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# Microinjection free delivery of miRNA inhibitor into zygotes

## SUBJECT AREAS:

MIRNAS  
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The development of gene delivery systems into embryos is challenging due to technical difficulties, delivery efficiency and toxicity. Here, we developed an organic compound (VisuFect)-mediated gene delivery system for zygotes. The VisuFect, which is hydrophilic and Cy5.5-labeled, was conjugated with poly(A) oligo (VFA). The VFA into CHO cells showed clathrin-mediated internalization and no toxicity. The VFA successfully penetrated through the zona pellucida of fertilized eggs of various species including pigs, zebrafish, drosophilas and mice. The experiment with VisuFect-mediated delivery of the miR34c inhibitor showed similar results with direct microinjection of the miR34c inhibitor by suppressing the development of zygotes up to the blastocyst stage. Noticeable features of the VisuFect will provide great benefits for further studies on gene function in sperms and embryos.

Transgenic techniques have provided a great potential in studying the regulation and function of genes through the observation of inherited characteristics. However, gene delivery into an oocyte and an embryo has been a challenge due to presence of the zona pellucida, a glycoprotein membrane surrounding the plasma membrane of an oocyte and an embryo with a function as a barrier to exogenous materials<sup>1</sup>. Therefore, a successful gene delivery procedure into an oocyte and an embryo should minimize the potential inhibitory response of the zona pellucida. At present, considering the availability and efficiency to deliver foreign genetic materials including DNA and RNA into zygotes, the two most common methods are microinjection and electroporation<sup>2,3</sup>. However, disadvantages of both gene delivery systems including physical damage on the plasma membrane and high skills to operate the equipment result in low efficiency of gene delivery into zygotes<sup>4</sup>. Moreover, both microinjection and electroporation should safely deliver genes into a large numbers of zygotes at one time.

Recent advances in inorganic and organic nanoparticles including iron, gold, magnesium, liposomes, polymer, dendrimer and cationic lipid have enabled many of biologists to successfully deliver genetic materials including DNA, non-coding small RNA, mRNA and protein into cells<sup>5-9</sup>. Although noticeable features of these nanoparticles with easy access, low cost and high internalization into many cells at one time make them good candidates as gene delivery systems, they are limited to primary cells including stem cells, sperms and oocytes due to their high toxicity and low delivery rate<sup>10</sup>. Unfortunately, there have been no studies on the development of gene delivery systems into zygotes using these nanoparticles.

Therefore, we studied the development of organic compound-mediated gene delivery into zygotes. The organic compound (VisuFect) with favourable characteristics suitable as gene delivery system are highly hydrophilic helpful for better cell binding<sup>11</sup> and labelled with Cy5.5 that is good for tracking and visualizing bioconjugates in cells<sup>12</sup>. We investigated VisuFect-mediated delivery of short DNA oligonucleotides into primary cells including human embryonic stem (ES) cells, human fibroblast cells, mouse sperms and zygotes of various species.

## Results

To examine the feasibility of VisuFect for gene delivery into various primary cells, the VisuFect was first conjugated with a poly(A)<sub>50</sub> oligonucleotides (non-functional oligo, used as a control) at a molar ratio of 1:0.8 (designated as VFA). Bands with slight mobility shifts and fluorescence signals by gel electrophoresis confirmed the formation of the VFA (Supplementary Fig. 1). When various concentrations of the VFA were transfected into CHO (Chinese hamster ovary) cells, the MTT assay showed no significant reduction of cell viability (Supplementary Fig. 2). After conjugation of 25  $\mu$ M of the poly(A) with the VisuFect, the VFA was incubated



