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Nongenetic control of receptor signaling dynamics with a DNA-based optochemical tool

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Optochemical tools that can modulate activity of the target protein provide an opportunity for studying and regulating the related biological processes. Here we present a DNA-based nongenetic optochemical tool that can control dynamics of growth factor signaling. This photo-caged mimicry of growth factor can be a promising tool for elucidating a linkage between dynamics of signaling and resulting biological outcomes, as well as for manipulating cellular functions and the fate of living cells.

Growth factors (GFs) are polypeptide ligands that regulate various cellular activities such as proliferation, migration, and differentiation. Binding of GFs to receptor tyrosine kinases (RTKs) on the cell membrane induces dimerization and subsequent phosphorylation of the RTKs and initiates phosphorylation cascades of intracellular kinases.[1] Intriguingly, the activation dynamics of kinases downstream of RTKs plays an important role in determining a cellular function and fate (Fig. 1).[2] For example, in rat PC12 cells, both epidermal growth factor (EGF) and nerve growth factor (NGF) activate the Ras-Raf-Mek-Erk pathway via activation of their cognate receptors, but the dynamics of kinase activation and the resulting cellular outcomes show unique patterns.[3] EGF causes transient activation of Erk that leads to cell proliferation, while NGF causes sustained activation of Erk that leads to cell differentiation. To date, much attention has been focused on the mechanisms that initiate such dynamics of GF signaling, thereby regulating cellular functions. Therefore, a method capable of controlling the activity of RTKs with a precise temporal resolution is highly sought after for studying receptor-level mechanisms guiding the dynamics of GF signaling and rewiring a cellular function of living cells by modulating the signaling dynamics.[4] Optochemical tools that target the interaction between GF and RTK represent a promising approach for the regulation of the dynamics of GF signaling without any gene transfer process. Luebke *et al.* reported synthesis of EGF containing a photo-caged glutamate at the Glu40 position, which is involved in the interaction with the receptor of EGF, using solid phase peptide synthesis (SPPS).[5] However, this strategy cannot be applied to the majority of GFs, as they consist of large and complex polypeptides with post-translational modifications, which render their preparation via SPPS challenging and labor-intensive.

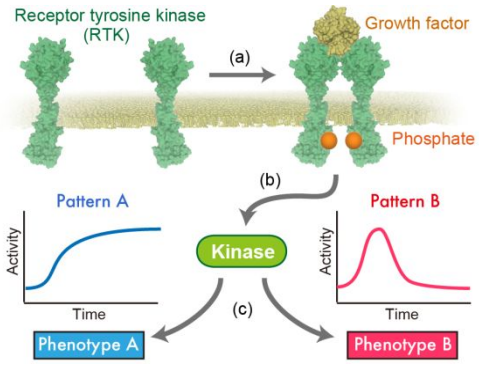

Fig.1 Schematic representation of GF signaling and its implication in determining cell function and fate. (a) Binding of GF induces dimerization and subsequent phosphorylation of receptor tyrosine kinase (RTK) on the cell membrane. (b) The activated RTK triggers phosphorylation cascades of intracellular kinases. (c) The dynamics (*e.g.* amplitude, duration, and frequency) of kinase activity determine the biological outputs.

In this work, we present a nongenetic temporal control of GF signaling, using a synthetic surrogate of GF. We adopted DNA aptamer as a building block due to its synthetic accessibility and amenability to chemical modifications. Previously, we demonstrated that the aptamer binding to an RTK[6] could be used as a synthetic switch, which can control dimerization state of the RTK, depending on external cues.[7a] The RTK-binding aptamer was designed to form a "sandwich" with a target molecule by tethering the aptamer with an appropriate ligand

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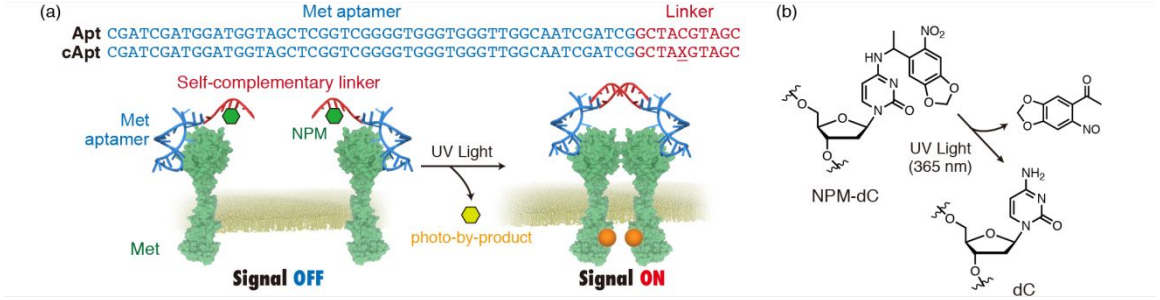

