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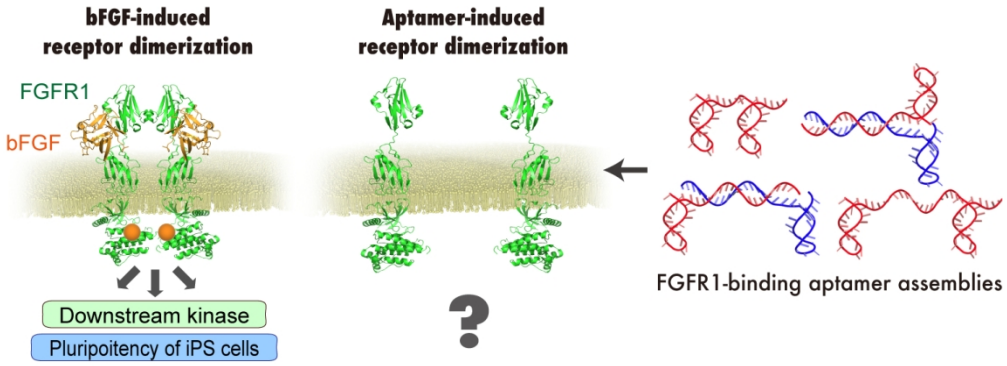
| Journal: | *ChemComm* |
| Manuscript ID | CC-COM-10-2018-008080.R1 |
| Article Type: | Communication |
| | |

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Chemical Communications

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# DNA Aptamer Assemblies as Fibroblast Growth Factor Mimics and Their Application in Stem Cell Culture

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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**Replacing expensive and thermally unstable growth factors with synthetic alteratives has been an important issue in stem cell-based regenerative medicines. Here we developed DNA aptamer-assemblies that act as functional mimics of basic fibroblast growth factor (bFGF), one of the essential factors for stem cell culture. The most potent aptamer assembly named TD0, composed solely of 76-mer single-stranded DNA, could support the self-renewal and pluripotency of induced pluripotent stem cells (iPSCs). This work presents the first application of DNA aptamer in the maintanence of iPSCs.**

Development of synthetic alternatives that mimic the biological activity of a native protein is fascinating albeit challenging tasks in the field of chemistry. As basic units for this aim, DNA is an attractive material owing to its thermal stability, synthetic scalability and structural programmability. The specificity of Watson-Crick base-pairing enables *de novo* design of molecular machineries with defined shapes and functions.[1] In addition, DNA aptamer can be used as an affinity agent for various target molecules such as proteins and small molecules.[2] A number of DNA nano-devices that can recognize target biomolecules have been developed based on DNA aptamers.[3]

In the past few decades, there has been a growing need for synthetic alternatives to basic fibroblast growth factor (bFGF) after establishing culture methods for embryonic stem cells (ESCs)[4] and the discovery of induced pluripotent stem cells (iPSCs).[5] bFGF is one of the most widely used growth factors in stem cell culture because of its ability to maintain pluripotency and the self-renewing activity of the stem cells,[6] which are hallmarks of stem cells and essential for expanding the cell population intended for various downstream applications. Despite its utility, intrinsic drawbacks of recombinant bFGF, such as low thermal stability[7] and batch-to-batch variation, often reduce the integrity of stem cell culture. In addition, the high cost of their production has limited the scalability of stem cell culture, creating an obstacle for stem cell-based regenerative therapy and relevant basic research. Although great efforts have been made to overcome the thermal instability of bFGF, [8] drawbacks remain with these approaches because they rely on protein expression systems.

As an alternative approach, artificial agonists of FGF receptor (FGFR) have been developed.[9] The bFGF binds to FGFR with the help of heparin or heparan sulfate proteoglycans to induce dimerization and subsequent phosphorylation of FGFR (Fig. 1a, left).[10] According to this mechanism, most of the reported FGFR agonists were designed to induce FGFR dimerization. However, none of these agonists has been practically used as an alternative for bFGF, presumably because of their low efficacy, and perhaps because these artificial agonists could not mimic the mode of dimerization and subsequent activation of the receptor induced by native ligands.