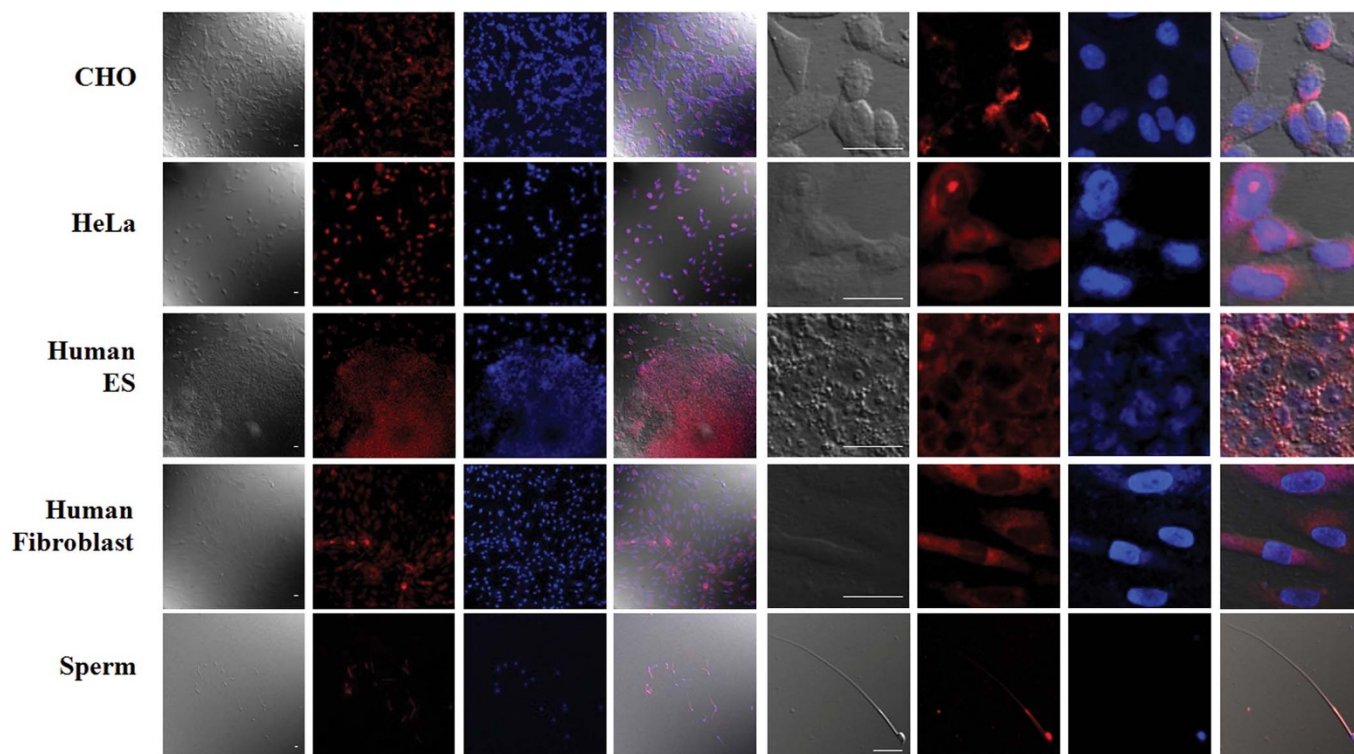
into various cells at 37°C for 12 hr. Confocal microscopy imaging at an excitation wavelength of 675 nm and an emission wavelength of 694 nm demonstrated strong fluorescence brightness in the cytoplasm of CHO and HeLa (human cervical cancer) cells (Fig. 1). Interestingly, most human ES and human fibroblast cells showed a great uptake of the VFA in the cytoplasm. Strong fluorescence signals of the VFA were detected in the head and midpiece of mouse sperms. Z-stack confocal images of CHO, HeLa, human ES, human fibroblasts and mouse sperms further confirmed the internalization of the VFA inside the cells (Supplementary Fig. 3a–e).

To verify the molecular mechanism VFA uptake, prior to incubation of the VFA at 37°C for 12 hr, CHO cells were pretreated at 4°C for 1 hr (endocytosis inhibition) or at 37°C for 1 hr with 6 different chemicals including dynasore (an inhibitor for the scission of clathrin-coated vesicles), cytochalasin D (an inhibitor of actin-based transport), amiloride (an inhibitor of macropinocytosis), filipin (an inhibitor of caveolae formation), nystatin (an inhibitor of caveolin-dependent uptake) and mannan (an inhibitor of mannose receptor-mediated phagocytosis)<sup>13</sup>. For a cellular uptake evaluation with 6 different endocytic inhibitors, the concentration range of each inhibitor beyond which there was no effect or low effect (less than 20%) on drug cytotoxicity was selected (Supplementary Fig. 4a)<sup>14</sup>. Fluorescence intensity of the VFA in CHO cells showed that the uptake of the VFA was nearly completely inhibited at 4°C compared to 37°C (Supplementary Fig. 4b)<sup>14</sup>. Among 6 inhibitors, only dynasore resulted in significant dose-dependent inhibition of VFA uptake in CHO cells. Similarly, a confocal microscopy image revealed that there was no clear fluorescence brightness of the VFA in CHO cells with the treatment of 4°C and dynasore (10 μM) while the treatment of cytochalasin D (2.5 μM), amiloride (0.5 mM), filipin (2.5 μM), nystatin (5 μg/ml) and mannan (0.5 mg/ml) visualized significant fluorescence signals of the VFA in CHO cells (Fig. 2). These results

showed that VFA uptake involved the process of clathrin-mediated internalization into cells.

To further investigate VisuFect-mediated gene delivery into zygotes, 10 μM of poly(A) conjugated with the VisuFect was incubated with zygotes from various species including pigs, zebrafish and drosophilas. After treatment of the VFA into zygotes of each species, confocal microscopy images were acquired at 12 and 48 hr for the pig, 8 and 24 hr for the zebrafish and 6 and 12 hr for the drosophila. VFA uptake was clearly visualized inside 1-cell and 4-cell embryos of the pig but not in the nucleus (Fig. 3a). Interestingly, similar with mouse sperms (Fig. 1), a few pig sperms on the zona pellucida of the 4-cell embryo were visualized in the head and midpiece by the VFA (Fig. 3a, black arrows), indicating great efficiency of VisuFect-mediated gene delivery into the sperms. A great internalization of the VFA was also visualized in the embryos of both drosophilas and zebrafish (Fig. 3a). Moreover, fluorescence signals 24 hr after incubation of the VFA into a zebrafish zygote were found even in the head and tail (Fig. 3a, blue arrows). Z-stack confocal images of the embryos of pigs, zebrafish and drosophilas further confirmed that the VFA was localized inside the embryo (Supplementary Fig. 5a–c). It was noted that the VFA did not affect the embryo development of pigs, zebrafish and drosophilas.