Fig.2 (a) DNA sequences used in the present study and schematic representation of light-induced receptor dimerization with a caged DNA aptamer. The Met-binding aptamer sequence (50-mer) and self-complementary linker sequence (10-mer) are shown in blue and red, respectively. The site of NPM-dC modification is denoted by X. The structure of Met was depicted by using the structural data of the extracellular domain and the kinase domain deposited in the Protein Data Bank (PDB IDs 2UZY and 3A4P) (b) Scheme of the photo-deprotection reaction of NPM-dC.

molecule, thereby enabling receptor dimerization in the presence of the target molecule.[7a] This concept has been extended to the design of aptamer-based tools for inducing receptor dimerization, responding to other external stimuli, including light irradiation.[7b-d] For example, Chen *et al.* reported multi-component system that enables light-induced receptor dimerization using DNA strand exchange reactions, initiated by cleavage of a photo-labile linker.[7c] However, none of these tools has demonstrated nongenetic temporal control of the GF signaling.

To realize optical control of dynamics of the GF signaling, we designed a single-component system that can turn on its RTK-activating potential, depending on a deprotection event of just one photo-caging group. As a proof-of-concept study, we selected Met, a receptor for hepatocyte growth factor (HGF), as the target.[8] As shown in Fig. 2, we designed a single-stranded DNA sequence composed of two functional parts; first, a 50-mer DNA aptamer that binds to Met (blue)[9] and a 10-mer self-complementary linker sequence (red), tethered to the 3′ terminal of the aptamer sequence. In the linker sequence, 6-nitropiperonyl-α-methyl (NPM)-caged deoxycytidine (dC)[10] was introduced to enable photo-induced dimerization of the aptamer upon irradiation of 365 nm light (cApt, Fig. 2).

The melting temperature (T_m) of the linker sequence with or without the photo-caging group was measured in the physiological conditions (in Dulbecco's phosphate-buffered saline). While T_m value of the linker without the photo-caging was 39.0 ˚C, the transition in the melting curve was not observed in the case of the linker, which was modified with the NPM-caging group, indicative of significant destabilization of the DNA duplex (Fig. S1). Importantly, a clear transition in the melting curve was observed (T_m = 40.7 ˚C) after an irradiation with 365 nm-peak UV light to the caged linker sequence, suggesting the light-dependent formation of duplex at the physiological conditions. To verify whether the cApt turns on its Met-activating potential depending on UV-irradiation, Met-expressing DU145 cells were stimulated with the photo-caged aptamer irradiated with a 365-nm peak UV light (8 mW, 0–60 s). The result indicated that the cApt showed increased Met-activating potential in an irradiation time-dependent manner (Fig. 3), indicative of the production of aptamer dimer upon the liberation of photo-caging group. It was also confirmed that the aptamer could induce activation of the downstream kinase, Erk, after the UV-irradiation.

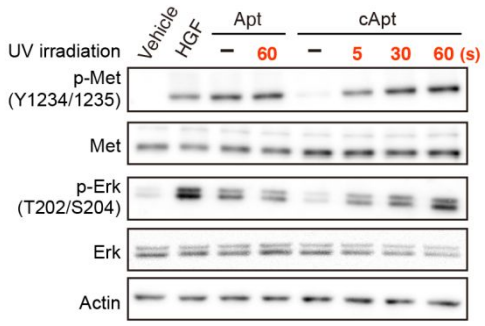

Fig.3 Western blotting of DU145 cell lysates. The cells were stimulated with HGF (1 nM) or aptamer (200 nM; monomer concentration) for 15 min. The samples were irradiated with a 365-nm peak UV irradiation (8 mW) for the indicated time before the addition to the medium.

We used the caged aptamer for optical control of the dynamics of GF signaling. In the present study, we focused on Erk activity, whose dynamics (rate, duration, and frequency) are implicated in the regulation of several cellular functions.[2] To date, genetically engineered proteins or specialized microfluidic devices have been used to study the roles of RTK signaling dynamics in determining cellular function and fate.[2,4] Here, we investigated whether our caged aptamer could generate unique signaling dynamics by merely changing the light input pattern without using a pipette or special microfluidic devices.

