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Bioimaging of microRNA-294 expression-dependent color change in cells by a dual fluorophore-based molecular beacon

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Experimental procedures

Design of the ColoR294 MB

The sequences of miR-294, 5'-AAAGUGCUUCCCUUUUGUGUGU-3', were obtained from the PicTar database (http://pictar.bio.nyu.edu). The long oligonucleotide was designed to contain miR-294 binding sequence which is reverse complementary against miR-294 and labeled with two different fluorophores; Cy3 at the 5' end and Cy5 at the 3' end. It was synthesized as follow; 5'-Cy5-ccgcgaACACACAAAAGGGAAGCACTTT aaaaaaatccaaacct ccc-Cy3-3' and purchased from Bionics (Bionics, Inc, Korea). A short oligonucleotide was designed to contain a sequence which is partially complementary to the 5' end of the long oligonucleotide and a black hole quencher 2 (BHQ2) at the 3' end. It was synthesized as follow; 5'-GTGTGTtcgcgg-BHQ2-3' and purchased from Bionics. Formation of the ColoR294 MB was simply achieved by annealing of two oligonucleotides (each at a concentration of 30 pmol).

Cell culture

CHO cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. P19 cells were maintained in DMEM containing 10% FBS, 1% penicillin/streptomycin, 1% non-essential amino acid solution (NEAA, Sigma, St Louis, MO) and β-mercaptoethanol. For the induction of neuronal differentiation of P19 cells, cells were cultured in DMEM/F12 (1:1) medium (Gibco, Grand Island, NY) containing 1% N2 supplement (Gibco, Grand Island, NY) and 0.5 mM all-trans-retinoic acid (RA, Sigma, St Louis, MO) and two days after differentiation induction, differentiation medium without RA was used.

Quantification of miRNA 294 expression

Quantitative reverse polymerase chain reaction (qRT-PCR) was performed to quantify the miR-294 expression. On the differentiation day, the total RNA was isolated from CHO cells and P19 cells using TRIzol® Reagent (Invitrogen, Grand Island, NY) and used for qRT-PCR analysis. For miRNA qRT-PCR, miR-Q-assay was used to analyze 200 ng of RNA sample.[1] The relative expression level of miRNA was normalized to small RNA 5S rRNA and for statistical comparison the student's t-test was used.

Stability of the ColoR294 MB

The ColoR294 MB (30 pmol) was incubated in phosphate buffered saline (PBS) and Opti-MEM media (Gibco, Grand Island, NY) at 37 °C up to 4 days. Four days after incubation 30 pmol of miR-294 was added and further incubated for 3 h and the fluorescence intensity of the ColoR294 MB was analyzed using Bio Tek Fluorescent Microplate Fluorometer (Synergy Mx, BioTeck Ltd, VT).

Immunofluorescence and confocal microscopy

CHO cells and P19 cells were seeded onto 24-well culture plates containing gelatin-coated glass cover slips at a density of 5 x 10^4 cells/well. After 24 hr, cells were transfected with the ColoR294 MB (30 pmol) and incubated for 3 hr at 37 °C. Cells were fixed with 4% paraformaldehyde phosphate buffer solution for 15 min and washed three times with PBS buffer. Glass cover slips were then mounted onto the slide glass with a solution containing a 40, 6-diamidino-2-phenylindole dihydrochloride (DAPI) (Vector Laboratories, Inc., CA). Imaging analysis was performed using confocal laser scanning microscopy (LSM 510; Carl Zeiss, Weimer, Germany).

For neuronal differentiation imaging, after fixation, cells were further treated with 0.25 % Triton X-100 (in PBS) for 10 min and washed three times with PBS buffer. Followed by

blocking for 30 min, cells were incubated with anti-Oct-4 antibody (1 : 100, Chemicon, Millipore, Watford, UK) and anti-TuJ-1 antibody (1 : 1000, Santa Cruz Biotechnology, Heidelberg, Germany) at 4 °C overnight. After washing three times with ice-cold PBS buffer, Alexa Fluor-conjugated secondary antibodies were added and incubated for 1 hr. The line scan analysis was performed by scanning of 10 μm distances using LSM image examiner.

Transfection and analysis of fluorescence intensity

CHO cells (5×10^4 cells/well) were seeded onto 24-well tissue culture plate approximately 24 h before transfection. 30 pmol of the ColoR294 MB was transfected using LipofectAMINE and Plus reagent (Invitrogen, Grand Island, NY). Mature miR-294 (0, 1, 10, and 30 pmol) and miR-1 (30 pmol, negative control) was co-transfected into each well. After 3 hr at 37 °C, cells was washed two times with PBS buffer and lysed with radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific Inc., Waltham, MA). The fluorescence intensity was analyzed using Bio Tek Fluorescent Microplate Fluorometer.