Here we developed the first synthetic bFGF-mimic that can support the pluripotency of stem cells. To our knowledge, there has been no report of a synthetic bFGF-mimic that was successfully applied in the maintenance of stem cells. We envisioned that DNA aptamers that work as RTK agonists would be ideal alternatives to recombinant growth factors (Fig. 1a, right).[11] Replacing native growth factors with DNA aptamers has some potential advantages, including eliminating the recombinant expression processes and minimizing the risk of thermal denaturation and contamination. DNA aptamers can be chemically synthesized in uniform quality at a much lower cost ($50 per gram for gram quantities) than recombinant proteins.[2] In addition, the structural programmability of DNA enables us to design synthetic FGFR agonists that present FGFR1-binding aptamers in a unique spatial arrangement, thereby allowing the clustering the

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Electronic Supplementary Information (ESI) available:
See DOI: 10.1039/x0xx00000x

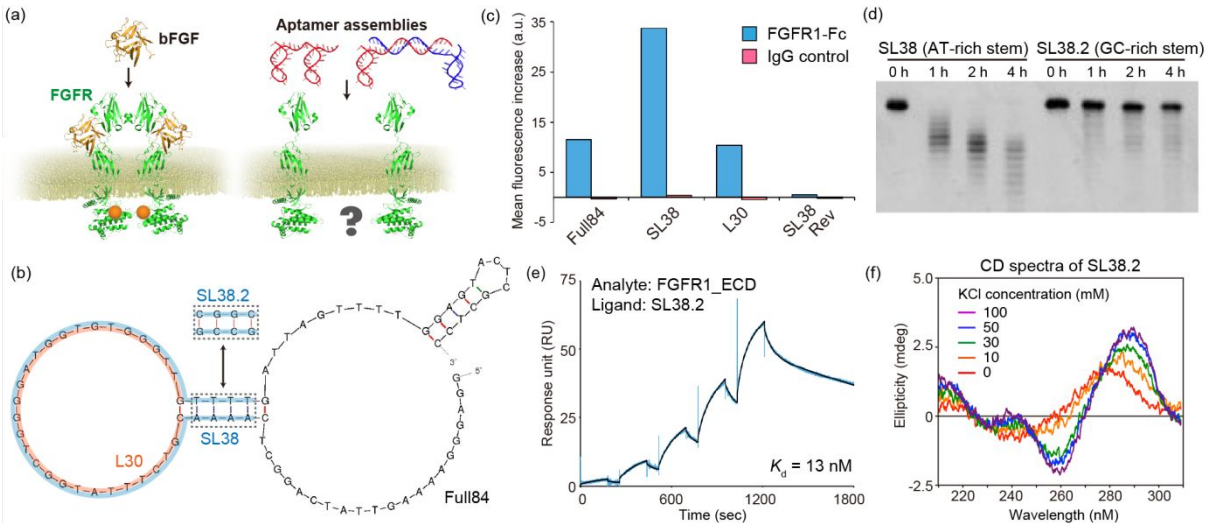


**Fig. 1** (a) FGFR activation induced by bFGF (left) or aptamer assemblies (right). The images are depicted using data from the Protein Data Bank (PDB IDs 1FQ9 and 5UR1). (b) A predicted secondary structure of Full84 that was calculated using Mfold (in 140 mM $Na^+$, 0.4 mM $Mg^{2+}$ at 37°C). (c) Flow cytometry of aptamer binding to protein-immobilized beads. A 5'-FITC-labeled aptamer (400 nM) was incubated with FGFR1-Fc (1 pmol) or human IgG (1 pmol)-immobilized beads for 30 min at 37°C. (d) Nuclease stability of SL38 (left) and SL38.2 (right). Each aptamer (2 μM) was incubated in phosphate-buffered saline containing 10% fetal bovine serum for the indicated time at 37 °C. (e) Surface plasmon resonance measurement of binding kinetics of SL38.2 against the extracellular domain of FGFR1 (2.5, 10, 25, 50 and 100 nM). The sensorgram was fitted to a two-state binding model. (f) CD spectra of SL38.2 (5 μM) in 20 mM Tris-HCl buffer (pH 7.6) supplemented with KCl (0-100 mM) at 37°C.

receptors in a designated manner for efficient receptor activation.