To visualize Erk activity in the living cells, we observed Met-expressing HeLa cells expressing Erk-kinase translocation reporter (KTR)-Clover,[11] a fluorescent reporter for the Erk activity, under time-lapse imaging using a fluorescence microscopy (Fig. 4a). This fluorescent reporter was designed to translocate from nucleus to cytosol upon phosphorylation, caused by the activated Erk.[11] Since basal Erk activity and the resulting nuclear localization propensity of the reporter may vary from cell to cell, the cells showing moderate nuclear localization of the reporter at the beginning of the analysis were used for the analysis (Fig. S2). We confirmed that the addition of recombinant HGF to the medium induced a prolonged (>60 min) translocation of the reporter from nucleus to the cytosol, indicating sustained Erk activation, which was consistent with observation in an immunoblotting analysis (Fig. S3).

First, the cells were incubated in the presence of the cApt (50 nM) and irradiated with a 365-nm peak UV light. When a short pulse (1 s) of UV irradiation was applied to the cells in 1 min interval for 6 times (input A), translocation of the reporter

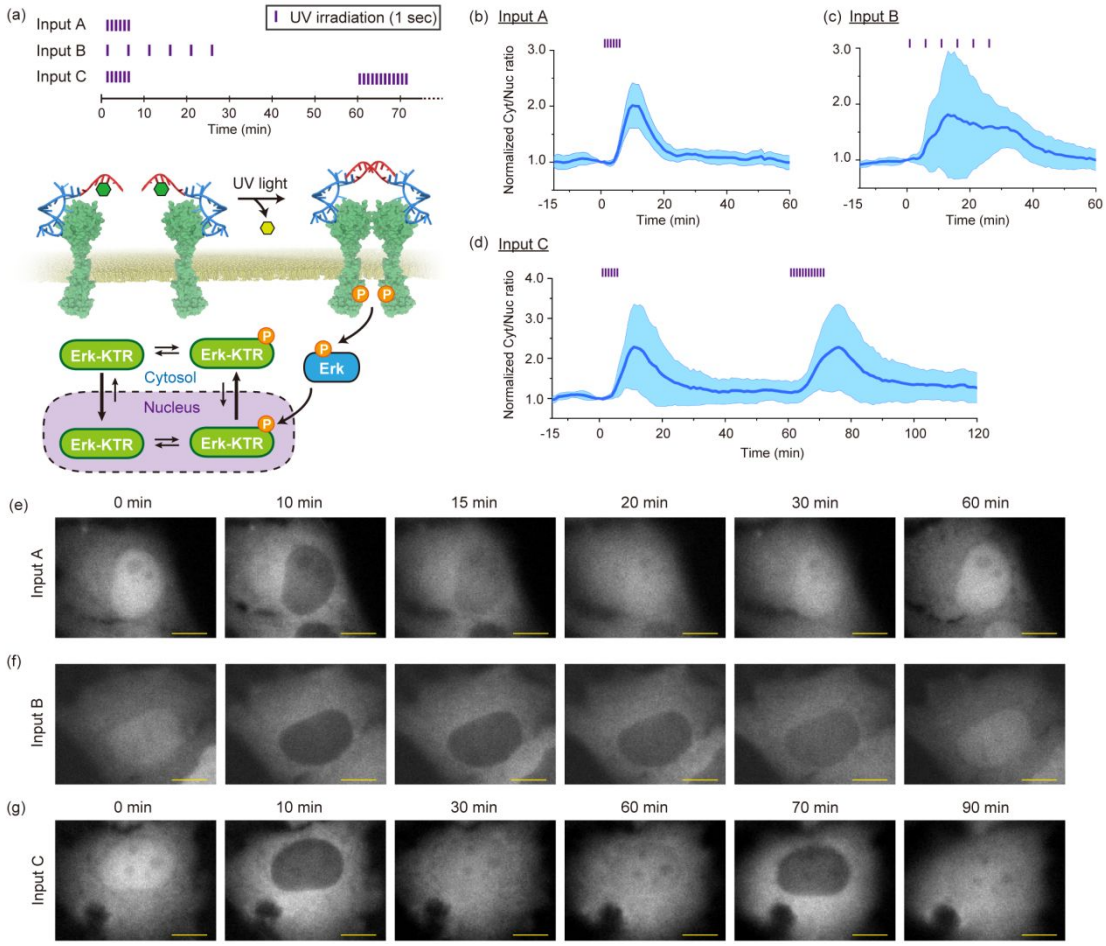

Fig.4 (a) Schematic representation of the experimental workflow. Erk-KTR-Clover (depicted as "Erk-KTR" in the figure) is a fluorescent reporter that changes its localization propensity according to Erk activity. Met activation induces the phosphorylation and activation of Erk, thus inducing the phosphorylation and translocation of Erk-KTR-Clover from the nucleus to cytosol. Temporal patterns of the light irradiation applied to the cells are shown at the top. (b)–(d) Normalized ratio of the fluorescence intensity of Erk-KTR-Clover in the cytosol and nucleus. HeLa cells, expressing Erk-KTR-Clover, were incubated with cApt (50 nM; monomer concentration) and a 365-nm peak UV light (0.2 W/cm^2) was irradiated according to the temporal pattern shown in Fig. 4a. The data are expressed as average ratio, calculated from 10 cells. The colored areas indicate SD. (e)–(g) Representative time-lapse imaging, showing translocation of Erk-KTR-Clover during time-lapse imaging analysis. Scale bars: 10 μm.

