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Utilizing bivalent aptamers as first DNA agonist to activate RTKs heterodimer of different families

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ABSTRACT

Heterodimerization in RTKs is of vital importance in the RTK signaling and cell functions. Heterodimerization between RTKs can result in diversity of downstream signals, increasing the ability of cells to respond to external experiments. Traditional RTKs heterodimerization always occur in the same families and is lack of agonists to activate the heterodimeric RTKs signaling pathway. Herein, we developed the DNA agonist based on bivalent aptamers for the heterodimerized RTKs of different families, AF/AM-1, which could simultaneously activate FGFR1 and c-Met signaling. It is the first agonist that realizing the heterodimerization and activation of FGFR1 and c-Met, two different RTK families. The activation of FGFR1/c-Met heterodimer result in the down-stream signals transduction, such as the phosphorylation of Akt and Erk, inducing the cell migration and proliferation. The DNA agonist for RTK heterodimer of different families would have potential applications in the fields of biomedicine.

Receptor tyrosine kinase, known as RTKs, are of great importance in cell survival and proliferation as well as differentiation [1,2]. Conventional activation of RTKs is based on the homodimerization between receptors through the binding of ligands which always be called agonists, such as various of growth factors [3]. However, the RTKs heterodimer remains insufficient agonists.

Heterodimerization is a common phenomenon among RTK receptors, and the cross-activation of signal pathways often occurs through heterodimerization [4,5]. For example, in hematopoietic cells, activation of the ErbB-3/ErbB-2 heterodimeric receptor triggers the formation and diffusion of PI3 kinase-dependent lamellipodia [6]. The degree of heterodimerization between RTK receptors depends on the concentration of receptors and ligands, which is not specific. In the ErbB family, EGF promotes the homodimerization of EGFR, but in fact, EGF has a higher affinity for the EGFR/ErbB2 heterodimer [7]. Heterodimerization between RTK receptors can alter their signal strength and duration, increase the diversity of downstream signals, and thereby increase the ability of cells to respond to external changes and stimuli [8–10]. Therefore, the development of RTK heterodimerization agonists has important implications in biomedical fields. However, most heterodimeric RTK receptors have insufficient corresponding agonists and the heterodimerization always occur between same RTK families. There is an urgent need for developing agonist of heterodimeric RTK receptors. Moreover, the realization of RTKs heterodimer between different families is waiting for new agonists. Currently, Inooka has successfully developed an artificial peptide agonist for the FGFR1c/KLB heterodimeric complex [11]. However, the preparation process of this dimeric peptide is complex and expensive. Moreover, peptides are not convenient to store and are easily degraded by proteases at the area of the wound. Therefore, developing novel heterodimeric receptor agonists is urgently needed.

Deoxyribonucleic acids (DNAs) are widely used in the fields of biosensing and cell regulation owing to their predictable hybridization and programmable assembly [12–17]. Functional DNAs, such as aptamers, could bind to the target with high specificity and affinity [18–23]. Based on the property of aptamers specifically binding to cell receptors, we and others have been developed using aptamers to construct molecular devices that activate cell receptor signal pathways [24–31]. We propose to use aptamers to construct novel DNA agonists for the activation of heterodimeric RTK receptors.

Herein, we developed a universal method to construct novel DNA agonist for the activation of heterodimeric RTK receptors between different families based on the bivalent aptamers. FGFR1 and c-Met, two different RTKs for fibroblast growth factor and hepatic growth factor respectively, were used as a model. FGFR1 and c-Met both could result in the activation of Akt and Erk signaling

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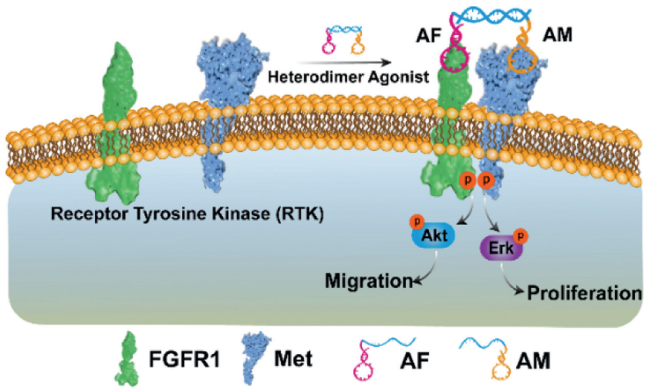

Fig. 1. Schematic representation of a bivalent aptamers induced c-Met and FGFR1 activation to regulate downstream signaling pathways and cellular behavior.

pathways, playing important role in cell migration and proliferation [24–31]. The research on simultaneously activation of FGFR1 and c-Met pathways is of great significance. Interestingly, the DNA agonist reported in current work realized the simultaneously activation of c-Met and FGFR1 based on the heterodimerization between c-Met and FGFR1. Moreover, the DNA agonist could result in the simultaneously activation of c-Met and EGFR [32], which shows good universality. We believe that the proposed DNA agonist will have broad applicability in cell therapy and regenerative medicine.

Our DNA agonist is composed of two subunits, AM and AF, hooking on the extracellular region of c-Met and FGFR1 receptor *via* the specific aptamer, respectively (Fig. 1). AM and AF could form a heterodimer through hybridization (Fig. 1 and Fig. S1 and Table S1 in Supporting information). The hybridization by AM and AF result in the heterodimerization of c-Met and FGFR1, following by the activation of the c-Met and FGFR1 signal pathway, which could induce the cell migration and proliferation.