In P19 cells, the ColoR294 MB (30 pmol) was transfected into undifferentiated (day 0) and differentiated (day 4) P19 cells, respectively. As a control experiment, miR-294 antagomir (30 pmol) was also co-transfected into undifferentiated P19 cells. The remaining transfection and analysis steps are all the same as for CHO cells. Student's t-test was performed for statistical analysis (p-value < 0.005).

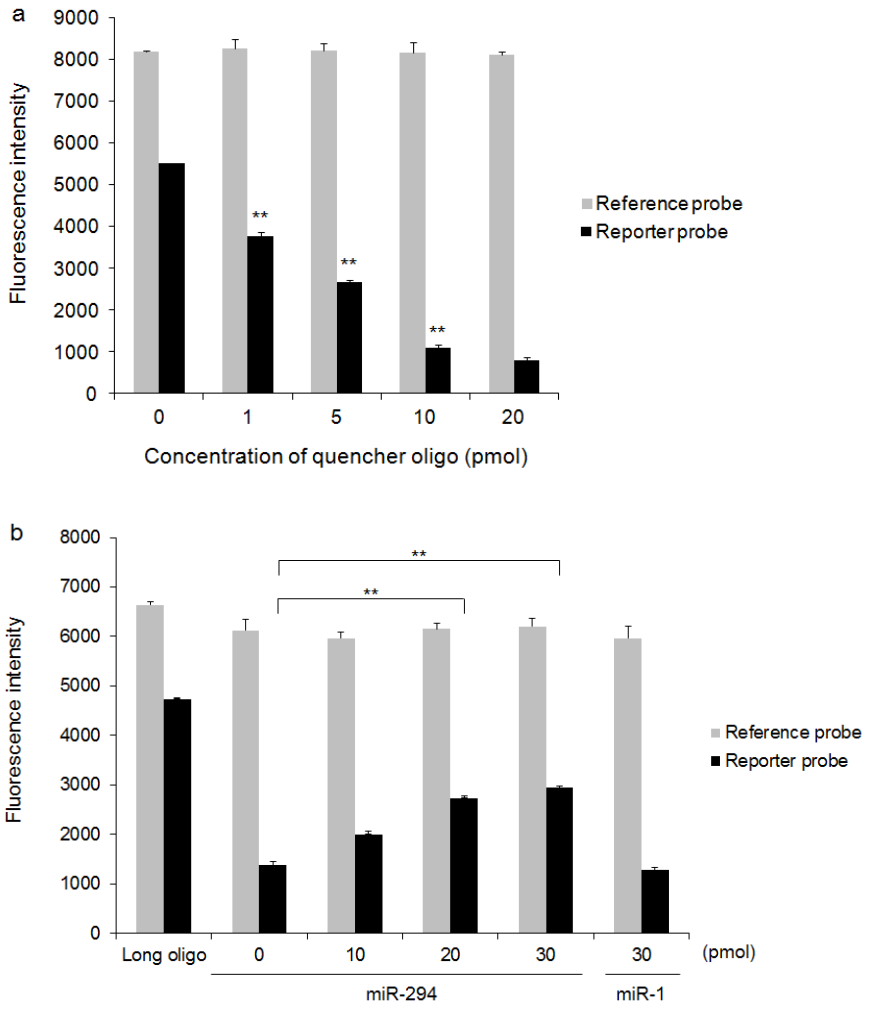

Figure S1. (a) Quantitative intensity of reporter probe and the reference probe in long oligo (10 pmol) was measured by incubating different concentrations (0, 1, 5, 10, and 20 pmol) of the quencher oligo. (b) quantitative intensity of the ColoR294 (30 pmol) against different concentrations (0, 10, 20, 30 pmol) of exogenous miR-294 and miR-1 (30 pmol, negative control). The long oligo (30 pmol) was used as a positive control. Data are represented as means ± standard deviations of triplicate samples (**P < 0.005).

