Aptamers Hot Paper

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Oligonucleotide-Based Mimetics of Hepatocyte Growth Factor

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Abstract: *Oligonucleotide-based hepatocyte growth factor (HGF) mimetics are described. A DNA aptamer to Met, a cognate receptor for HGF, was shown to induce Met activation when used in dimer form. The most potent aptamer dimer, ss-0, which was composed solely of 100-mer single-stranded DNA, exhibited nanomolar potency. Aptamer ss-0 reproduced HGF-induced cellular behaviors, including migration and proliferation. The present work sheds light on oligonucleotides as a novel chemical entity for the design of growth factor mimetics.*

The hepatocyte growth factor (HGF) is a polypeptide ligand for the receptor Met, which belongs to the receptor tyrosine kinase family.[1] Binding of HGF to Met at the cell surface induces dimerization and subsequent autophosphorylation of Met. This phosphorylation event initiates intracellular signal transduction that regulates various cellular functions, including proliferation, differentiation, and migration (Figure 1).[1]

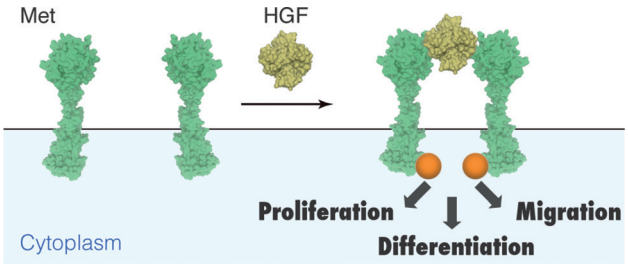

Figure 1. Schematic representation of HGF-induced Met signaling. The structures of Met and HGF are taken from the structures in the Protein Data Bank (PDB IDs 2UZY, 3A4P, and 1NK1).

Because of these biological activities, recombinant human HGF (rhHGF) has attracted much attention in regenerative therapy. Since HGF promotes the survival and proliferation of a broad spectrum of cells, rhHGF has been applied to the treatment of various diseases, wound healing, and tissue regeneration.[1a] In addition, rhHGF is often used as a supplement in cell culture media to reproduce biological environments in vitro. One example is induction of the differentiation of embryonic stem (ES) cells or induced pluripotent stem (iPS) cells into functional hepatocyte cells.[2,3] This approach has attracted much attention as an unlimited potential source of patient-specific functional hepatocytes.[3]

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Despite its potential, however, the adoption of rhHGF for such applications is often impeded by its limited scalability. rhHGF is a large (80 kDa) and complex multidomain polypeptide; the production hosts are thus limited to insect or mammalian cells.[4a] This fact renders the production of rhHGF laborious and expensive. To circumvent this limitation, great efforts have been made to replace rhHGF with alternative Met agonists. To date, a major approach has relied on protein engineering, focusing on splice variants of HGF or a Met-binding antibody.[4] However, these protein Met agonists still hold some inherent drawbacks, such as the risk of thermal denaturation or contamination.

Recently, synthetic compounds have emerged as a promising alternative. Ito et al. reported the first example of HGF mimetics based on non-protein molecules.[5] Those authors screened artificial macrocyclic peptides that bind to Met, and tethered two Met-biding peptides through a chemical linker to induce Met activation. That work clearly demonstrated the feasibility and potential of HGF mimetics. However, this method requires multiple preparation steps, including macrocyclic peptide synthesis and subsequent chemical conjugation. Moreover, access to such unique material is limited for most researchers, especially non-chemists.

In this context, we envisioned that oligonucleotides would be an ideal chemical entity for the design of "accessible" growth factor mimetics. Oligonucleotides can be synthesized by using the well-established automated solid-phase synthesis in a simple, fast, and cost-effective manner.[6] The SELEX methodology[7] can generate a receptor-binding aptamer that would be a good starting point for the design of an artificial ligand that controls the activity of target receptors.[8]

Herein, we describe the development of oligonucleotide-based HGF mimetics that function as Met agonists. We focused on CLN0003_SL1 (SL1), a previously reported 50-mer DNA aptamer that binds to Met.[9] Taking into account the mechanism of activation of Met,[10] we hypothesized that a dimerized SL1 (Di-SL1) binds to two Met molecules, thereby allowing Met dimerization and activation (Figure 2a).[11]

