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Bioimaging of microRNA34c in a single sperm by molecular beacon

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Experimental procedures

Cell culture

Human embryonic kidney (HEK) 293 cells and mouse testicular Sertoli TM4 cells were purchased from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Thermo Scientific Hyclone, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (Gibco) at 5% CO$_2$ and 37°C.

Design and preparation of the VisuFect (VF)-conjugated microRNA (mRNA or miR) imaging molecular beacons (VF miR34c MB and VF miR427 MB)

The VF miR34c MB and the VF miR427 MB consisted of two functionalized oligonucleotides. To prepare the VF miR34c MB, the linear long oligonucleotide containing the miR34c binding sequence (long-oligo: 5’-ccgcga_gcaatcagctaactacactgcct-3’, italicized sequence indicates miR34c binding sequence) was synthesized by Bioneer (Daejeon, Korea). The mature miR34c sequence (5’-aggcaguguaguuagcugauugc-3’) was obtained from the miRBase database (www.mirbase.org). According to the manufacturer’s protocol, the VisuFect (SeouLin Bioscience, Gyeonggi-do, Korea) was conjugated with the long-oligo (designated as VF-long-oligo) at a molar ratio of 1: 0.8 in PBS buffer (pH 7.4) for 4 h at room temperature. The short oligonucleotide with sequence partially complementary to the 5’-end of the long-oligo and a black hole quencher molecule (BHQ2) at the 3’-end was synthesized (quencher-oligo: 5’-gattgctcgcgg-BHQ2-3’). 30 pM of the VF-long-oligo was annealed with 60 pM of the quencher-oligo to construct the VF miR34c MB.

To prepare the VF miR427 MB, the linear long oligonucleotide containing the miR427 binding sequence (427-long-oligo: 5’-ccgcga_gcccaaaacagaaagcacttt-3’, italicized sequence indicates miR427 binding sequence) was synthesized by Bioneer. The mature miR427
sequence (5’- aaagcucuuucuuugggcg-3’) was obtained from the miRBase database. The VisuFect was conjugated with the long-oligo (VF-427-long-oligo). The short oligonucleotide with sequence partially complementary to the 5’-end of the long-oligo and a black hole quencher molecule (BHQ2) at the 3’-end was synthesized (427-quencher-oligo: 5’-gggctcgcgg-BHQ2-3’). 30 pM of the VF-427-long-oligo was annealed with 60 pM of the 427-quencher-oligo to construct the VF miR427 MB.

**Analysis of the formation of the VF-long-oligo**

Electrophoretic mobility shift assay was performed to verify the formation of the VF-long-oligo using urea-denaturing polyacrylamide gel (15% polyacrylamide and 8 M urea in nuclease-free TBE buffer). 50 pM of the long-oligo and the VF-long-oligo were loaded. The conjugation pattern on polyacrylamide gel was further analyzed by Cy5.5 fluorescence signal (excitation: 675 m, and emission: 694 nm) using IVIS® spectrum imaging system (Caliper Life Sciences, MA).

**Quenching efficiency of the VF-long-oligo in the VF miR34c MB**

Oligonucleotide mixtures consisted of a fixed concentration (10 pM) of the VF-long-oligo and various concentrations (0, 5, 10, and 20 pM) of the quencher-oligo in 100 μl of annealing buffer incubated on a black plate for 5 h at room temperature. The fluorescence intensity was measured using Bio Tek Fluorescent Microplate Fluorometer (Synergy Mx, BioTeck Ltd, VT) and fluorescence image was acquired by IVIS® spectrum imaging system (Caliper Life Sciences, MA).

**Specificity of the VF miR34c MB for sensing miR34c**

The quenched VF miR34c MB (30 pM) was incubation with various concentrations (0, 1, 10,
30, and 50 pM) of exogenous miR34c on a black plate for 5 h at room temperature. Signal recovery of the quenched VF miR34c MB was evaluated by fluorescence intensity and image.

**Quantification of miRNA expression**

The miR34c expression was quantified and compared by quantitative reverse polymerase chain reaction (qRT-PCR) using total RNA isolated from HEK293 cells, TM4 cells, and mouse sperm. For miRNA qRT-PCR, a miR-Q-assay was performed. Relative miRNA expression was normalized by 5S rRNA. qRT-PCR was performed in triplicate using an iCycler (Bio-Rad, Hercules, CA, USA) and SYBR Premix Ex Taq™ (2×; Takara, Japan) at 95°C for 3 min followed by 40 cycles of 95°C for 15 s and 62°C for 30 s. Expression data are presented as means of relative expression values obtained from three samples with standard deviations. To compare means, a t-test was performed; a p-value less than 0.005 was considered statistically significant.

