Suppressing mosaicism by Au nanowire injector-driven direct delivery of plasmids into mouse embryos

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A B S T R A C T

Transgenic animals have become key tools in a variety of biomedical research areas. However, microinjection commonly used for producing transgenic animals has several challenges such as physical and chemical damage to the embryos due to microinjector with buffer, and low transgene integration rates with frequent mosaicism. Here, we report direct delivery of plasmids into mouse embryos using a Au nanowire injector (NWI) that significantly improved transgene integration efficiency and suppressed mosaicism. The Au NWI could deliver plasmid into the pronucleus (PN) of a mouse zygote without buffer and rapidly release it with electric pulse. Because zygote, which is a fertilized 1-cell stage embryo, has two physical barriers (cytoplasmic membrane and zona pellucida), direct delivery of plasmids into PN of zygote is more difficult than into a normal cell type. To penetrate the two physical barriers with minimal disruption of the embryo, we optimized the diameter and length of Au NWI. The mosaicism is more reduced in the Au NWI injected embryos than in micropipette injected embryos, which was determined by the expression of transgene in a blastocyst stage. We suggest that Au NWI can increase the efficiency of gene delivery into zygote with suppressed mosaicism and become a useful alternative.

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1. Introduction

Transgenic animals, subjected to the artificial introduction of a transgene, are widely used in a biomedical research. The transgenic animals can be used to study the functions of transgenes and to provide models of various human diseases, including cancer, diabetes, heart disease, obesity, and Alzheimer’s disease [1–7]. They can also become useful bioreactors to produce high-value biomaterials, such as proteins and peptides [8–10]. The transgenic animals are primarily generated using artificial gene introduction via microinjection, although retrovirus-mediated gene transfer and embryonic stem cell-mediated gene transfer are also commonly used [11–13]. The efficiency of transgenic animal production is, however, quite low because of low embryo viability rates, low transgene integration rates, complex experimental processes, and, most importantly, frequent mosaicism in the resulting transgenic animals [14–18].

Mosaicism is one of the primary issues in transgenic animal production, indicating the presence of two or more populations of cells with different genotypes in one individual [19]. The occurrence of mosaicism in an embryo indicates that the delivered transgene is expressed only in a subset of cells; thus, generate the mosaic transgenic animal and the transgene may not be transmitted to the next generation [20,21]. Therefore, mosaicism hampers the efficient production of transgenic animals. It has been known that the mosaicism may occur when the transgene is...
integrated after the 1-cell stage of an embryo [20]. As transgene integration is difficult to predict or control using conventional gene transfer methods, mosaicism frequently occurs in the transgenic animals. Consequently, the development of gene transfer method for efficient integration of transgene into embryos without occurrence of mosaicism is currently one of the major challenges in the production of transgenic animals [22,23].

Recently, various nanomaterials including nanoparticles, nanorods, nanowires that interface with living cells or organisms have substantially advanced the analysis and/or control of biological events, nanowires that interface with living cells or organisms have been used as powerful delivery tools for delivering therapeutic DNAs or drugs with increased transfection efficiency to advance regenerative medicine [24–26]. In particular, one-dimensional (1D) nanomaterials have been widely used to efficiently transport electrical and optical signals, stimuli, and biological cargo into living cells with minimal invasiveness and with nanoscale spatial resolution through the formation of nano-sized functional biointerfaces [28–32]. 1D nanomaterials have also been used to detect electrical signals, chemicals, or biomolecules from cells [33–36]. For example, VanDersarl et al. reported the nanostraw–based delivery of molecules, ions, and plasmids into cells. Robinson et al. reported nanowire (NW) electrode arrays that interfaced with neurons to stimulate and record neuronal activity. Yan et al. demonstrated that NWs can transport light into the intracellular compartments of a cell [37–39]. We previously reported the use of a Au NW nanoinjector to precisely deliver DNA into the nucleus of a mammalian cell [40]. Single-crystalline Au NWs have superb characteristics that positively distinguish themselves from other nanomaterials; i) superelasticity and super strength, ii) well-defined geometry with atomically flat Au (111) surfaces, iii) extremely narrow diameters, iv) high biocompatibility, and v) excellent electrical conductivity [35,40,41]. These exceptional properties make a Au NW injector (NWI) an ideal gene transfer tool for the generation of transgenic mouse embryos.