As a starting point, we selected FGFR-binding DNA aptamers *in vitro*. Among four subtypes of FGFR (FGFR1, 2, 3, and 4), we targeted the FGFR1 because this subtype is most abundantly expressed in several human ES cell lines.[12] The *in vitro* selection was conducted using an $N_{40}$ random DNA pool (N40_lib) and PCR primers, which were used in previous work.[13] Some candidate sequences were identified after the sixth round of selection (Fig. S1). Jurek *et al.* have reported an FGFR1-binding-DNA aptamer (named A11) as an agent targeting FGFR1-overexpressing cancer cells.[14] This aptamer sequence, however, was not enriched in our *in vitro* selection. This is probably because of the difference in the diversity of the initial DNA pools, as they used $N_{30}$ random DNA pool.

Among candidate aptamers, we focused on an 84-mer sequence named Full84 based on the secondary structure prediction by Mfold.[15] As shown in Fig.1b, Full84 was predicted to adopt a stem-loop sequence whose loop sequence contains guanine tracts. This structural motif is analogous to a previously reported receptor binding DNA aptamer.[16] To minimize the aptamer sequence, we synthesized two truncated aptamers, a 38-mer stem-loop sequence (SL38) and a 30-mer loop sequence (L30). The binding activity of these truncated sequences was analysed by flow cytometry using FGFR1-Fc-immobilized magnetic beads. As shown in Fig. 1c, SL38 exhibited the highest binding activity among tested aptamers, suggesting that this stem-loop structure is a minimal high-affinity-binding motif for binding to FGFR1. The binding of the reverse sequence of SL38 (SL38Rev) was negligible. While the recombinant FGFR1 was expressed as an Fc-fusion protein, the observed binding was specific to the extracellular domain of FGFR1, as aptamers did not bind to human IgG-immobilized beads. We also confirmed binding of these aptamers to FGFR1-expressing A204 cells using flow cytometer (Fig. S2).

We checked the nuclease resistance of SL38 by incubating the aptamer in 10% fetal bovine serum solution (Fig. 1d). Unfortunately, SL38 was digested in a few hours. We hypothesized that degradation of SL38 was attributed to exonuclease, which is abundant in the serum. The stem sequence of SL38 was substituted with thermally stable GC-rich duplex to improve resistance to the exonuclease[17] (we named this mutant aptamer "SL38.2"). As expected, SL38.2 exhibited higher nuclease stability both in 10% FBS (Fig. 1d) and exonuclease-containing buffer (Fig. S3). We note that SL38.2 has affinity for FGFR1 with a $K_d$ of 13 nM (Fig. 1e), which is almost comparable to that of SL38 ($K_d$ = 4.5 nM, Fig. S4). We focused on SL38.2 in the following experiments because of its higher nuclease stability in typical cell culture conditions.

We elucidated structure-affinity relationships of SL38.2. We anticipated that SL38.2 adopts a G-quadruplex (G4) structure, which has been often found in aptamer sequences, since its loop sequence contains guanine tracts. CD spectra suggested formation of anti-parallel G4 structures as the addition of $K^+$ cation increased the positive peak around 295 nm and the negative peak around 260 nm (Fig. 1f).[18] The ability to adopt the G4 structure correlates with affinity to FGFR1, as introduction of a mutation to the guanine tracts inhibited formation of G4 structures and significantly deteriorated affinity of SL38.2 to FGFR1 (Fig. S5).