To test this hypothesis, we produced a Di-SL1 oligonucleotide through hybridization of two SL1 monomers with a complimentary strand at their 5' termini (SL1 A and SL1 B in Figure 2b; full sequences in Figure S1 in the Supporting Information). The Met activation potential of Di-SL1 was confirmed by immunoblotting (Figure 2b). The Met-expressing A549 cells were treated with Di-SL1 (1 nM), and phosphorylated Met was detected. While neither of the SL1 monomers affected the Met phosphorylation level compared to the vehicle control (lanes 1–3, Figure 2b), a significant signal enhancement was observed in the sample treated with Di-SL1 (lane 4, Figure 2b). Importantly, Met phosphorylation was suppressed when the formation of Di-SL1 was

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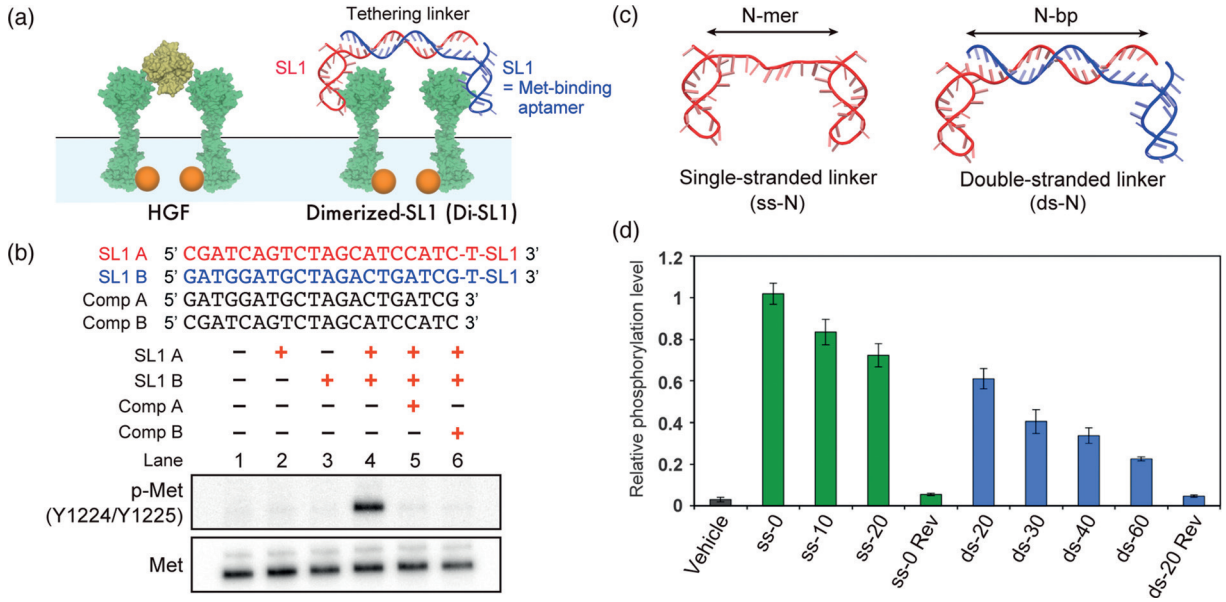

Figure 2. a) Schematic representation of oligonucleotide-based HGF mimetics. b) Immunoblotting of A549 cell lysates treated with SL1 A and/or SL1 B (1 nM each). Lanes 5 and 6 show samples to which Comp A or Comp B (50 nM each) was added to inhibit the formation of Di-SL1. c) Schematic representation of Di-SL1 with an N-mer single-stranded linker (ss-N) or N-bp double-stranded linker (ds-N). d) The level of Met phosphorylation in A549 cell lysates treated with Di-SL1s (10 nM) for 15 min. The data are expressed as mean relative values ($n=3$) compared to the level of Met phosphorylation induced by treatment with HGF (1 nM). The error bars indicate the standard deviation (SD).

inhibited by the addition of excess amount (50 nM) of complimentary strand to the hybridization sites (lanes 5 and 6 in Figure 2b). Moreover, the addition of SU11274, a Met-selective kinase inhibitor, suppressed the Met phosphorylation that was induced by Di-SL1 (Figure S2). Together, these results strongly support the hypothesis that Di-SL1 works as a Met agonist.

Next, we optimized the linker moiety of Di-SL1 to achieve efficient activation of Met. We designed Di-SL1s with a single-stranded N-mer linker (ss-N) or a double-stranded N-bp linker (ds-N, Figure 2c and Figure S3; full sequences in the Supporting Information). The Met phosphorylation level in A549 cell lysates treated with these Di-SL1s (10 nM) was evaluated by ELISA. As shown in Figure 2d, all of the Di-SL1s exhibited an increased Met activation potential compared to vehicle control and Di-SL1s constructed by using the reverse sequence of SL1 (ss-0 Rev, and ds-20 Rev). In addition, this experiment revealed that Di-SL1 with shorter linkers tended to exhibit higher activation potentials at this concentration (10 nM).