Here, we report the direct delivery of plasmids into a mouse embryo using a Au NWI for mosaicism-free transformation. The plasmid–loaded Au NWI was injected into the pronucleus (PN) of a mouse zygote (the 1-cell stage embryo of a fertilized egg), and the plasmid was released by applying an electric pulse to the Au NWI. Because zygotes have two physical barriers, namely, the cytoplasmic membrane and the zona pellucida (ZP), the direct delivery of plasmids into the 1-cell stage of embryos is more difficult than in mammalian cells. We optimized the diameter and length of the Au NWI to penetrate the ZP with minimal invasiveness and to precisely reach the PN. The optimized Au NWI successfully delivered transgenes into embryos, and transgene expression was clearly verified by polymerase chain reaction (PCR) analysis and fluorescence (FL) measurements. Importantly, the Au NWI increased the transgene integration efficiency and reduced mosaicism at the blastocyst (BL) or morula (M) stage of embryos compared to the microinjection.

These improvements reflect two critical advantages of Au NWIs. First, Au NWIs are sufficiently rigid to penetrate the ZP but sufficiently thin and superelastic to avoid the destruction of the nucleolus which is in the PN. The exceptional mechanical properties of Au NWIs help maintain the membrane integrity of embryos and minimize damage to embryo viability and development [42]. Second, Au NWIs can deliver plasmids directly into the 1-cell stage of embryos upon an electric pulse without the need for extracellular buffer. By contrast, the microinjection method necessarily transfers plasmids with buffer solution into embryos. Notably, the buffer solution adversely affects embryo development and transgene genomic integration [43,44]. Taken together, Au NWIs can minimize the physical and chemical damage to zygotes during gene transfer, thereby improving transgene integration efficiency. Most importantly, Au NWIs enable plasmid to integrate into a 1-cell embryo without delay, largely suppressing the occurrence of mosaicism [21,45,46]. Moreover, this is the first report on reducing mosaicism by using nanomaterials for delivering transgene into the 1-cell embryo by confirming expression of the GFP at M- or BL-stage embryos in vitro. Thus, the use of Au NWIs would make transgenic animal production more accurate and efficient. We suggest that Au NWIs will extend the application of transgenic animals to achieve significant advances in related studies. This work indeed shows the potential for critical improvement by introducing nanotechnology into a biological system.

2. Materials and methods

2.1. Fabrication of plasmid-attached Au NWIs

Au NWs were synthesized using a previously reported vapor transport method [47]. To fabricate a Au NWI, a W tip mounted on a piezoelectric stage (Sigma Koki, Japan) was used. The tip was brought close to a single Au NW grown on a sapphire substrate, and the Au NW was attached to the W tip by van der Waals attraction. Next, the entire W tip was insulated with a UV-curing polymer and nail varnish to prevent the interruptive electrochemical reactions, exposing only the Au NW. For plasmid attachment, the Au NWI was incubated in a 20 mM cysteamine (CA, HSH2CH2NH2) solution for 30 min, and excess CA was washed with distilled water (DW). The CA-modified Au NWI was then incubated overnight with 100 nM phMGFP plasmid in 1 M KH2PO4 at room temperature. After plasmid attachment, excess plasmids remaining on the Au NWI were washed off with DW.

2.2. Preparation of phMGFP plasmids and transfection into 293T cells

phMGFP vector (E6421; Promega, USA) was amplified via heat shock transformation of DH5α (18263012; Life Technology Co., USA). Plasmid DNA was prepared according to the protocol provided in the manual (MACHEY-NADEL, Germany). The DNA concentration was measured using a NanoDrop spectrophotometer (Thermo, USA). Transfection into 293T cells was performed using Metafectene PRO (T040-1.0; Biontex, Germany). Transfection was detected using green fluorescent protein (GFP) FL on a fluorescence microscope (Leica, Germany) after 2 days of treatment.