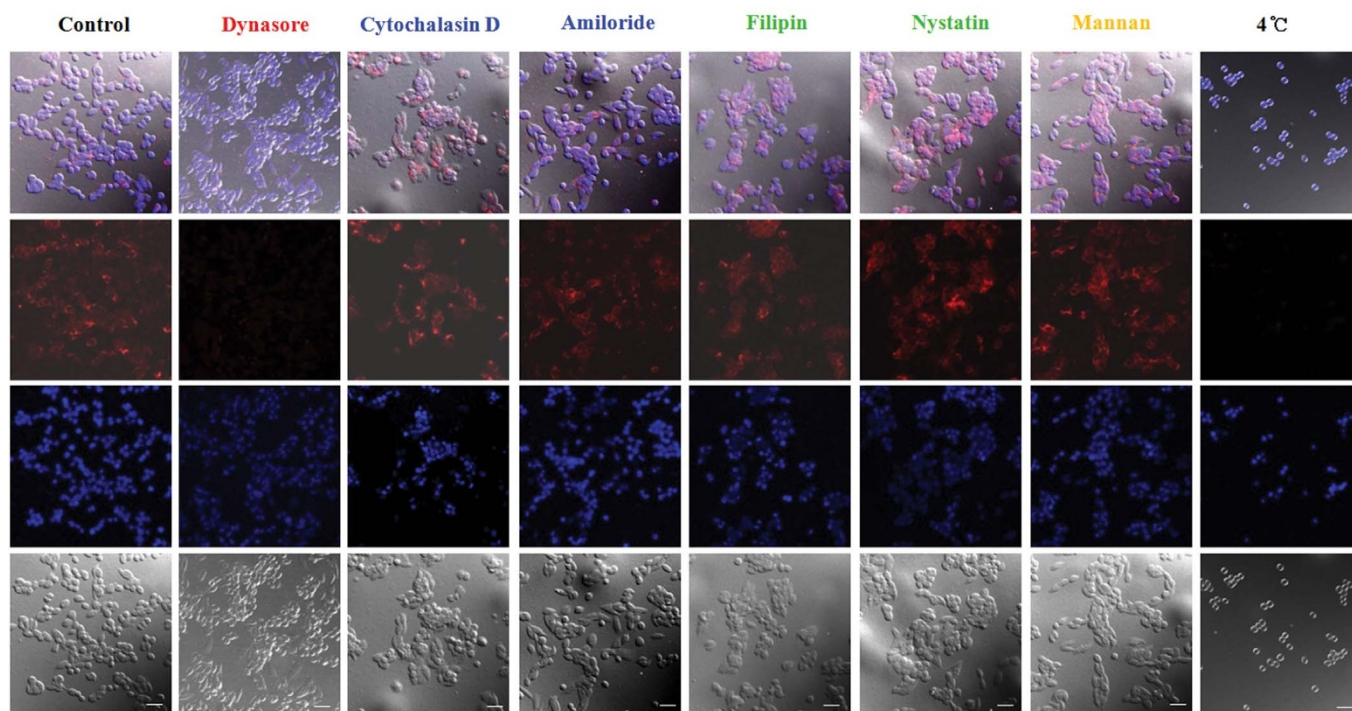
Live cell imaging with additional incubation of the VFA into mouse zygotes was conducted for 11 hr 20 min. Time-lapse microscopy showed that the VFA initially interacted with the polar body of mouse zygotes, then quickly accumulated inside the zygotes after 10 hr and finally completely internalized into all zygotes (Fig. 3b and Supplementary Movie 1). The results of live cell imaging demonstrated that VisuFect-mediated gene deliver system could effectively deliver genes into a large number of zygotes at a time. To see the possibility of delivering genes into oocytes and various stages of mouse embryos by the VisuFect, the VFA was incubated with imma-



**Figure 1** | Confocal microscopy imaging of the VFA in various cells. The VisuFect conjugated with 25 μM of the poly(A) was incubated with CHO cells, HeLa cells, human ES cells, human fibroblast cells and mouse sperms at 37°C for 12 hr. The left panel shows a magnification of 200× and the right panel a magnification of 800×. For each panel, column 1: cellular morphology; Column 2: Cy5.5 signals of the VFA (red, excitation: 675 nm and emission: 694 nm); Column 3: 4', 6-diamidino-2-phenylindole (DAPI) imaging (blue, nucleus staining, emission: 460 nm); Column 4: merged image. Scale bars indicate 20 μm.