We further evaluated the concentration-dependent Met activation profiles of HGF, ss-0, ss-20, and ds-20 (Figure 3a). Among these Di-SL1s, ss-0 exhibited the highest Met activation potential at the range of concentrations tested. Interestingly, while the apparent half-maximum effective concentration (EC_{50}) values of these Di-SL1s were comparable (1–5 nM), the maximal Met phosphorylation level induced by ss-0 was significantly higher than that induced by ss-20 or ds-20. Given the fact that two Met molecules should be in proximity during the activation process,[10] this result may stem from the varied dimerization geometry of Met receptors mediated by these Di-SL1s.

Under our experimental conditions, the apparent EC_{50} value of HGF was estimated to be in the range of 100–250 pM (Figure 3a). Although the apparent EC_{50} value of ss-0 (1–5 nM) was slightly higher than that of HGF, it is lower than or comparable to that of previously reported non-protein Met agonists.[5] Based on the results in Figure 2d and Figure 3a, we focused on ss-0 as the most potent Met agonist among the designed Di-SL1s. A comparison of ss-0 and its equivalent monomeric aptamers in terms of Met activation potential clearly indicated that dimerization is critical to induce Met activation (Figure S4). The immunoblotting experiments revealed that ss-0 triggered downstream signaling from Met (Figure 3b). The cells treated with ss-0 or HGF showed phosphorylation profiles that were similar to those of molecules downstream of Met, such as Gab1, Akt, and ERK1/2.

To validate the applicability of ss-0 as a Met agonist, we checked whether ss-0 could reproduce HGF-induced cellular behaviors. Cell migration is one of the representative biological phenotypes that are promoted by HGF/Met signaling.[1] In the DU145 cell-scattering assay[12] (Figure 3c, Figure S5, and videos in the Supporting Information), cells treated with HGF (500 pM) or ss-0 (5 nM) exhibited intensive dissociation of colonies as a result of increased cell motility. The dissociation of colonies was scarcely observed in cells treated with the vehicle control or ss-0 Rev (5 nM).

To further confirm this effect on cell motility, we performed a SUIT-2 cell-migration assay.[9a] In this assay, the cells were seeded on culture inserts equipped with a PET membrane with 8 μm pores. The migrating cells that reach the pore can pass through the membrane. The result was consistent with the DU145 cell scattering: the addition of

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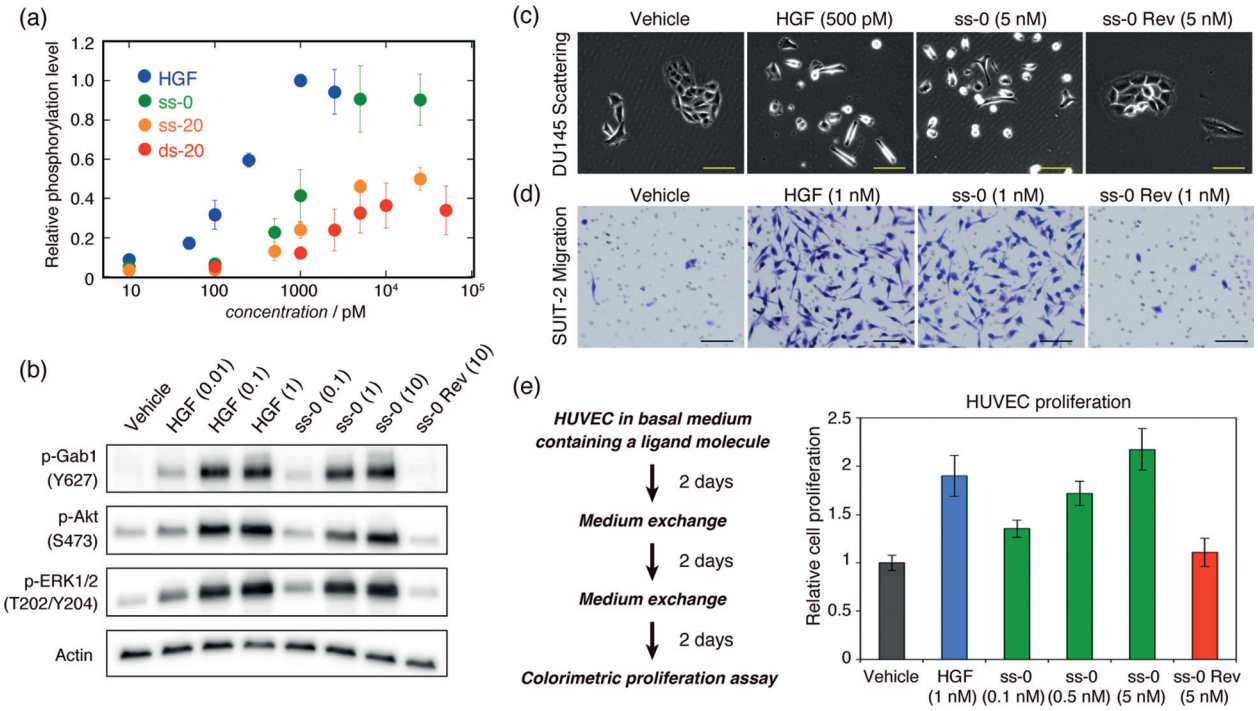