2.3. Mouse embryo preparation and incubation

To induce superovulation, female C57BL/6 mice over 4 weeks of age were treated with pregnant mare serum gonadotropin (PMSG) (G-4877; Sigma, USA). After 48 h, human chorionic gonadotropin (hCG) (CG-5; Sigma, USA) was injected. The female mice were then individually housed with C57BL/6 males. The next morning, the females were examined for the presence of a vaginal plug, and those with a vaginal plug were sacrificed via cervical dislocation. The oviduct was then exposed, and the embryos were collected after being flushed from a swollen section of the oviduct. The cumulus cells were removed via exposure to 300 mg/mL hyaluronidase (H3884; Sigma, USA) and washed with M2 medium (M7167; Sigma, USA). The collected embryos were incubated in M16 medium (M7292; Sigma, USA) overlaid with mineral oil at 37 °C and 5% CO2 until Au NWI- or micropipette-based injection was performed. All animals were maintained according to the guidelines of an approved protocol from the Institutional Animal Care and Use Committee (IACUC) of the KAIST (Republic of Korea).
2.4. Delivery of plasmids into embryos via Au NWI or micropipette

Mouse embryos were placed in a 35 mm cell culture dish containing M2 medium, and the dish was placed on the stage of an optical microscope (DMI 3000B) (Leica, Germany). For Au NWI injection, a single 1-cell stage of embryo was transferred to M2 medium. The embryo was held with a conventional glass micropipette (Micro-hematocrit capillary tubes, plain) and manipulated using a conventional microinjection system (Sutter Instruments, USA) mounted on a Leica micromanipulator for successful PN injection. A Au NWI connected to an electrochemical workstation (CHI 660D) (CH Instruments, USA) was then lowered into the medium until reaching the same focal plane of the embryo. For electro-triggered plasmid release from the Au NWI, the reference electrode and counter electrode were also immersed in M2 medium. The Au NWI was then penetrated into the ZP, cytoplasmic membrane, and PN. After insertion of the Au NWI into the PN, a −0.8 V electric pulse was immediately applied for 2 min to trigger plasmid detachment from the Au NWI. The plasmid-delivered embryo was subsequently transferred to another cell culture dish filled with M16 medium and placed under the lens of a Leica MZ16 microscope and incubated at 37 °C with 5% CO2 for 4 days for subsequent development and further analysis. For micro-injection, phMGFP plasmids were prepared by diluting the plasmid stock to a final concentration of 5 ng/μL in microinjection buffer (1 M KH2PO4). The micropipettes were prepared with borosilicate glass capillaries (Sutter Instruments, USA; inner diameter: 0.75 mm, outer diameter: 1.0 mm) and pulled using a pipette puller (P-97) (Sutter Instruments, USA). A holding pipette was polished with a microforge (Alcatel, France). Microinjection was conducted in M2 medium under mineral oil. Several picoliters of 5 ng/μL plasmids were injected until slight swelling of the injected PN was observed. Both Au NWI injection and microinjection were performed at room temperature.

2.5. SEM and FL imaging

Scanning electron microscopy (SEM) images of Au NWIs were obtained using a FEI Nova 230. The FL images of embryos at M- or BL-stage were acquired on a confocal microscope (Carl Zeiss, Jena, Germany) installed at the Korea Basic Science Institute (Daejeon, Korea). To obtain the FL images, photographs were taken using a Carl Zeiss LSM 710 with excitation and emission filters of 488 nm and 598 nm, respectively.

2.6. PCR analysis

Embryos were transferred to PCR tubes containing 20 μL of medium and boiled at 99 °C to expose the plasmid and genomic DNA from the embryos. PCR was then performed using the isolated genomic DNA. Primers specific to the phMGFP plasmid were designed using Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0; forward: 5'-AGCCCTAGGACGACAGT-3' and reverse: 5'-GCTGCTGTCAGCTGGTCTTGC-3'). The general reaction mixture for PCR utilized EX Taq polymerase (RR001A; TaKaRa, Japan) and PCR was carried out on a PCT-100 instrument (Bio-Rad, USA). The PCR products were detected by agarose gel electrophoresis on 1.2% agarose gels and observed using RedSafe (21141; Intron, Korea) on a Gel Doc XR+ System (Bio-Rad, USA).