Uptake mechanism inhibited: **Clathrin**, **Macropinocytosis**, **Caveolin**, **Mannose receptor**

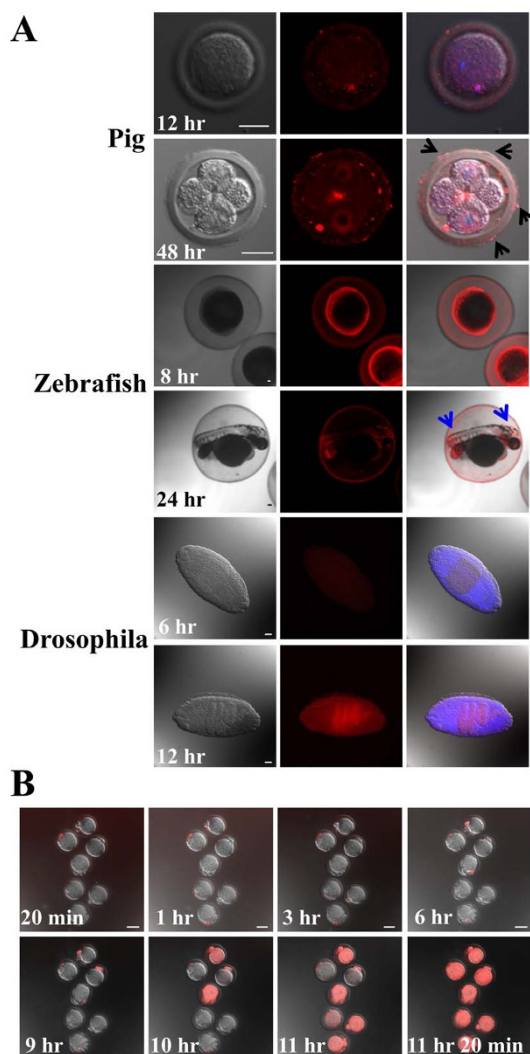


**Figure 2 | Confocal microscopy imaging for the uptake mechanism of the VFA in CHO cells.** CHO cells were pretreated at 37°C for 1 hr with dynasore (10  $\mu$ M), cytochalasin D (2.5  $\mu$ M), amiloride (0.5 mM), filipin (2.5  $\mu$ M), nystatin (5  $\mu$ g/ml) and mannan (0.5 mg/ml) before the incubation with the VisuFect conjugated with 25  $\mu$ M of the poly(A) at 37°C for 12 hr. In addition, 4°C incubation for 1 hr instead of 37°C for 1 hr was used as control. For each group, 1<sup>st</sup> row: merged image; 2<sup>nd</sup> row: Cy5.5 signals of the VFA (red, excitation: 675 nm and emission: 694 nm); 3<sup>rd</sup> row: DAPI image (blue, nucleus staining, emission: 460 nm); 4<sup>th</sup> row: cellular morphology. Confocal microscopy images of all experiments show a magnification of 200 $\times$ . Scale bars indicate 75  $\mu$ m.

ture oocytes, mature oocytes, zygotes and embryos of 2-cell, 4-cell, morula and blastocyst stages for 12 to 16 hr. The VFA incubation did not have any effect on oocyte maturation or further embryonic development (Supplementary Fig. 6). Confocal microscopy images showed strong fluorescence brightness of the VFA inside the oocytes and embryos 12 to 16 hr after the incubation of the VFA, indicating successful delivery of the VFA into oocytes and embryos of the mice.

To study VisuFect-mediated delivery of microRNA (miR) inhibitor into zygotes, miR34c, that is highly expressed in a zygote and important for the first cell division, was selected<sup>15</sup>. qRT-PCR demonstrated high expression of miR34c at 1-cell embryo (E0.5) and then it abruptly decreased after subsequent embryonic developments (Supplementary Fig. 7). Microinjection of the miR34c inhibitor (0.5 and 1 nM) into mouse zygotes suppressed embryonic development (Fig. 4a)<sup>15</sup>. Compared with control zygotes without injection or those injected with DEPC only of which 39 and 37% of zygotes succeeded in cleaving to blastocysts (E3.5), respectively, mouse zygotes injected with 0.5 nM of the miR34c inhibitor showed that 87% of zygotes arrested at the 2- (E1.5) or 4- cell stage (E2.0), and the remaining 13% stopped at the compacted morula stage (E3.0) (Supplementary Fig. 8). In the case of zygotes injected with 1 nM of the miR34c inhibitor, 69% was arrested at the 1-cell stage, and the remaining 31% of 2-cell embryos failed to cleave to the 4-cell stage. To examine if VisuFect-mediated delivery of the miR34c inhibitor into zygotes arrests mouse embryo development, various concentrations (2.5, 5 and 10  $\mu$ M) of the miR34c inhibitor was conjugated with the VisuFect at a molar ratio of 1:0.8 (designated as VFm34cI). Confocal microscopy analysis of the VFA or VFm34cI demonstrated dose-dependent quantitative fluorescence brightness at the 2-cell stage of mouse embryos (Supplementary Fig. 9). Control mouse zygotes incubated with null or various concentrations of VFA did