**Confocal microscopy**

HEK293 and TM4 cells were seeded onto 4-well culture dishes (5 x 10^4 cells/well), covered with a 13-mm diameter cover glass, and cultured for 24 h. Cells were treated with 30 pM of the VF miR34c MB or 30 pM of the VF-long-oligo. Following two washes with phosphate-buffered saline (PBS) for 15 min, cells were fixed in 3.7% paraformaldehyde (Sigma-Aldrich, St Louis, MO, USA). After washing three times with PBS for 10 min, mounting solution containing 4, 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc., CA, USA) was applied. Cells were imaged by confocal laser scanning microscopy (Carl Zeiss LSM 510, Weimer, Germany).

**Transfection and fluorescence intensity**
HEK293 and TM4 cells were seeded onto 24-well plates at 5 x 10^4 cells per well. For HEK293 cells, 30 pM of the VF miR34c MB or 30 pM of the VF-long-oligo was directly added after 1 day, and then 3 h after treatment with the VF miR34c MB, various concentrations (0, 150, and 300 pM) of mature miR34c were transfected using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA). The mature miR9 (300 pM) was used as a negative control. After incubation for 16 h at 37°C, cells were washed twice for 10 min at room temperature under mild shaking and lysed with radio-immunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Fluorescence was measured using a BioTek Fluorescent Microplate Fluorometer (Synergy Mx, BioTeck Ltd, VT, USA).

TM4 cells were treated with various concentrations (0, 15, 30, 60, and 90 pM) of the VF miR34c MB. After incubation for 3 h at 37°C, cells treated with 90 pM of the VF miR34c MB were transfected with miR34c inhibitor (5’-gcaatcagtaactacactgcttg-3’), which was a synthetic oligonucleotide with a sequence complementary to miR34c. After incubation for 16 h at 37°C, fluorescence intensity was measured as described above.

Treatment of mouse sperm with the VF miR34c MB
Sperm (1 x 10^7) were isolated from the cauda epididymis of a C57BL/6 male mouse (8-weeks-old), extracted, washed with pre-warmed M2 media (Sigma-Aldrich), and then further incubated in pre-warmed KSOM mouse embryo culture media (Milipore, Billerica, MA, USA) containing 0.4% bovine serum albumin (BSA, Sigma-Aldrich) at 37°C prior to use.

Preparation of the VF-conjugated quantum dots (VF-QDs) and cellular uptake
10 pM of carboxy-terminated quantum dot QD565 (Invitrogen) and 50 pM of the VF was conjugated using [N-(3-dimethylaminopropyl)-N-(ethyl-carbodiimide hydrochloride] (EDC, Sigma-Aldrich) and characterized by transmission electron microscopy (TEM, JEM-3010...
(JEOL Ltd., Japan). 10 pM of QDs, VF-QDs and 50 pM of the VF were treated into HEK293 cells and mouse sperm. After 5-h incubation, samples were washed with PBS buffer three times and then cellular uptake was evaluated by confocal laser scanning microscopy. The internalization of the VF-QDs in sperm were evaluated by the Tecnai™ G2 Spirit Twin transmission electron microscope (FEI, Hillsboro, USA).

**Cytotoxicity test**

Isolated mouse sperm were incubated with different concentrations (0, 5, 10, 25, 50, and 100 pM) of the VF miR34c MB. Lipofectamine 2000 was also used to deliver the miR34c MB to compare the cytotoxicity and delivery efficiency of VF and Lipofectamine. After 5-h incubation, the LIVE/DEAD® Sperm Viability Kit (Molecular Probes, Thermo Fisher Scientific Inc., USA) was used to analyze the viability of sperm. Sperm were treated with SYBR 14 reagent according to the manufacturer’s protocol and incubated for 10 min at 36°C. SYBR-stained sperm were analyzed by flow cytometry.

**In situ hybridization**

Isolated mouse sperm were air-dried at 37°C for 30 min on a slide glass and fixed with 4% paraformaldehyde (Sigma-Aldrich) for 20 min. Isolated mouse testes were fixed with 4% paraformaldehyde and embedded in paraffin. In situ hybridization was performed using a miR34c- detection probe and scrambled probe (Bioneer) according to previously reported protocols [27, 28]. Cy5.5 was conjugated at the 5’-end of the miR34c antisense oligonucleotide (5’-Cy5.5-gcaatcagctaactacactgcct-3’) and scrambled probe (5’-Cy5.5-aaaaaaaaaaaaaaaaaaaaaaa-3’; used as a control) for direct imaging analysis.