Figure 3. a) The level of Met phosphorylation in A549 cell lysates treated with HGF (blue), ss-0 (green), ss-20 (orange), or ds-20 (red). The cells were treated with the ligands for 15 min. The data are expressed as mean relative values ($n=3$) compared to the level of Met phosphorylation level induced by treatment with HGF (1 nM). The error bars show the SD. b) Immunoblotting analysis of the molecules downstream of Met. The cells were treated with the ligands for 15 min. The numbers in parentheses indicate the concentration (nM) of the ligands. c) DU145 cell scattering. The cells were cultured in the presence of the ligands for 24 h. Scale bars: 100 μm. d) SUIT-2 Migration. The cells were cultured in the presence of the ligands for 18 h. Scale bars: 100 μm. e) HUVEC proliferation. The cells were culture in the presence of the ligands for 6 days. The medium was replaced every 2 days. The cell proliferation in each well ($n=8$) was evaluated by using Cell Counting Kit-8 (Dojindo). The error bars show the SD.

HGF (1 nM) and ss-0 (1 nM) enhanced SUIT-2 migration compared to ss-0 Rev (1 nM) and the vehicle control (Figure 3d and Figure S6). Migration was also evaluated quantitatively by using a live-cell-specific fluorescent dye. The fluorescence intensity detected in the cells that migrated was enhanced significantly in the presence of HGF or ss-0, whereas the addition of ss-0 Rev did not affect the results (Figure S7). These cell-based experiments clearly indicate that ss-0 not only induces Met activation, but also reproduces HGF-induced cellular behaviors.

Encouraged by these results, we used ss-0 to induce the proliferation of normal human cells. As a model system, we cultured human umbilical vein endothelial cells (HUVECs) in the presence of HGF, ss-0, or ss-0 Rev (Figure 3e). HGF induces the proliferation of HUVECs. Under our experimental conditions, the addition of HGF (1 nM) stimulated proliferation by 1.9-fold compared to the vehicle control. In cells treated with ss-0, proliferation was also promoted in a concentration-dependent manner. The cell proliferation enhancement reached 2.2-fold (vs. vehicle control) after incubation with ss-0 (5 nM). Conversely, ss-0 Rev (5 nM) did not affect HUVEC proliferation. We thus conclude that ss-0 stimulates HUVEC proliferation in a Met-signaling-dependent manner.

In summary, we present ss-0, the first oligonucleotide-based HGF mimetic. The ss-0 oligonucleotide stimulates the migration and proliferation of cells and exhibits biological potential as an artificial growth factor. This fact implies the feasibility of regenerative medicine based on ss-0. Another powerful application of ss-0 is its use as an additive in cell culture media, as demonstrated herein. A series of cell-based experiments support the compatibility of ss-0 with cell culture conditions (Figure 3c,d,e). In denaturing PAGE, the full-length ss-0 oligonucleotide could be observed after 24 h incubation in the presence of 10% heat-inactivated fetal bovine serum (Figure S8). Although rhHGF has been adopted in primary cell cultures and for the induction of stem cell differentiation in vitro, its high production cost has been a major obstacle to its application to large-scale cell culture. The ss-0 oligonucleotide, which is a 100-mer ssDNA that can be produced through typical solid-phase synthesis, would be an ideal replacement for rhHGF in these applications.

One noteworthy fact in the present work is that the linker design of Bi-SL1 affects its Met activation potential (Figure 2d and Figure 3a). Previous studies have demonstrated that nucleic acid based ligand assemblies can be used to control protein activities.[13] These studies suggest it is feasible to design molecular scaffolds to optimize the geometry of receptor clustering mediated by Met-binding aptamers. Future studies along these lines would provide deeper insight to the design of more productive HGF mimetics.

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