The qPCR analysis was performed using an ABI Prism 7000 sequence detection system (Applied Biosystems, USA) with SYBR Green PCR Master Mix (4309155; Applied Biosystems, USA). To quantify the amount of phMGFP plasmids delivered into embryos, we compared phMGFP-loaded Au NWIs before and after the application of an electric pulse. Several concentrations of phMGFP (0.01, 0.05, 0.1, 0.5, and 1 ng) were prepared as standards. For realtime PCR, the threshold cycle (CT) was defined as the fractional cycle number at which the amount of amplified target reached a certain threshold. The amount of phMGFP plasmids in each group was then calculated using the CT based on those of the standards. Each test was performed in triplicate. By using CT value of various concentrations of phMGFP plasmid, a standard curve formula was made for estimating the plasmid concentration in each sample.

\[
\text{phMGFP plasmid (ng)} = 64.874e^{-0.6(C_T \text{ value})}
\]

2.7. Statistical analysis

GraphPad Prism 5 (GraphPad Software, USA) was used to perform statistical analysis. All results were expressed as the mean ± standard error of the mean. According to post-hoc testing using Bonferroni analysis, comparisons between two groups were conducted using unpaired t-tests or analysis of variance (ANOVA). Differences with P values of less than 0.05 were considered significant.

3. Result and discussion

Scheme 1 illustrates the delivery of a plasmid into a 1-cell embryo using a Au NWI. To facilitate transgene loading, the surface of the Au NW was modified with CA. As shown in Scheme 1a, CA binds to Au NWs through a Au−S bond. Because the CA−attached Au NWI provides positively charged amine groups (NH3+), neutral solution, the negatively charged plasmids are electrostatically attracted to the Au NWI [48−50] (see Figs. 1 and 2 in Ref. [51]). The plasmid-loaded Au NWI is then injected into the PN of an embryo without destroying the nucleolus, and an electric pulse is applied to the Au NWI to release the plasmid (Scheme 1b). Approximately 4 days after transgene delivery, the 1-cell embryo develops to the M- or BL-stage. At this stage, we used FL imaging to examine whether plasmid-derivd GFP was expressed in all blastomeres.

3.1. Fabrication of Au NWIs and confirmation of phMGFP plasmid expression in cells

Au NWIs were fabricated by attaching a single Au NW to a W tip and subsequently insulating the W tip with a UV-curable polymer and nail varnish. Fig. 1a shows a SEM image of a Au NWI before insulation. The vapor-phase-grown Au NW has well-defined facets and an elongated octahedron structure with a rhombic cross-section ( inset of Fig. 1a) [47]. The Au NW is several hundred nanometers in diameter and the tip displays a half-octahedron shape. Fig. 1b presents an optical image of a Au NWI after insulation. The insulating layer is clearly observed. The insulation process prevents undesirable chemical and electrochemical reactions with the W tip.

The structure of the phMGFP plasmid (4707 bp) is illustrated in Fig. 1c. The plasmid contains a GFP-encoding fragment, enabling the easy confirmation of transgene expression. We validated the plasmid using 293T cells with Metafectene PRO (transfectant). Fig. 1d shows the merged FL and optical image of 293T cells after phMGFP plasmid transfection. GFP-expressing cells are clearly observed, suggesting that the phMGFP plasmid can be used to examine the successful delivery of plasmids into a zygote using a Au NWI. Following the Au NWI delivery of phMGFP plasmids into a 1-cell stage of embryo, if the plasmids successfully integrate into the host genome, GFP expression will be observed in all blastomeres at the M- or BL-stage [45,52].
3.2. Optimization of Au NWIs for injection into the PN of mouse embryos