Having a potent FGFR1-binding aptamer SL38.2, we next designed four types of assemblies of SL38.2 based on the contention that a dimer ligand for FGFR1 should work as an FGFR1 agonist. As depicted in Fig. 2b, aptamer assemblies were designed to present two aptamers in a different geometry in terms of distance, orientation, and flexibility (see

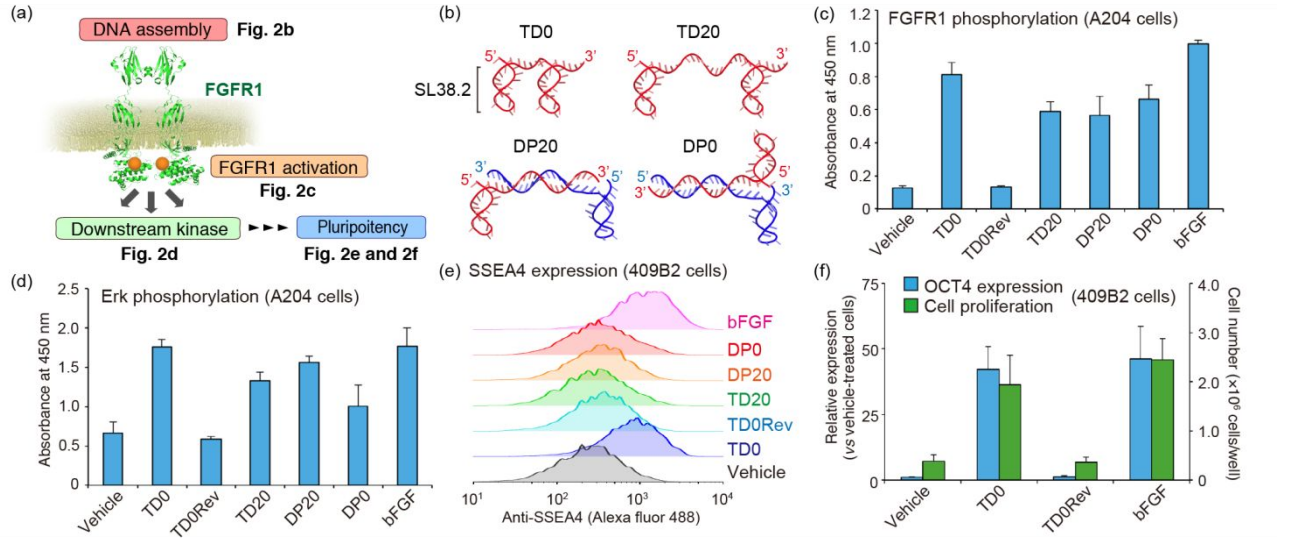


**Fig. 2 Design of aptamer assemblies and their agonistic functions** (a) Overview of experiments shown in Fig. 2. (b) Schematic representation of the FGFR1 agonists. (c) and (d) FGFR1 and Erk phosphorylation induced by bFGF or an aptamer assembly. A204 cells were stimulated by bFGF (2 nM) in the presence of heparin (10 μg/mL) or each aptamer assembly (500 nM) in the absence of heparin for 5 min. The data were expressed as mean values (n = 3) of absorbance at 450 nm. (e) and (f, blue) Expression of pluripotent markers in 409B2 hiPSCs maintained in the presence of bFGF (3 nM) or an aptamer assembly (500 nM) for 7 days. Error bars indicate standard deviation (n = 3). (f, green) Cell proliferation during the culture experiment. The hiPSCs were maintained using the same culture protocol as described in Fig. 3e. Error bars indicate standard deviation (n = 3).

Fig. S6). Briefly, the "TD0" and "TD20" were synthesized as single-stranded, tandem dimer forms. In the case of TD20, a 20-mer thymidine linker was inserted between two aptamers. "DP0" and "DP20" were prepared as an assembly of two aptamer strands with a 20-mer hybridization linker. In these assemblies, the two aptamers are presented in the same (DP0) or opposite (DP20) directions. Formation of these aptamer assemblies was confirmed by native-PAGE (Fig. S6).

The agonism by these ligands was tested using FGFR1-expressing A204 cells. The cells were incubated with an aptamer assembly and the level of phosphorylation of FGFR1 was evaluated by ELISA (Fig. 2c). Among the agonists designed, TD0 exhibited the highest FGFR1-activating potential, while its reverse sequence (TD0Rev) did not affect the level of phosphorylation. We also checked the phosphorylation of Erk induced by these FGFR1 agonists (Fig. 2d). This kinase downstream of FGFR1 has been implicated in the maintenance of pluripotency and the self-renewing activity of human ES cells and human iPSCs (hiPSCs).[19] The data revealed that the level of phosphorylation of Erk was significantly increased after adding bFGF or aptamer assemblies. These data indicate that the aptamer assemblies can induce intracellular signalling as well as FGFR1 phosphorylation.