**Statistical analysis**
All data are presented as the means ± standard deviation (SD). The significance of difference was evaluated using Student’s t-test (*P<0.05 and **P < 0.005).
Figure S1. Gel electrophoresis of the VisuFect conjugated long oligonucleotide (VF-long-oligo). Conjugation between the VF and the long oligonucleotide (long-oligo) was verified by gel electrophoresis (left panel). The VF-long-oligo (lane 1) and long-oligo (lane 2) were loaded onto a 15% polyacrylamide gel and immersed in electrophoresis buffer (1x Tris-Borate-EDTA). After gel electrophoresis the conjugation pattern was evaluated by IVIS® spectrum imaging system (Ex/Em, 683/705 nm, right panel).
Figure S2. Specificity of the VF miR34c MB to sense exogenous miR34c. (A) qRT-PCR analysis of a mature miR34c expression in HEK293, mouse sperm, and TM4 cells. (B) Fluorescence intensity of the VF miR34c MB in HEK293 cells. (C) Confocal microscope images of the VF miR34c MB-treated HEK293 cells. Fluorescence images of Cy5.5 of the first row were acquired at an excitation of 683 nm and emission of 705 nm. The second row is merged with the 4',6-diamidino-2-phenylindole (DAPI) image (nucleus staining, 460 nm) and cellular morphology image, respectively. Scale bars, 25 μm. Statistical data are displayed as means ± standard deviations of triplicate samples (** P < 0.005).
Figure S3. Stability of the VF miR34c MB to sense exogenous or endogenous miR34c. The VF miR34c MB was added to the HEK293 cell and TM4 cell culture plate and incubated for 2 days. (A) Exogenous miR34c (300 pM) was transfected into HEK293 cells each day and fluorescence intensity of the VF miR34c MB was measured. (B) miR34c inhibitor was transfected into TM4 cells and fluorescence intensity of the VF miR34c MB was measured.
Figure S4. RT-PCR analysis of the expression of p27, which is an endogenous target of miR34c. Total RNA isolated from TM4 cells treated with 0, 10, 25, 50, and 100 pM of the VF miR34c MB. Total RNA from C6 cell was used as positive control.
**Figure S5.** Transfection of the miR34c MB using a transfection reagent. (A) Schematic illustration of the miR34c MB. The miR34c MB consisted of two functionalized oligonucleotides. First, the linear structured long oligonucleotide with a miR34c binding sequence complementary to the mature miR34c (long-oligo) directly conjugated to the Cy5.5 (Cy5.5-long-oligo). Second, the short oligonucleotide with sequence partially complementary to the 5’-end of the long oligonucleotide and a black hole quencher molecule (BHQ2) at the 3’-end was synthesized (quencher-oligo). These two functionalized oligonucleotides were partially hybridized to form the miR34c MB. In the absence of miR34c, the miR34c MB did not show any fluorescence signal due to proximal quenching of Cy5.5 by the quencher-oligo. In the presence of miR34c, through the selective binding of miR34c to the miR34c binding sequence, the quencher-oligo detached from the miR34c MB, resulting in signal-on of a red fluorescence signal from Cy5.5. (B) Fluorescence intensity of the miR34c MB, which was conjugated with Cy5.5 instead of the VF at the 5’-end of the long oligonucleotide. HEK293 cells were co-transfected with 30 pM of the miR34c MB and four different concentrations (0, 30, 90, and 150 pM) of miR34c and 90 pM of miR9 using Lipofectamine 2000. 30 pM of the Cy5.5-long oligonucleotide was transfected into HEK293 cells as a control. Fluorescence intensities of Cy5.5 are displayed as means ± standard deviations of triplicate samples (** *P* < 0.005). (C) Confocal microscope images of the miR34c MB in HEK293 cells. The first column displays an image merged with the 4’,6-diamidino-2-phenylindole (DAPI) image (nucleus staining, 460 nm) and cellular morphology image. Fluorescence images of Cy5.5 (red, 2nd column) were acquired at an excitation wavelength of 683 nm and emission wavelength of 705 nm. Scale bars, 10 μm.
Figure S6. Comparison of VF-mediated delivery method and transfection reagent-mediated delivery method in mouse sperm. Sperm were directly treated with the VF miR34c MB, while miR34c MB was transfected into sperm using Lipofectamine 2000. (A) Transfection efficiency. Fluorescence intensities of Cy5.5 are displayed as means ± standard deviations of triplicate samples (** P < 0.005).
(B) Sperm viability. SYBR-stained sperm were analyzed by flow cytometry (* P < 0.05, ** P < 0.005).
Figure S7. Delivery of VF-conjugated QDs (VF-QDs) into HEK293 cells and mouse sperm. (A) TEM images of unconjugated QDs and prepared VF-QDs. Fluorescence intensity of unconjugated QDs, VF-QDs, and VF in (B) HEK293 cells and (C) mouse sperm. Cellular uptake was confirmed by fluorescence intensity after 5-h incubation.