To integrate a transgene into the host genome while maintaining its stability in the cell, the transgene should be delivered directly into the nucleus \([53^{−57}]\). If transgenes are unloaded in the cytoplasm instead of the nucleus, they may be degraded by various enzymes and metabolic mechanisms \([58^{−61}]\). This effect greatly lowers the penetration rate of transgenes into the nuclear membrane and thus decreases the transgene integration efficiency. Directly delivering plasmids into zygote is particularly difficult because zygotes have both a ZP and a cytoplasmic membrane. As single-crystalline Au NWs are super strong and superelastic, Au NWIs can effectively penetrate into 1-cell stage of embryos. For the precise unloading of plasmids into the PN of mouse zygotes, we optimized the length and diameter of Au NWIs. A mouse zygote is \(\sim 100\) \(\mu\)m in diameter; therefore, the optimal length of an Au NWI to reach the PN would be \(20^{−50}\) \(\mu\)m \([62]\). Furthermore, for the Au NWI to penetrate the ZP of a zygote, the Au NWI should be mechanically strong. The mechanical strength of the Au NWI is primarily determined by its diameter. We fabricated several Au NWIs with a broad range of diameters by adjusting the synthetic
conditions and then tested the ability of these Au NWIs to insert into zygotes. Fig. 2a shows that a thin, 170 nm Au NWI slides over the surface of the ZP and bends into an L-shape, probably due to insufficient mechanical strength. Fig. 2b shows that a thick, 1.5 μm Au NWI penetrates the ZP but not the cytoplasmic membrane, reflecting the dissipation of the cutting force and resulting in insufficient mechanical force. SEM images of the Au NWIs in Fig. 2a and b are shown in Fig. 2e and f, respectively. We observed that ~500 nm Au NWI of is optimal for injection into the ZP and cytoplasmic membrane of an embryo. The entire procedure for Au NWI injection into the PN of a zygote is presented in Movie 1 in supplementary data and a representative image is shown in Fig. 2c. The SEM image of the optimized Au NWI is also shown in Fig. 2g. The optimized Au NWI can penetrate into the ZP and reach the PN of an embryo without bending or slipping. The sharp tip of the Au NWI, with its half-octahedron geometry, high mechanical strength, and thin diameter, enabled the successful penetration of the ZP and cytoplasmic membrane. Because the insertion force can be concentrated on the sharp tip, the Au NWI can efficiently pierce and enter the ZP and membrane [63]. Furthermore, the thin Au NWI forms only a small hole at the ZP and membrane of the zygote, which is easily sealed upon withdrawal of the Au NWI, maintaining the viability of the zygote [62]. More importantly, the Au NWI prevents the deformation of the nucleolus. The magnified image of Fig. 2c shows that the Au NWI passes by the nucleolus (red circles of Fig. 2d). Avoiding the destruction of the nucleolus during injection is critical to maintaining embryo viability [42]. Indeed, the thin and superelastic Au NWI used in the present study can move into the PN while effectively passing by the nucleolus.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2017.05.044.