not affect zygotes' cleavage up to the blastocyst stage (Fig. 4b and Supplementary Fig. 10a). However, mouse zygotes treated with the VFm34cI showed dose-dependent cleavage arrest. Zygotes incubated with 5  $\mu$ M of miR34c inhibitor-conjugated VisuFect showed that 82% of zygotes stopped at the 2- or 4-cell stage, and the remaining 18% of morula failed to cleave to the blastocyst stage (Supplementary Fig. 10b, c). 78% of zygotes incubated with 10  $\mu$ M of miR34c inhibitor-conjugated VisuFect failed to cleave to the 2-cell stage, and the remaining 22% of 2-cell embryos failed to further progress into cell cleavage division. Confocal microscopy images demonstrated that the treatment of 10  $\mu$ M of poly(A)-conjugated VisuFect visualized by the strong fluorescence signals in embryos normally drove cell cleavage progression of zygotes up to blastocyst (Fig. 4c). Meanwhile, treatment with 10  $\mu$ M of miR34c inhibitor-conjugated VisuFect visualized by clear fluorescence brightness resulted in developmental arrest of the fertilized embryo. The developmental arrest of the zygote by the VFm34cI was associated with the failure of syngamy formation, which is important for the mingling of male and female genomes<sup>16</sup>. To determine whether the VFm34cI inhibits miR34c function, the gene expression of Bcl-2, which is one of miR34c targets<sup>15</sup> was evaluated by RT-PCR. Messenger RNA for RT-PCR was collected from non-treated E0.5 and E1.5 embryos and embryos at day 1 after the treatment with the VFA (10  $\mu$ M) or the VFm34cI (10  $\mu$ M). The gene expression of pluripotency biomarkers including Oct4 and Sox2<sup>17</sup> showed no significant difference among E1.5 control embryos and embryos 1 day after the treatment with the VFA or the VFm34cI (Fig. 4d). However, the gene expression of Bcl-2 was highly expressed only in the embryos at day 1 after the treatment with the VFm34cI due to the inhibition of miR34c function<sup>15</sup>, implying VisuFect-mediated successful delivery of the miR34c inhibitor into zygotes and successful function of delivered miR34c inhibitor in the zygotes.



**Figure 3 | The incubation of the VFA into zygotes of various species.** (a), The VisuFect conjugated with 10  $\mu$ M of the poly(A) was incubated at 37°C with pig zygotes for 12 and 48 hr, zebrafish zygotes for 8 and 24 hr and drosophila zygotes for 6 and 12 hr, and then confocal microscopy images of each embryo were obtained. For each embryo, 1<sup>st</sup> column: embryo morphology; 2<sup>nd</sup> column: Cy5.5 signals of the VFA (red, excitation: 675 nm and emission: 694 nm); 3<sup>rd</sup> column: merged image with DAPI staining (blue, nucleus staining, emission: 460 nm). DAPI images of zebrafish embryos were deleted because they gave poor images of the VFA in zebrafish embryos. Confocal microscopy images of all embryos show a magnification of 800 $\times$ . Black arrows indicate localization of the VFA internalized into pig sperms, and blue arrows the localization of the VFA internalized into the head and tail of zebrafish embryos. Scale bars indicate 75  $\mu$ m. (b), Time-lapse confocal microscopy images of mouse zygotes treated with the VisuFect conjugated with 10  $\mu$ M of the poly(A) were recorded sequentially in 20-min intervals for 11 hr 20 min. The red fluorescence signals indicate the locomotion of the VFA. Scale bars indicate 75  $\mu$ m.

## Discussion

Current gene delivery techniques for embryos including viral-based and non-viral based systems need further improvement in terms of its efficiency and safety. Although the viral-based gene delivery system has relatively higher delivery efficiency than the non-viral-based gene delivery system, it is commonly seen with the risks of working with viruses and problems of immunogenicity<sup>18</sup>. Furthermore, the delivery efficiency and toxicity of non-viral systems including phys-