We evaluated the potential of these aptamer assemblies to maintain the pluripotency of hiPSCs. Culture medium that requires supplementation with bFGF to maintain the pluripotency of hiPSCs (see supporting information) was supplemented with each aptamer assembly (500 nM). After 7 days culture, we analysed the level of expression of a pluripotent cell marker (SSEA-4) using flow cytometry (Fig. 2e). Compared with bFGF-treated cells, the expression of SSEA-4 was significantly decreased in the cells cultured with vehicle control, indicating the necessity for bFGF-supplementation to maintain the pluripotency of hiPSCs under these culture conditions. Among the aptamer assemblies tested, TD0 could sustain the expression of SSEA-4, while its reverse sequence, TD0Rev, could not support the pluripotency of hiPSCs. Interestingly, no aptamer assemblies except for TD0 could sustain the expression of SSEA-4, despite activating intracellular signalling molecule (Fig. 2e). Although the actual mechanism remains unclear, this result implies that the mode of FGFR dimerization may dictate resulting biological outcomes.

Finally, we asked whether TD0 could replace the role of bFGF in maintaining hiPSCs. In immunostaining, the expression of SSEA-4 was confirmed throughout the colonies of hiPSCs that were cultured with bFGF (3 nM) or TD0 (500 nM) (Fig. S7). By contrast, the expression was decreased significantly in cells cultured with TD0Rev (500 nM). We next determined the expression of pluripotent marker OCT4 quantitatively using RT-qPCR (Fig. 2f). As expected, the expression of OCT4 was decreased significantly when the cells were cultured in medium with vehicle control alone. However, the level of expression of OCT4 was significantly up-regulated when the cells were cultured in the presence of TD0 or bFGF. We also evaluated the self-renewing activity of the TD0-treated cells by cell counting after the culture (Fig. 2f). We found that cell proliferation was inhibited significantly in the absence of bFGF. By contrast, adding TD0 could rescue the absence of bFGF. Taken together, these findings indicate that TD0 could support both the self-renewing activity and pluripotency of hiPSCs.

## Conclusions

To summarize, we developed DNA aptamer assemblies as a novel class of FGFR1 agonists. Our data indicate that the spatial arrangement of the FGFR-binding aptamers in a DNA assembly is a determining factor for both their efficacy in FGFR1 phosphorylation and their ability to maintain the pluripotency of hiPSCs. Importantly, this work presents the

first application of agonistic aptamers in the maintenance of stem cell culture. Taking the synthetic scalability of oligonucleotides into account,[2] it may facilitate large-scale stem cell culture, which is not practical because of the costliness of recombinant growth factors.[20]

We note that the current culture protocol using TD0 could not sustain the expression of pluripotent markers in hiPSCs in long-term culture (data not shown). This implies that the agonists may not perfectly mimic the pattern of activation of intracellular signalling induced by bFGF. Possible solutions may include (1) optimizing the culture protocol in terms of composition of basal medium and frequency of medium change, or (2) supplementing with small molecule inhibitors targeting the downstream signalling molecules.[21] Another point should be noted is higher concentrations of TD0 were required for the maintenance of hiPSCs compared to that of bFGF. This is consistent with the fact that half-maximal effective concentration ($EC_{50}$, A204 cells) of TD0 is higher than that of bFGF (2.7 nM *vs* 0.59 nM, Fig. S8). Although we also tested the performance of tandem dimer of SL38 without linker (TD0_SL38), it was less potent in both FGFR1 activation ($EC_{50}$ = 8.0 nM, Fig. S8) and maintenance of hiPSC (Fig. S9). This issue may be tackled by further evolution of SL38.2 or other candidate aptamers found in the current *in vitro* selection (Fig. S10). Our future work will be conducted along these lines.

## Conflicts of interest

The authors (R.U., S.A., A.U. and S.S.) have filed a provisional patent application (No. 62/584,755).