3.3. Release of plasmids from Au NWIs using electric pulses and embryo viability test

Au NWIs can selectively and rapidly release plasmids upon the application of an electric pulse. To examine the release of plasmids from Au NWIs, we constructed an aqueous three-electrode system that could be observed by optical microscopy; the system consisted of a plasmid-loaded Au NWI (working electrode), a saturated calomel electrode (SCE, reference electrode), and a Pt wire (counter electrode) (see Fig. 3a in Ref. [51]). The optical image of the Au NWI immersed DW is presented (see Fig. 3b in Ref. [51]). Using this electrode system, we applied an electric potential of ~0.8 V to the Au NWI for 2 min to release the plasmids into the DW through the dissociation of the Au-S bonds. Next, PCR and gel electrophoresis were performed to detect the plasmids released from the Au NWI. A 218-bp fragment of the phMGFP plasmid was amplified using PCR.
and Fig. 3a shows representative PCR and gel electrophoresis results from phMGFP plasmids at 0, 0.1, and 1 ng. Fig. 3b depicts the PCR and gel electrophoresis results from the DW sample of the three-electrode system with and without the application of an electric pulse to the plasmid-loaded Au NWI. Only after the application of an electric pulse, the DW sample showed strong bands corresponding to PCR products. When the plasmid-loaded Au NWIs were dipped in DW for 2 min without the application of an electric pulse, no plasmid was detected. This result indicates that the plasmids were successfully released from the Au NWI only when the electric pulse was applied. By comparing the band intensities of the PCR products in Fig. 3a and b, the amount of plasmid released from a Au NWI was estimated to be less than 0.1 ng. We further investigated the amount of plasmid released from Au NWIs using quantitative real-time PCR (qPCR). Two plasmid-attached Au NWIs were prepared, after which one Au NWI was triggered to release the plasmids while the other remained intact (see Fig. 4 in Ref. [51]). By analyzing the amount of plasmid released from these two Au NWIs, we validated that a single Au NWI injected ~0.014 pg of plasmid into a zygote (see Fig. 5 in Ref. [51]). It is important to deliver an optimal amount of plasmid into a zygote because injecting an excessive amount of transgenes can reduce 1-cell stage of embryo viability and hamper development [44,64,65]. Our experimental result suggests that the amount of plasmid delivered by the Au NWI does not affect the development of embryo from 1 cell to morula/blastocyst.

Next, for efficient transgenic animal production, the viability of 1-cell stage of embryos after the gene delivery procedure is critical. We investigated whether Au NWI injection and electric pulse application altered the viability of embryo. First, we injected a bare Au NWI (without plasmids) into a 1-cell stage of embryo and removed it without electric pulse application. Five Au NWIs were used to inject, and the diameters and lengths of the Au NWIs are presented in Table 1 in Ref. [51]. After injection of the Au NWIs, the 1-cell stage of embryos were incubated for 3 days, and we observed that all embryos developed normally to the M stage (see Fig. 6 in Ref. [51]). Second, we examined whether the application of an electric pulse affects the development of 1-cell stage embryo to 2-cell stage. A total of 59 zygotes were injected with Au NWIs, and the plasmids were delivered into the PN of zygotes via the application of an electric pulse. Of the 59 zygotes, 54 were viable and successfully developed into 2-cell embryos after Au NWI-based plasmid delivery (92%, see Table 2 in Ref. [51]). Only 2 zygotes were degraded, and 3 zygotes showed 1-cell block after plasmid delivery. The high survival rate after using a Au NWI suggests that a Au NWI reduces physical and chemical damage to the zygotes. Thin and superelastic Au NWIs can maintain the membrane integrity of the 1-cell embryo and protect the nucleolus from damage. Moreover, the minuscule amount of current (1−10 nA) flowing between the Au NWI and the counter electrode during the pulse application did not disturb the cellular signaling system for embryonic development, nor did it physically damage the embryos [66]. Additionally, the Au NWI causes minimal chemical damage to the embryos because it delivers plasmids without any buffer solution, which adversely affects embryo development and the genomic integration of transgenes [43,66]. We verified that Au NWIs can deliver plasmids into the PN of zygotes with minimal damage and that 92% of plasmid-delivered embryos developed to the 2-cell stage. It indicates that Au NWIs reduce the embryo loss incurred by the injection process, which is one of issue in transgenic animal production.

