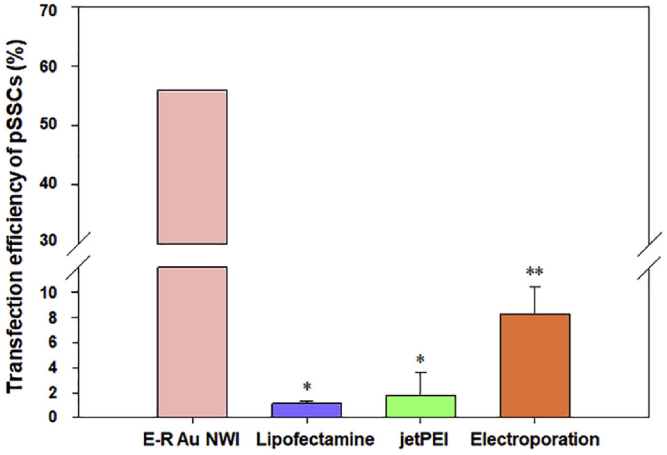


Fig. 3. Quantitative transfection efficiencies obtained by transfecting pSSCs by the E-R Au NWI, Lipofectamine, jetPEI, and electroporation methods. Each method was independently repeated at least three times. Data represent the mean plus standard deviation from each method. n = 3. *, **$P < 0.05$.

3.4. Production of transgenic pSSCs applicable to production of bone regenerative medicines-manufacturing animals using an E-R Au NWI-based gene delivery system

Finally, we tried to produce pSSCs that are capable of expressing hBMP2 by using E-R Au NWIs. The BMPs have been known as bone growth factors that could form bone and cartilage [82–84]. Since the hBMP2 is marketed by the Food and Drug Administration as a biosimilar, the hBMP2-expressing pSSCs can be used in biopharmaceutical applications [85,86]. Fig. 4a shows a schematic illustration of the vector designed for hBMP2 expression. The pUPKII promoter was inserted to express hBMP2 only in the bladder of transgenic pigs. Since confirming the genetic modification of pSSCs with the pUPKII promoter alone is difficult, a poly A signal and SV40 promoter were inserted between hBMP2 and the EGFP gene. The designed hBMP2 plasmids

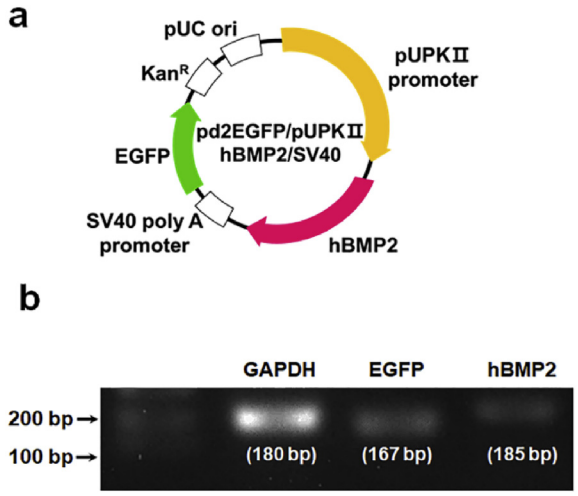


Fig. 4. (a) Schematic illustration of the vector designed for hBMP2 expression. (b) PCR and gel electrophoresis analysis of pSSCs after plasmid delivery using E-R Au NWIs. The hBMP2 gene, EGFP gene, and GAPDH gene were detected.

were electrostatically attached to E-R Au NWIs and delivered into the nuclei of pSSCs. One day after hBMP2 plasmid delivery, we extracted the genomic DNA of transformed pSSCs and performed PCR and gel electrophoresis analysis to verify the integration of the exogenous gene. Fig. 4b shows the PCR and gel electrophoresis analysis results, revealing that the hBMP2 gene was inserted into the genomic DNA of pSSCs. The amplified EGFP gene (167 bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (180 bp) were also detected. The GAPDH gene is a housekeeping gene and was used as an internal control. The results demonstrate that the functional gene modification of pSSCs by E-R Au NWI is feasible. To advance this study, we will try to differentiate the transfected pSSCs into sperm cells and produce transgenic pigs.

4. Conclusion

Spermatogonial stem cells are a promising gene carrier for the production of transgenic pigs due to their abilities of infinite self-renewal and of differentiation into sperm cells through spermatogenesis, enabling the transfer of genetic heritability to the next generation. In this study, gene delivery into pSSCs based on a 1-dimensional nanomaterial was successfully achieved and reported for the first time. The E-R Au NWI readily formed a noninvasive interface with the pSSC nucleus, and a plasmid was delivered without cell damage by the application of an electrical stimulus. Comparing the pSSC transfection efficiency with that of conventional nonviral gene delivery systems including Lipofectamine, jetPEI and electroporation revealed that the E-R Au NWI can greatly improve the transfection efficiency of pSSCs. Additionally, we successfully delivered hBMP2, which is known as a biosimilar, into pSSCs by using E-R Au NWIs and confirmed its genomic integration. These results indicate the feasibility of the E-R Au NWI-based gene delivery method for biopharmaceutical applications. Many issues remain in the production of transgenic pigs from transfected pSSCs. A high priority is to enhance the transfection efficiency of pSSCs. In this regard, the E-R Au NWI-based pSSC transfection technique can bring us one step closer to the facile production of transgenic pigs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biomaterials.2018.12.005.

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