to the cytosol started to increase within a few minutes (Fig. 4b, 4e and Supplementary video 1). The translocation of reporter reached its maximum after 15 min from initial irradiation, and decayed to the basal level after another 10 min. This pulse-like Erk activation pattern may not be attributed to the nature of the agonist but to the intermittent uncaging of the aptamer in a short time, as a sustained stimulation with Apt resulted in prolonged ERK activation (Fig. S4). One possible means is the passive diffusion of uncaged aptamers to the non-irradiated area. In the current experimental setup, the UV light was applied through the optical path of the fluorescence microscope. Therefore, uncaging of the aptamer occurs only in and near the observation area, and the local concentration of the activated (uncaged) aptamer would decrease with time. The translocation of reporter was negligible by the irradiation of the cells in the absence of cApt, thereby discarding the possibility of UV irradiation-dependent activation of the signaling under these experimental conditions (Fig. S5).

We found that the dynamics of Erk signaling can be modulated by changing temporal pattern of UV irradiation. When the interval of irradiation pulse was elongated from 1 to 5 min (input B), the reporter showed a prolonged translocation (Fig. 4c, 4f and Supplementary video 2). While the duration for the translocation reached a plateau (~15 min) was almost comparable to that observed in the cells, stimulated with the irradiation pattern A, it took longer time (>30 min) until the translocation level returned to the basal level. A recent study suggested that Erk signaling dynamics are defined, at least in part, by the activation kinetics of the upstream RTKs.[12] Therefore, the observed differences in signaling dynamics could be attributed to the modulation of the temporal pattern of Met activation.

We also confirmed that a repetitive UV-irradiation pattern (input C) could induce a pulsatile Erk activation pattern (Fig. 4d, 4g and Supplementary video 3). During the optimization of irradiation conditions for repetitive cell signaling activation, we noticed that an increased second UV irradiation (1 s × 12 times) was required to evoke the same level of Erk activation pulse as the first time; When the second UV pulses (1 s × 6 times) were applied 60 min later after the first pulses, the induced reporter translocation level was substantially decreased (Fig. S6). It should be also noted that the current system can suffer from certain limitations consequent on repetitive manipulation. When the cells were irradiated with the third UV irradiation cycle, the reporter translocation level was slightly decreased compared with that induced by the first and second pulses (Fig.

S7). The possible mechanisms underlying these observations may include negative feedback regulation of Erk,[2a] receptor internalization, and consumption of the caged aptamer in the system. The extent to which the caged aptamer can activate signaling over repetitive cycles would depend on the experimental conditions, such as the interval between UV pulses and the UV irradiation duration.

We also elucidated spatial resolution of the current system. The irradiation pattern A (Fig. 4a) was applied to a selected area and the translocation of reporter in the cells, either inside or outside of the irradiated area, was monitored (Fig. S8 and Supplementary video 4). In the cells located in the irradiated area, Erk-reporter showed a pulse-like translocation pattern when the cells were stimulated with the irradiation scheme A. On the contrary, the translocation of reporter was negligible in the cells outside of the area. These results indicate that activation of Met receptor occurred only in the light-irradiated area and suggest that the influence of diffusion of uncaged aptamers was negligible. This excellent spatial resolution is presumably due to the excess amount of uncaged aptamers, existing in the non-irradiated area, which can compete for the receptor binding with the active aptamer produced in the irradiated area.

To summarize, we demonstrated nongenetic temporal control of GF signaling using a non-proteinaceous growth factor surrogate. Unlike precedent optochemical tools based on a combination of chemically inducible dimerization proteins and an appropriate photo-caged dimerizer,[13] this nongenetic approach applies only a photocaged-aptamer that can directly control protein dimerization in living cells. The caged DNA aptamer could be observed to generate unique cell signaling dynamics by merely changing the light input pattern without using a pipette or special microfluidic devices. The current system represents a promising approach for studying relationships involving signaling dynamics and the resultant biological outcomes, with the aim of manipulating cellular functions and fates of living cells. One intrinsic limitation of the current system is that UV irradiation may cause cytotoxicity depending on the irradiation conditions. Replacing the caging group with other photo-labile groups that can be photolyzed at longer wavelengths would overcome this limitation.

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Conflicts of interest

There are no conflicts to declare.

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