## Acknowledgements

This work was supported by a Core Research for Evolutional Science and Technology (CREST) of Molecular Technology (No. JPMJCR13L4), Japan Science and Technology Agency, grant to S.S and KAKENHI (No. 17K14512), Japan Society for the Promotion of Science, grant to R.U.

## References

1 (a) X. Ouyang, M. De Stefano, A. Krissanaprasit, A. L. Bank Kodal, C. Bech Rosen, T. Liu, S. Helmig, C. Fan and K. V. Gothelf, *Angew. Chem. Int. Ed.* 2017, ***56,*** 14423; (b) F. Praetorius and H. Dietz, *Science* 2017, ***355,*** eaam5488; (c) H. G. Franquelim, A. Khmelinskaia, J. P. Sobczak, H. Dietz and P. Schwille, *Nat. Commun.* 2018, ***9,*** 811; (d) T. Kurokawa, S. Kiyonaka, E. Nakata, M. Endo, S. Koyama, E. Mori, N. H. Tran, H. Dinh, Y. Suzuki, K. Hidaka, M. Kawata, C. Sato, H. Sugiyama, T. Morii and Y. Mori, *Angew. Chem. Int. Ed.* 2018, ***57,*** 2586.

2 J. Zhou and J. Rossi, *Nat. Rev. Drug Discov*. 2017, ***16,*** 181.

3 (a) J. Zhao, J. Gao, W. Xue, Z. Di, H. Xing, Y. Lu and L. Li, *J. Am. Chem. Soc.* 2018, ***140,*** 578; (b) H. Liang, S. Chen, P. Li, L. Wang, J. Li, H. H. Yang and W. Tan, *J. Am. Chem. Soc.* 2018, ***140,*** 4186; (c) H. Li, M. Wang, T. Shi, S. Yang, J. Zhang, H. H. Wang and Z. Nie *Angew. Chem. Int. Ed.* 2018, ***57,*** 10226; (d) C. Shan, J. Li, H. Liang, L. Xia-Hui, L. Juan, and H. H. Yang, *Chem. Eur. J.* 2018, **24,** 15988.

4 J. A. Thomson, J. Itskovitz-Eldor, S. S. Shapiro, M. A. Waknitz, J. J. Swiergiel, V. S. Marshall and J. M. Jones, *Science* 1998, ***282,*** 1145.

5 K. Takahashi and S. Yamanaka, *Cell* 2006, ***126,*** 663.

6 (a) R. H. Xu, R. M. Peck, D. S. Li, X. Feng, T. Ludwig and J. A. Thomson, *Nat. Methods* 2005, ***2,*** 185; (b) M. E. Levenstein, T. E. Ludwig, R. H. Xu, R. A. Llanas, K. VanDenHeuvel-Kramer, D. Manning, and J. A. Thomson, *Stem Cells* 2006, ***24,*** 568.

7 G. Chen, D. R. Gulbranson, P. Yu, Z. Hou and J. A. Thomson, *Stem Cells* 2012, ***30,*** 623.

8 (a) K. Motomura, A. Hagiwara, A. Komi-Kuramochi, Y. Hanyu, E. Honda, M. Suzuki, M. Kimura, J. Oki, M. Asada, N. Sakaguchi, F. Nakayama, M. Akashi and T. Imamura, *Biochim Biophys Acta.* 2008, ***1780,*** 1432; (b) T. H. Nguyen, S. H. Kim, C. G. Decker, D. Wong, J. A. Loo and H. D. Maynard *Nat. Chem.* 2013, ***5,*** 221; (c) S. Lotz, S. Goderie, N. Tokas, S. E. Hirsch, F. Ahmad, B. Corneo, S. Le, A. Banerjee, R. S. Kane, J. H. Stern, S. Temple and C. A. Fasano, *PLoS One* 2013, ***8,*** e56289; (d) U. Han, H. Park, Y. J. Kim, T. H. Park, J. H. Park and J. Hong, *ACS Appl. Mater. Interfaces* 2017, ***9,*** 25087.