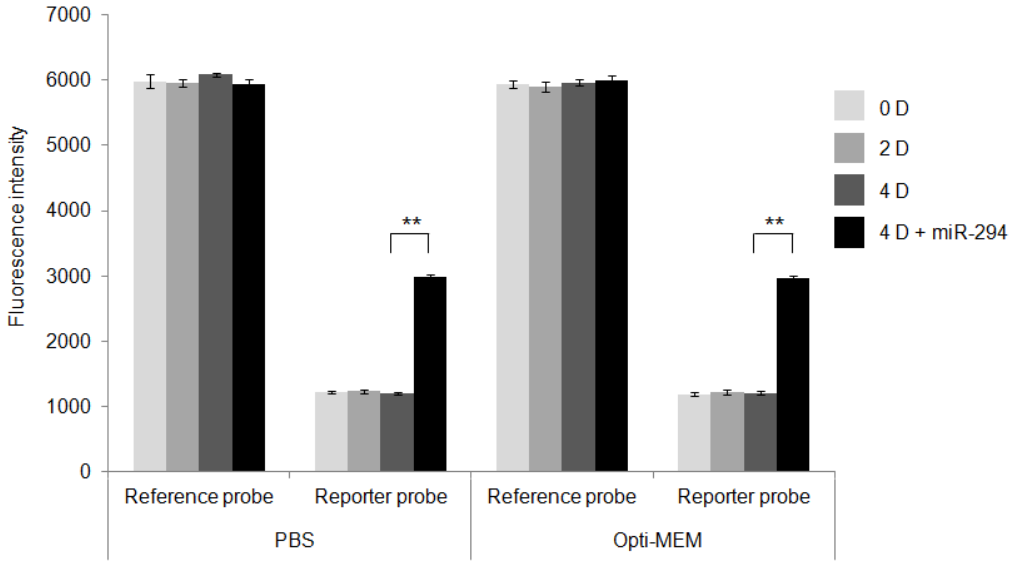

Figure S2. Stability of the ColoR294 MB. 30 pmol of the ColoR294 MB was incubated in PBS buffer and Opti-MEM medium up to 4 days and fluorescence intensities of the reference probe and the reporter probe was measured. Data are represented as means ± standard deviations of triplicate samples (**P < 0.005).

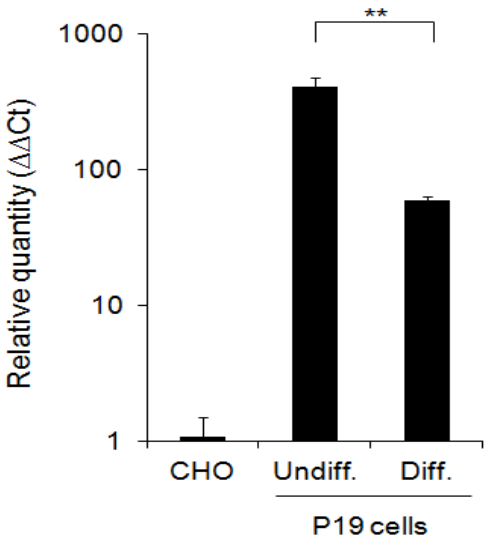

Figure S3. qRT-PCR analysis of an endogenous miR-294 expression both in undifferentiated and differentiated P19 cells. CHO cell was used as a negative control. Data are presented as means ± standard deviations of triplicate samples (**P < 0.005).

