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

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ABSTRACT

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.

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

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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 behaviors [24–27]. Moreover, nanomaterials 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 W 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, $HSCH_2CH_2NH_2$) 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 KH_2PO_4 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 (MACHEREY-NAGEL, 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% CO_2 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).