3.4. Efficient delivery of plasmids into the PN of 1-cell stage embryos using Au NWIs

To deliver plasmids directly into the 1-cell stage of embryos using Au NWIs, we placed SCE and Pt wire in medium including an embryo and captured the embryo using a standard holding pipette. After placing a plasmid-loaded Au NWI in the medium and properly adjusting its position under optical monitoring, we injected the Au NWI into the PN of the embryo. Next, a −0.8 V electric pulse was applied for 2 min to release the plasmids into the PN of the embryo, and the Au NWI was withdrawn from the embryo. The transgene-delivered embryo was individually incubated in a droplet of M16 medium covered with mineral oil for 3−4 days at 37 °C in an atmosphere containing 5% CO2. After the development of the embryo to the M- or BL-stage, the FL signals were measured. If the phMGFP plasmids delivered into the PN of the embryo were successfully integrated into the host genome at the 1-cell stage, and if the embryo developed normally, then GFP expression should be observed in all blastomeres [45]. However, if the phMGFP plasmids failed to integrate into the host genome, then the unintegrated plasmids would naturally degrade within a few days through an intracellular mechanism [11,67−69]. Thus, any GFP FL observed in embryos at the M- or BL-stage was most likely derived from genomically integrated phMGFP. Fig. 4 shows a representative z-stack image of a M-stage embryo after the delivery of phMGFP using a Au NWI. The step size was 2.6 μm. GFP FL was observed in all blastomeres, suggesting the successful production of a transgenic mouse embryo (see Movie 1 in Ref. [51]). To further verify the genomic integration of Au NWI-injected phMGFP, we analyzed the genomic DNA of the embryo at M- or BL-stage using PCR and gel electrophoresis (Fig. 5). Genomically integrated plasmids were detected only in embryos showing GFP FL after Au NWI injection. No plasmids were detected via PCR and gel electrophoresis in the embryos without GFP FL or in intact embryos. Notably, Au NWIs can deliver plasmids into the PN of an embryo and lead to successful genomic integration without plasmid linearization. Conventional gene transfer methods generally employ linearized plasmids to overcome the low efficiency of transgene integration, although this process is complicated and requires extra time and cost [21,44,65,70,71]. Au NWIs can deliver plasmids to embryo with an acceptable integration efficiency without plasmid linearization.

3.5. Suppressing of mosaicism using Au NWI-based plasmid delivery

It was reported that a nano-lance improved the embryo survival
rate and the efficiency of transgene integration compared to the microinjection [66]. However, not only improvement of survival rate but also suppression of mosaicism is a critical factor in the efficient production of transgenic animals. This study aimed to investigate the feasibility of a Au NWI in suppressing mosaicism by using the extent of GFP-expression in the blastocyst.

Mosaicism is one of the primary issues in transgenic animal production. Despite the successful delivery and integration of a transgene, mosaicism can still occur during embryonic development. Mosaicism typically occurs when the transgene is integrated after the 1-cell stage of an embryo [21]. When mosaic embryos are transferred into recipient female mice, the founders derived from

Fig. 4. Representative z-stack images of a M-stage embryo after the delivery of phMGFP plasmids using a Au NWI. The step size was 2.6 μm. GFP FL was observed in all blastomeres, suggesting the successful production of a transgenic mouse embryo.

Fig. 5. PCR and gel electrophoresis results from the phMGFP plasmids (gray) and genomic DNA of embryos at M- or BL-stage after Au NWI-based plasmid delivery (red) and from an intact embryo (green). A 218-bp fragment of the phMGFP plasmid was amplified by PCR. Prior to PCR analysis, the GFP FL of the embryos at M- or BL-stage was measured by confocal microscopy. The plasmids were detected only in the embryos that showed GFP FL after Au NWI injection. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
these embryos will exhibit mosaicism, hampering the efficiency of transgenic animal production [72]. To prevent mosaicism, the transgene should be immediately integrated into the host genome before the first chromosomal replication, resulting in transgene integration into the genome of every cell in the embryo. Fig. 6a and b shows FL and merged images of GFP-expressing M-stage embryos with/without mosaicism after Au NWI- or micropipette-based plasmid delivery, respectively. GFP FL was clearly observed in all blastomeres of the embryo after Au NWI-based gene delivery, whereas GFP FL was observed in only two blastomeres of the microinjected embryo.