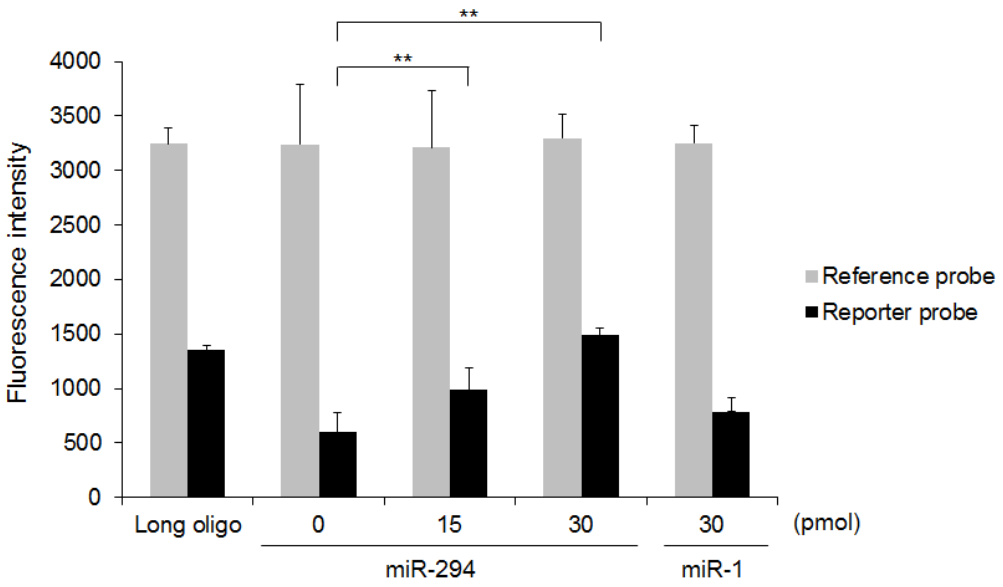

Figure S4. qRT-PCR analysis of an endogenous miR-294 expression both in undifferentiated and differentiated P19 cells. CHO cell was used as a negative control. Data are presented as means ± standard deviations of triplicate samples (**P < 0.005). Analysis of the miR-294 Sensing specificity of the ColoR294 MB in CHO cells by fluorescence intensity. CHO cells were co-transfected with the ColoR294 MB (30 pmol) and either different concentrations (0, 15, and 30 pmol) of exogenous miR-294 or miR-1 (30 pmol, negative control). The long oligo (30 pmol) was used as a positive control. Data are represented as means ± standard deviations of triplicate samples (**P < 0.005).

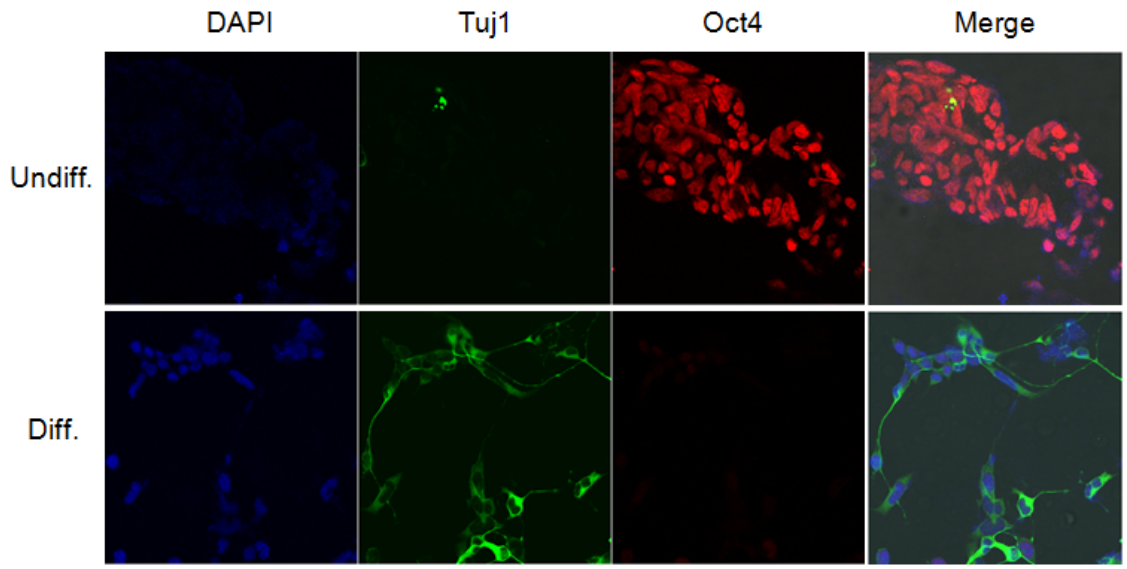

Figure S5. Immunofluorescence analysis of undifferentiated and differentiated P19 cells. For neuronal differentiation, retinoic acid was treated to P19 cells for 4 days. Green signal indicates Tuj1 (neuronal marker), red signal indicates Oct4 (stem cell marker), and blue signal indicates nuclei.