Figure S8. The specificity of the VF miR34c MB in mouse sperm. The VF miR34c MB-treated sperm were additionally incubated without (left panel) or with exogenous miR34c (middle panel) or with miR9 (right panel) without changing the culture media. Fluorescence signals of the VF miR34c MB were clearly visualized inside the sperm regardless of the incubation with exogenous miR34c or miR9. The exogenous miR34c treated culture media displayed with red fluorescence signals due to the specific hybridization between exogenous miR34c and the remaining VF miR34c MB in culture media, and sequential signal-on of Cy5.5 in the VF. However, Cy5.5 fluorescence signals were not detected in the exogenous miR9 treated culture media. The first row displays the fluorescence images of Cy5.5 and the second raw images are merged with the DAPI images and cellular morphology images. Scale bars, 10 μm.
Figure S9. *In vitro* single sperm imaging of the VF miR34c MB. (A) Confocal microscopy analysis of mouse sperm treated with various concentrations (0, 5, 10, 25, 5, and 100 pM) of the VF miR34c MB or (B) the VF miR427 MB (negative control), and sperm treated with 50 pM of (C) the VF miR34c MB or (D) the VF miR427 MB and incubated for 7 h. Scale bars, 25 μm. Figure (A) and (C), and the second rows in Figure (B) and (D) are Cy5.5 images merged with DAPI images. The first rows in Figure (B) and (D) are Cy5.5 images merged with the DAPI images and cellular morphology images.
Figure S10. Characterization of the VF miR427 MB. (A) qRT-PCR was used to analyze the expression of miR427 in HEK293 cells (negative control), mouse sperm, and *Xenopus* blastula (positive control). (B) Quenching efficiency of the VF-427-long-oligo in the VF miR427 MB. Fluorescence intensity of Cy5.5 in the VF-427-long-oligo in a black plate (upper panel) and fluorescence images obtained using the IVIS® spectrum imaging system (bottom panel).
Oligonucleotide mixtures consisting of a fixed concentration (10 pM) of the VF-427-long-oligo and various concentrations (0, 5, 10, and 20 pM) of the 427-quencher-oligo were incubated on a black plate for 5 h at room temperature. As the 427-quencher-oligo concentration increased, the fluorescence intensity of the VF miR427 MB decreased gradually. (C) Specificity of the VF miR427 MB for sensing miR427 in plate. Fluorescence intensity of Cy5.5 in the VF-427-long-oligo in a black plate (upper panel) and fluorescence images (bottom panel). The quenched VF miR427 MB (30 pM) was incubated with various concentrations (0, 1, 10, 30, and 50 pM) of exogenous miR427 on a black plate. The fluorescence signal significantly and gradually increased. As a control experiment, 30 pM of miR9 was used and no increase in fluorescence signal was observed. (D) Specificity of the VF miR427 MB for sensing miR427 in HEK cells. HEK293 cells were treated with different concentration (0, 15, 30, and 60 pM) of exogenous miR427 or 60 pM of miR9. The Cy5.5 red fluorescence signal of the VF miR427 MB increased in an exogenous miR427 concentration-dependent manner. In contrast, in the presence of 60 pM of exogenous miR9, the Cy5.5 fluorescence was quenched. Fluorescence intensities of Cy5.5 are displayed as means ± standard deviations of triplicate samples (** P < 0.005).
**Figure S11.** *In situ* hybridization of miR34c in sperm. Sperm were fixed and then hybridized with a Cy5.5-labeled miR34c-detection probe (left column) or scrambled probe (right column; negative control). The first row images merged with Cy5.5 and DAPI and the second row images are fluorescence images of Cy5.5. Scale bars, 25 μm.
Figure S12. (A) Z-stack images of Cy5.5 fluorescence in a single mouse sperm. The fluorescence images of Cy5.5 were merged with cellular morphology images. After incubation of sperm with 50 pM of the VF miR34c MB for 7 h, a total of 10 confocal images were collected, with a distance between planes of 1.5 µm. Scale bars, 10 µm. (B) Z-stack images of sperm heads. Six confocal images were collected at a distance between planes of 1.0 µm. The first and the third columns are Cy5.5 fluorescence images merged with the DAPI images and cellular morphology images. The second and the fourth columns are images of Cy5.5 fluorescence. Scale bars, 25 µm.
**Figure S13.** *In situ* hybridization of miR-34c in adult mouse seminiferous tubule sections. Adult mouse seminiferous tubule sections were treated with MiR34c-detection probe (upper panel) or scrambled probe (negative control, bottom panel). The first columns are images of Cy5.5 fluorescence merged with the DAPI images and cellular morphology images. The second columns are images of Cy5.5 fluorescence. Scale bars, 50 μm.