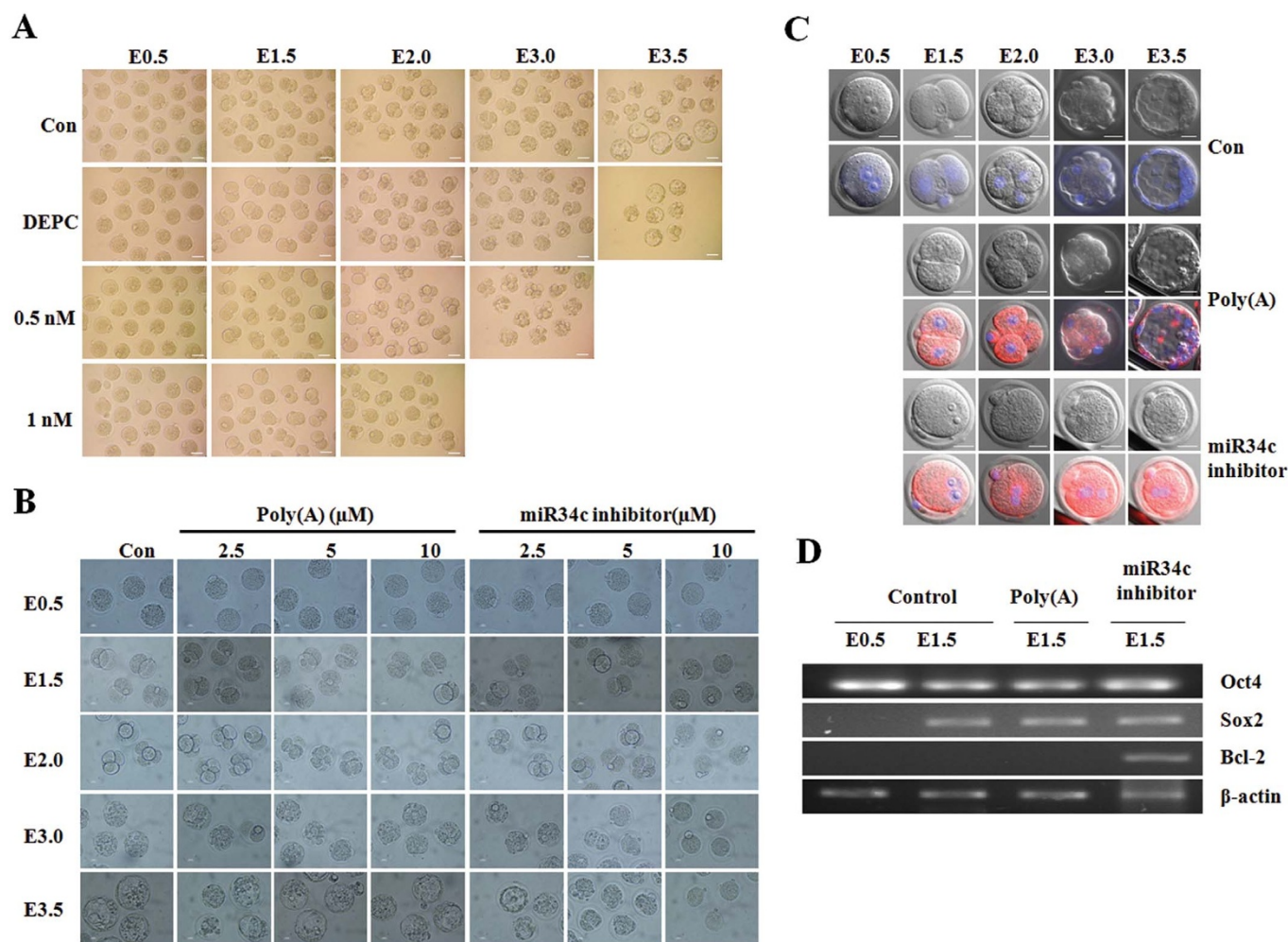
ical (microinjection, electroporation and gene gun) and chemical methods (organic and inorganic nanoparticles) with many advantages such as easy of synthesis, low immune response still need to be improved<sup>18</sup>. In this study, the poly(A) or miR34c inhibitor-conjugated VisuFect showed great potential in providing easy to use, great penetration through the zona pellucida, low toxicity, easy monitoring of delivered genes into zygotes. However, confocal microscopy images with DAPI staining showed that the VisuFect in zygotes was localized in the cytoplasm but not in nucleus. Interestingly, the VisuFect showed high delivery efficiency with seven zygotes at a time. Considering high delivery efficiency of the VisuFect from other cells including CHO, HeLa, human ES and human fibroblast cells, the number of zygotes for the simultaneous delivery by the VisuFect will be much greater than seven. The results of VisuFect-mediated delivery of the miR34c inhibitor into zygotes that suppressed cell cleavage progression of mouse zygotes provide successful expression of delivered genes into zygotes. Furthermore, the interesting discovery of internalization of the VFA in sperms of mice and pigs will elicit great attention from sperm biologists. Unlike other inorganic or organic compounds, the VisuFect was challenging to deliver a plasmid containing the coding region of green fluorescence protein (pEGFP-c1) into cells (data now shown). Since the VisuFect has a few of limitation of delivering a genetic material into nucleus of zygotes or large DNA into zygotes, it should be technically advanced for transgenic animal production. However, the invaluable features of the VisuFect will be useful to study function of genes in sperms, oocytes and embryos using small RNAs or DNAs.

## Methods

**Conjugation of VisuFect with DNA oligos.** The VisuFect-mediated conjugation with DNA oligonucleotides including the miR34c inhibitor and poly(A)<sub>50</sub> manufactured by Bioneer (Bioneer, Daejeon, Korea) was customized at SeoulLin Bioscience (Seongnam-si, Gyeonggi-do, Korea). The miR34c inhibitor sequences (5'-GCAATCAGCTAACTACTGCT-3'), which were perfectly complementary against mature miR34c, were obtained from the miRBase database (www.mirbase.org). According to the manufacturer's protocol, the VisuFect was conjugated with the poly(A)<sub>50</sub> (designated as VFA) or the miR34c inhibitor (designated as VFm34cI) at a molar ratio of 1 : 0.8 in PBS buffer (pH 7.4) for 4 hr at room temperature. To confirm the conjugation between the VisuFect and DNA oligos, the poly(A) and the conjugated VFA were loaded onto each well of 15% SDS/PAGE gel with electrophoresis buffer (0.5 $\times$  Tris-Borate-EDTA).