9 (a) M. D. Ballinger, V. Shyamala, L. D. Forrest, M. Deuter-Reinhard, L. V. Doyle, J. X. Wang, L. Panganiban-Lustan, J. R. Stratton, G. Apell, J. A. Winter, M. V. Doyle, S. Rosenberg and W. M. Kavanaugh, *Nat. Biotechnol.* 1999, ***17,*** 1199; (b) X. Lin, K. Takahashi, S. L. Campion, Y. Liu, G. G. Gustavsen, L. A. Peña and P. O. Zamora, *Int. J. Mol. Med.* 2006, ***17,*** 833; (c) S. Li, E. Bock and V. Berezin, *Int. J. Mol. Sci.* 2010, ***11,*** 2291; (d) A. L. Wu, G. Kolumam, S. Stawicki, Y. Chen, J. Li, J. Zavala-Solorio, K. Phamluong, B. Feng, L. Li, S. Marsters, L. Kates, N. van Bruggen, M. Leabman, A. Wong, D. West, H. Stern, E. Luis, H. S. Kim, D. Yansura, A. S. Peterson, E. Filvaroff, Y. Wu and J. Sonoda, *Sci. Transl. Med.* 2011, ***3***:113ra126; (e) S. Pankratova, H. Bjornsdottir, C. Christensen, L. Zhang, S. Li, O. Dmytriyeva, E. Bock and V. Berezin, *Mol. Neurobiol.* 2016, ***53,*** 584.

10 (a) M. A. Lemmon and J. Schlessinger, *Cell* 2010, ***141,*** 1117; (b) D. M. Ornitz and N. Itoh, *WIREs Dev. Biol.* 2015, ***4,*** 215.

11 (a) N. O. Yunn, A. Koh, S. Han, J. H. Lim, S. Park, J. Lee, E. Kim, S. K. Jang, P. O. Berggren and S. H. Ryu, *Nucleic Acids Res*. 2015, ***43,*** 7688; (b) V. Ramaswary, A. Monsalve, L. Sautina, M. S. Segal, J. Dobson and J. B. Allen, *Nucleic Acid Ther.* 2015, ***25,*** 227; (c) R. Ueki, A. Ueki, N. Kanda and S. Sando, *Angew. Chem. Int. Ed.* 2016, ***55,*** 579; (d) R. Ueki, S. Atsuta, A. Ueki and S. Sando, *J. Am. Chem. Soc.* 2017, ***139,*** 6554.

12 P. Dvorak, D. Dvorakova, S. Koskova, M. Vodinska, M. Najvirtova, D. Krekac and A. Hampl, *Stem Cells.* 2005, ***23,*** 1200.

13 A. Boltz, B. Piater, L. Toleikis, R. Guenther, H. Kolmar and B. Hock, *J. Biol. Chem.*, 2011, **286**, 21896.

14 P. M. Jurek, K. Zabłocki, U. Waśko, M. P. Mazurek, J. Otlewski and F. Jeleń, *Int. J. Nanomed.* 2017, ***12,*** 2941.

15 M. Zuker *Nucleic Acids Res.* 2003, ***31,*** 3406.

16 R. Ueki and S. Sando, *Chem. Commun.* 2014, ***50,*** 13131.

17 I. M. Khan and J. M. Coulson, *Nucleic Acids Res.* 1993, **21** 2957.

18 M. Vorlíčková, I. Kejnovská, J. Sagi, D. Renčiuk, K. Bednářová, J. Motlová and J. Kypr, *Methods* 2012, **57**, 64.

19 J. Li, G. Wang, C. Wang, Y. Zhao, H. Zhang, Z. Tan, Z. Song, M. Ding and H. Deng, *Differentiation* 2007, ***75,*** 299.

20 S. Yasuda, T. Ikeda, H. Shahsavarani, N. Yoshida, B. Nayer, M. Hino, N. Vartak-Sharma, H. Suemori and K. Hasegawa, *Nat. Biomed. Eng.* 2018, ***2,*** 173.

21 M. Kinehara, S. Kawamura. D. Tateyama, M. Suga, H. Matsumura, S. Mimura, N. Hirayama, M. Hirata, K. Uchio-Yamada, A. Kohara, K. Yanagihara and M. K. Furue, *PLoS One* 2013, ***8,*** e54122.