We quantitatively compared the transgene integration efficiency and mosaicism rate of Au NWIs and micropipettes. Table 1 presents the number of GFP-expressing M+BL-stage embryos without (w/o) mosaicism, mosaic M+BL-stage embryos, and degraded or non-GFP-expressing embryos after Au NWI- and micropipette-based plasmid delivery. The transgene integration efficiency of Au NWIs was 53% whereas the transgene integration efficiency of microinjection was 25% (Fig. 7a). The efficiency was calculated by dividing the number of GFP-expressing M+BL-stage embryos by the number of plasmid-delivered embryos. We counted GFP-expressing embryos at the M+BL-stage and counted partially GFP-expressing embryos as successful plasmid integrations. Au NWIs doubled the transgene integration rate compared to conventional micropipettes. Importantly, the generation efficiency of GFP-expressing M+BL-stage embryos without mosaicism using Au NWIs was 5 times higher than that with micropipettes (37% vs 7%) (Fig. 7b). Au NWI-based gene delivery promoted transgene integration into the genome, leading to the expression of GFP in all blastomeres of M+BL-stage embryos with far greater efficiency than that achieved using micropipette-based gene delivery. Given that suppressing mosaicism is critical for the efficient production of transgenic animals, the dramatic enhancement of gene transfer efficiency without mosaicism using Au NWIs is highly impressive.

We hypothesize that the cause of this efficient transgenesis with suppressed mosaicism using Au NWI is as follows. First, the thin Au NWIs minimize the deformation of 1-cell embryos and the stress imposed on the embryos. Furthermore, this tool produces only a small hole in the ZP and cytoplasmic membrane during entry into the embryo. This hole can be readily sealed after removal of the Au NWI.

<table>
<thead>
<tr>
<th>Delivery tool</th>
<th>Number of GFP-expressing M+BL-stage embryos with/without mosaicism</th>
<th>Number of degraded or non-GFP-expressing embryos</th>
<th>Total number of plasmid-delivered embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au NWI</td>
<td>7 (with mosaicism) 3 (without mosaicism)</td>
<td>9</td>
<td>19</td>
</tr>
<tr>
<td>Micropipette</td>
<td>3 (with mosaicism) 8 (without mosaicism)</td>
<td>33</td>
<td>44</td>
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Fig. 6. (a) FL and (b) merged images of a GFP-expressing morula (M)-stage embryo after Au NWI-based phMGFP plasmid delivery. Clear GFP FL was observed in all blastomeres of the M-stage embryo. (c) FL and (d) merged images of a partially GFP-expressing M-stage embryo after phMGFP microinjection. GFP FL was observed in only two blastomeres of the embryo.

Fig. 7. (a) GFP-expression efficiency in M+BL-stage embryos (%) of Au NWI (magenta) and micropipette (blue). The efficiency was calculated by dividing the number of GFP-expressing M+BL-stage embryos with and without (w/o) mosaicism by the number of plasmid-delivered embryos. The GFP-expressing in M+BL-stage embryos with mosaicism was counted as successful plasmid integration. (b) GFP-expression efficiency in M+BL-stage embryos without (w/o) mosaicism (%) using Au NWI (magenta) and micropipette (blue). The efficiency was calculated by dividing the number of GFP-expressing M+BL-stage embryos without mosaicism by the number of plasmid-delivered embryos. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
In the present study, we introduced thin and superelastic Au NWIs with a well-defined single-crystalline structure for the delivery of exogenous genes into 1-cell stage of embryos, as the first step in the production of transgenic animals. The optimized Au NWIs readily penetrated the ZP and membrane of embryos with minimal damage and effectively avoided the destruction of nucleolus in the PN. Moreover, Au NWIs deliver only plasmids (without buffer) upon the application of an electric pulse, facilitating the efficient genomic integration of exogenous plasmids by avoiding the detrimental effects of buffer on embryo viability. These advantages of Au NWIs led to successful gene delivery into the PN with markedly reduced mosaicism. We verified that Au NWIs, which improve the occurrence of mosaicism, will eventually be the efficient tool compared with general methods for delivering gene into the 1-cell embryos. Next, to advance this study, we would employ considerably efficient genetic tools such as CRISPR/Cas9, which could be highly efficient in producing animal models in the Au NWI system [75]. Furthermore, a Au NWI could improve the production efficiency of transgenic animals by modifying elements of DNA in a plasmid and thus increasing the gene delivery efficiency without interfering host genome [76]. Employment of such powerful genetic tools in further studies could clearly verify the feasibility of Au NWI in transgenic animal production. 

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