**Cell culture and incubation with the VFA.** CHO (Chinese hamster ovary) cells, HeLa (human cervical cancer) cells, human fibroblast cells and human embryonic stem (ES, H9) cells were used to evaluate the uptake efficiency of the VFA. CHO, HeLa and fibroblast cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Grand Island, NY) containing 10% fetal bovine serum (FBS, Gibco, Lot Number, 1312606) and 1% antibiotics (Invitrogen, Grand Island, NY) at 37°C in 5% CO<sub>2</sub> atmosphere. Human ES cells were cultured according to the previously reported protocols<sup>19</sup>. Briefly, H9 human ES cells were cultured on mouse embryonic fibroblast feeder layers in DMEM/F-12 medium (Gibco, Gaithersburg, MD) supplemented with 20% knockout serum replacement (Gibco, Lot Number, 731340), 1 mM L-glutamine (Gibco), 0.1 mM  $\beta$ -mercaptoethanol (Gibco), 0.1 mM nonessential amino acids (Gibco), and 4 ng/ml human recombinant bFGF (Invitrogen). Human H9 ES cell clumps were split into separated colonies using the micro-dissection method and transferred onto new three replicate feeder layers every five days. The three replicate cell culture experiments were conducted to grow ES cell colonies at 37°C in 5% CO<sub>2</sub> atmosphere. These cells including CHO, HeLa, human fibroblast and human ES cells were plated at 1  $\times$  10<sup>5</sup> cells/well into 24-well plates and passaged to 70–80% confluence before the incubation of the VFA. Mouse sperms (1  $\times$  10<sup>7</sup>) were collected from the cauda epididymis from one male (C57BL/6, 8 weeks old), extracted and washed with M2 media (Sigma, St Louis, MO) and further incubated in KSOM media (Millipore, Billerica, MA) containing 0.4% bovine serum albumin (BSA Sigma, St Louis, MO) at 37°C prior to use. The seven replicate experiments were conducted with various cells transfected with 25  $\mu$ M of the poly(A) conjugated VisuFect. Cells were incubated further 12 hr at 37°C.

**Oocytes and embryos collection and the incubation of the VFm34cI or the VFA.** In order to induce ovulation of the mice to collect oocytes and embryos, B6D2F<sub>1</sub> (C57BL/6  $\times$  DBA/2) female mice (4 to 8 weeks old) were first injected with 7.5–10 IU PMSG (Sigma, St Louis, MO); 48 hr later, injection of 7.5–10 IU hCG was performed. Fresh germinal vesicle (GV) oocytes were obtained from the rupture of follicles from isolated ovaries with a 26-gauge needle. Metaphase II (MII) oocytes were collected from mice 14 hr after the priming injection of the hCG. The GV and MII oocytes were maintained in KSOM media containing 0.4% BSA prior to use. To collect fertilized



**Figure 4** | VisuFect-mediated delivery of miR34c inhibitor into mouse zygotes. (a), Microphotographs (60 $\times$ ) of mouse embryo developments with or without microinjection of DEPC, 0.5 and 1 nM of the miR34c inhibitor. Scale bars indicate 50  $\mu$ m. (b), Mouse zygotes were incubated at 37 $^{\circ}$ C for 3 days with the VisuFect conjugated with 2.5, 5 and 10  $\mu$ M of poly(A) or miR34c inhibitor. Non-treated mouse zygotes were used as control. Microphotographs (400 $\times$ ) showed that the VisuFect-mediated delivery of the miR34c inhibitor resulted in the arrest of embryo development. Scale bars indicate 50  $\mu$ m. (c), Confocal microscopy images of embryos treated with the VisuFect conjugated with 10  $\mu$ M of poly(A) or miR34c inhibitor for 3 days. All images (800 $\times$ ) were merged with DAPI signals (blue), Cy5.5 brightness (red) of the VFA or VFm34cI and cellular morphology. Non-treated mouse embryos were used as control. Scale bars indicate 25  $\mu$ m. (d), Gene expression of Bcl-2 which is one of miR34c targets. Messenger RNAs for RT-PCR were collected from non-treated E0.5 and E1.5 embryos and embryos 1 day after the treatment of the VisuFect conjugated with poly(A) (10  $\mu$ M) or miR34c inhibitor (10  $\mu$ M). Sox2 and Oct4 were used as control for pluripotency and  $\beta$ -actin control for RT-PCR.

eggs, female mice injected with 7.5–10 IU hCG were mated with male mice overnight. In the presence of a vaginal plug from the female mice, fertilized eggs were obtained from the oviducts and then cumulus cells were removed by the treatment of 300 U/ml hyaluronidase to isolate 1-cell embryos. The isolated zygotes were further cultured up to the blastocyst stage in KSOM media containing 0.4% BSA at 37 $^{\circ}$ C under 5% CO<sub>2</sub> in air. Oocytes and various stages of embryos were incubated in various concentrations of the VFA or various concentrations of the VFm34cI at 37 $^{\circ}$ C for 12 to 36 hr.

Wild-type zebrafish were purchased from local pet stores (E-mart, Seoul, Korea) and used for breeding. The collected fertilized eggs of zebrafish were grown at a density of approximately 50–100 embryos per 50 ml of egg water (60  $\mu$ g/ml, sea salt, Korea) in a plastic 90-mm culture dish and were maintained in an incubator at 28.5 $^{\circ}$ C with a 14 hr light on/10 hr light off cycle under approximately 3000 Lux without any colored material inside. The fertilized eggs (n = 200) were incubated for 8 and 24 hr with the VisuFect conjugated with 10  $\mu$ M of the poly(A).

A *w<sup>1118</sup>* drosophila strain was obtained from the Bloomington Stock Center (stock number BL-5905) and reared on standard cornmeal/agar media under non-crowded conditions at 25 $^{\circ}$ C. Embryos of *w<sup>1118</sup>* were collected for 20 min on standard egg laying plates with yeast paste. The fertilized drosophila embryos (n = 100) were dechorionated manually and incubated at 25 $^{\circ}$ C for 6 and 12 hr with the VisuFect conjugated with 10  $\mu$ M of the poly(A).