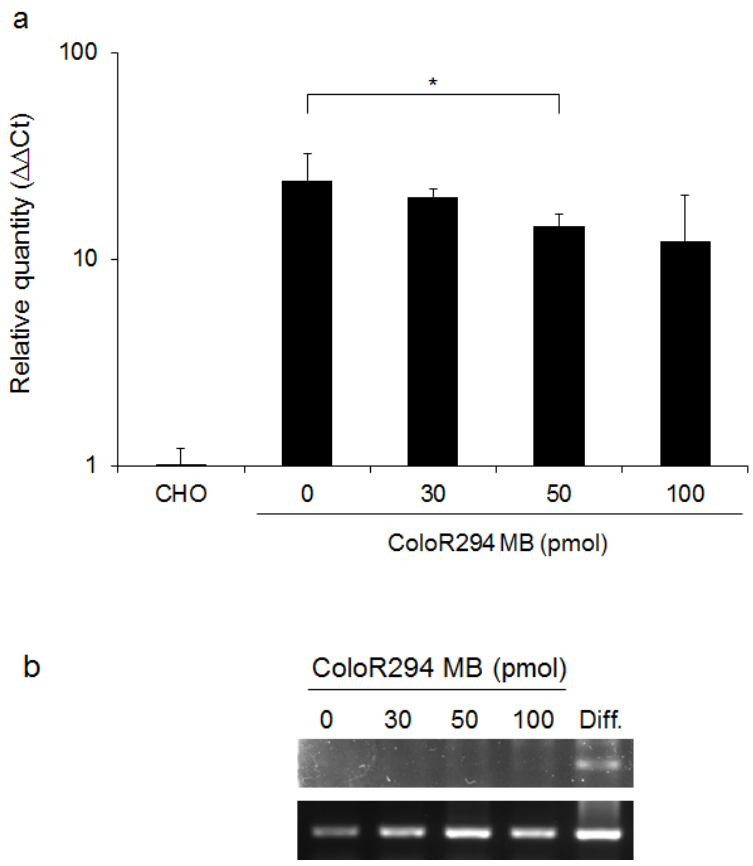

Figure S6. (a) qRT-PCR analysis of miR-294 expression in undifferentiated P19 cells transfected with various concentrations (0, 30, 50, and 100 pmol) of the ColoR294 MB. CHO cells were used as a negative control. Data are represented as means ± standard deviations of triplicate samples (*$P < 0.05$). (b) RT-PCR analysis of Rbl2, which is an endogenous target of miR-294.

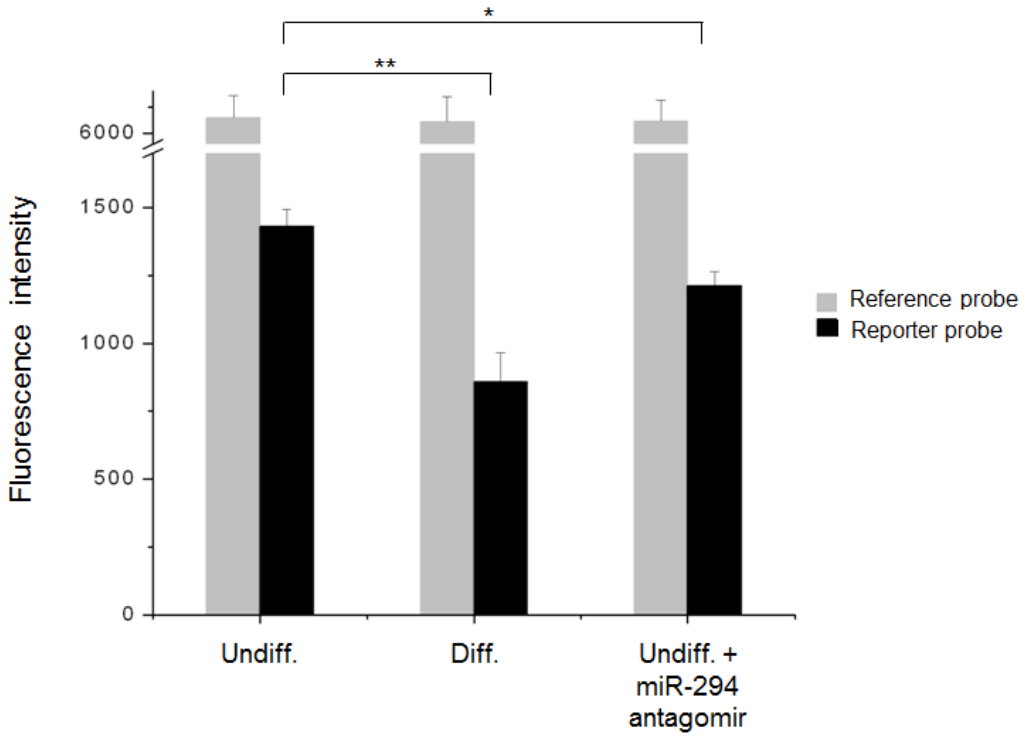

Figure S7. Analysis of the coloR294 MB (30 pmol) during neuronal differentiation in P19 cells. In differentiated P19 cells, the Cy5 signal was decreased because of lower expression of an endogenous miR-294 in differentiated state. The Cy3 signal was constant regardless of neuronal differentiation. Binding inhibition between miR-294 and the ColoR294 MB was confirmed by co-transfection of miR-294 antagomir. Fluorescence intensities are measured as means ± standard deviations of triplicate samples (*$P < 0.05$, **$P < 0.005$).

References

1. S. Sharbati-Tehrani, B. Kutz-Lohroff, R. Bergbauer, J. Scholven and R. Einspanier, *BMC Mol. Biol.*, 2008, **9**, 34.