Porcine ovaries (n = 15) were collected from a commercial slaughterhouse. Immature oocytes (n = 300) were obtained from pre-ovulated follicles by an 18-gauge needle and syringe. To further develop follicles into MII oocytes *in vitro*, immature oocytes were cultured in tissue culture medium (TCM)-199 (Gibco)

containing 0.57 mM cysteine, 0.91 mM sodium pyruvate, 0.1% polyvinyl alcohol (PVA, w/v), 10 ng/ml epidermal growth factor, 3.05 mM D-glucose, 10 IU/ml PMSG, 10 IU/ml hCG, 75 mg/ml penicillin G and 50 mg/ml streptomycin sulphate under mineral oil for 44 hr at 39 $^{\circ}$ C in a 5% CO<sub>2</sub> (v/v) in air. 42 hr after *in vitro* maturation of immature oocytes, the sperms extracted by squeezing an epididymis of boars were then added into the MII oocytes for fertilization. The fertilized eggs were incubated for 12 and 48 hr with the VisuFect conjugated with 10  $\mu$ M of the poly(A).

**Uptake mechanism.** To evaluate the uptake mechanism of the VisuFect-mediated gene delivery, CHO cells (5  $\times$  10<sup>3</sup>) were seeded into 96-well plates with 200  $\mu$ l of culture medium. A broad concentration range of 6 different uptake inhibitors including dynasore (0 to 40  $\mu$ M), cytochalasin D (0 to 40  $\mu$ M), amiloride (0 to 4 mM), filipin (0 to 40  $\mu$ M), nystatin (0 to 40  $\mu$ g/ml) and mannan (0 to 2 mg/ml). MTT was then added into CHO cells for 24 hr. The optical density (OD) of the MTT of each sample was evaluated in triplicate to assay cell viability as described above. The concentration range of 6 different uptake inhibitors showing no or low effect (less than 20%) on drug cytotoxicity in CHO cells was further used to study uptake inhibition. After CHO cells (1  $\times$  10<sup>4</sup>) were plated into 24-well plates with 500  $\mu$ l of culture medium, CHO cells were incubated at 37 $^{\circ}$ C for 1 h with dynasore (0 to 40  $\mu$ M), cytochalasin D (0 to 10  $\mu$ M), amiloride (0 to 2 mM), filipin (0 to 7.5  $\mu$ M), nystatin (0 to 20  $\mu$ g/ml) and mannan (0 to 2 mg/ml) and then further incubated at 37 $^{\circ}$ C for 12 hr with the VisuFect conjugated with 25  $\mu$ M of the poly(A). In addition, the treatment of incubation at 4 $^{\circ}$ C for 1 hr was conducted with CHO cells without the treatment of 6 different endocytosis inhibitors. 12 hr after the incubation of the VFA,



CHO cells were washed two times for 10 min each at RT under mild shaking. These cells were lysed with RIPA buffer (Thermo Fisher Scientific Inc., Waltham, MA) and then transferred into a dark 96-well plate (Chemicell GmbH, Germany) to measure the fluorescence intensity (50  $\mu$ L) at an excitation wavelength of 675 nm and an emission wavelength of 694 nm using SynergyMx (BiotEK, USA). The fluorescence intensity of the VFA in CHO cells treated with no inhibitor was first acquired and used as a control to analyze other fluorescence intensities of the VFA co-incubated with various concentrations of each inhibitor in CHO cells. The fluorescence ratio for the uptake mechanism of the VFA with 6 different inhibitors was normalized against the non-treated CHO cells with the incubation of the VFA. All the experiments were carried out in triplicate. To quantify the uptake efficiency of the VisuFect into zygote, the VisuFect conjugated with 0, 2.5, 5 and 10  $\mu$ M of poly(A) or miR34c inhibitor was treated with mouse zygotes and then region of interest (ROI) measurement was performed using confocal microscopy images of 2-cell stage embryos ( $n = 3$ ).

**Microinjection.** About 100 fertilized mouse embryos were microinjected with 10  $\mu$ L of the DEPC or miR34c inhibitor (50 or 100 pmol/ $\mu$ L) in M2 medium using a constant flow system (Transjector, Eppendorf, Hamburg, Germany). An injection pipette containing the miR34c inhibitor solution was inserted into the cytoplasm of the zygotes. The microinjected zygotes were further cultured up to the blastocyst stage for 3 days in KSOM media containing 0.4% BSA at 37°C under 5% CO<sub>2</sub> in air.

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## Author contributions

J.Y.J. and S.K. designed the study. J.Y.J., J.L., H.Y.K. and Y.S.L. conducted experiments related to mouse, D.H.L. and Y.S.L. experiments related to drosophilas, S.L. experiments related to zebrafish and J.J.K. and K.S.H. experiments related to stem cells and pigs. E.Y.K. and K.A.L. performed microinjection. S.C., Y.C., K.A.L. and S.K. analyzed data. J.Y.J., J.L. and S.K. wrote the manuscript. All authors discussed the results and commented on the manuscript.

## Additional information

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