We have developed a method that assembling AF/AM-R on the cell membrane (Fig. 2a Figs. S2 and S3 in Supporting information). As shown in Fig. 2a, the strands AF and AMR-TAMRA (AMRT) were both blocked by a complementary strand, AFRC and AMR-BHQ2 (AMRB), respectively, called AFR and AMR. The strands AF and AM could hook on the FGFR1 and c-Met [22,29]. In the presence of an initiate strand (AFRS), the AFR and AMR could interaction with each other, which could form the agonist AF/AM and bring the FGFR1 and c-Met heterodimerization. As shown in Fig. 2b, with the formation of AF/AM, the quenched TAMRA fluorescence significantly recovered. These results indicated that the agonist AF/AM could successfully assembling and hooking on the c-Met and FGFR1. The results were also demonstrated by turn-off confocal and flow cytometry experiments (Figs. S4 and S5 in Supporting information). The agonist, AF/AM-1, would bring anchored c-Met and FGFR1 in close proximity for cross phosphorylation. Indeed, AF/AM-1 quickly increased the c-Met and FGFR1 phosphorylation levels of A549 cells in 5 min (Fig. S6 in Supporting information). We have investigated the results about simply mixing FGFR1 and c-Met aptamers without complementary sequence. As shown in Fig. S7 (Supporting information), AF/AM-1 exhibit the highest c-Met and FGFR1 phosphorylation level, compared to the control that simply mixing FGFR1 and c-Met aptamers without complementary sequence. The length of complementary bases plays important role in phosphorylation of c-Met and FGFR1. As shown in Fig. S8 (Supporting information), the samples of different length, 12, 16, 23 (AF/AM-1), 30 and 40 were investigated. The 23 bp (AF/AM-1) length of complementary bases exhibited the highest phosphorylation level of FGFR1 and c-Met. Moreover, AF/AM-1 resulted in the FGFR1 and c-Met activation in a dose-dependent manner, with an effective dose as low as 10 nmol/L (Figs. 2c and d, Figs. S9 and S10 in Supporting information). Together, we have

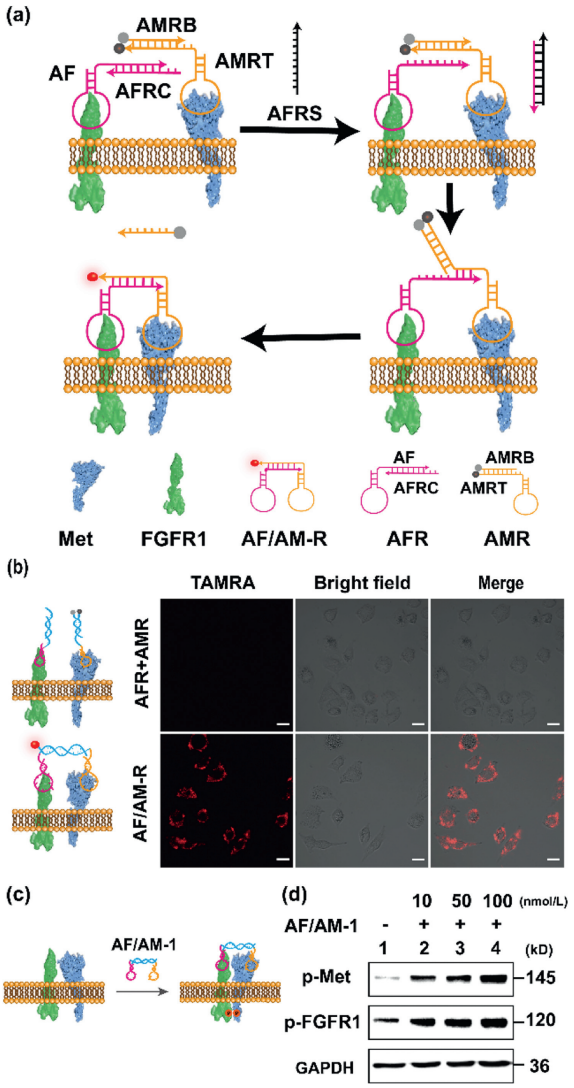

Fig. 2. (a) The scheme of fluorescence chain reaction. After AFRS was added, it will form a double chain with AFRC. AF's toehold will be exposed with the departure of the AFRC, which in turn will form a double chain with the AMRT, resulting in the separation of the AMRB and the fluorescence recovery of AMRT. (b) CLSM images of chain reaction. The A549 cells were incubated with 300 nmol/L AMR and AFR with or without AFRS (300 nmol/L). Red channel: TAMRA excitation 560 nm/emission 582 nm. Scale bars = 20 μm. (c) Schematic illustration of AF/AM-1 bivalent aptamers induced protein-pairing on the cell membrane. (d) Western blot analysis of p-Met and p-FGFR1 expression in A549 cells with different concentration AF/AM-1 treatment.

developed and characterized a novel heterodimerization agonist AF/AM-1 simultaneously activating FGFR1 and c-Met.

Next, we evaluated the downstream activation of Akt and Erk, key regulators in cell motility and cell proliferation (Fig. 3a). The A549 cells responded to AF/AM-1 with increased phosphorylation of both Akt and Erk in a linear manner (Fig. 3b, Figs. S11 and S12 in Supporting information). In response to AF/AM-1, GFP-actin transfected A549 cells generated large amounts of the actin lamellipodium (Fig. 3c), which indicate the potential increased motility, whereas control cells did not respond. The proliferation of AF/AM-1 treated A549 cells was 1.2x than untreated cells in 24 h (Fig. 3d). As expected, AF/AM-1 improved the cell motility compared with untreated cells (Fig. 3e). The AF/AM-1 treated cell motility rate was 16.4%, which was 3.1x compared with the control group (Fig. 3f). Above all, we have demonstrated the DNA agonist AF/AM-1 could induce the phosphorylation of both Akt and Erk, which successfully result in the enhanced cell motility and proliferation.

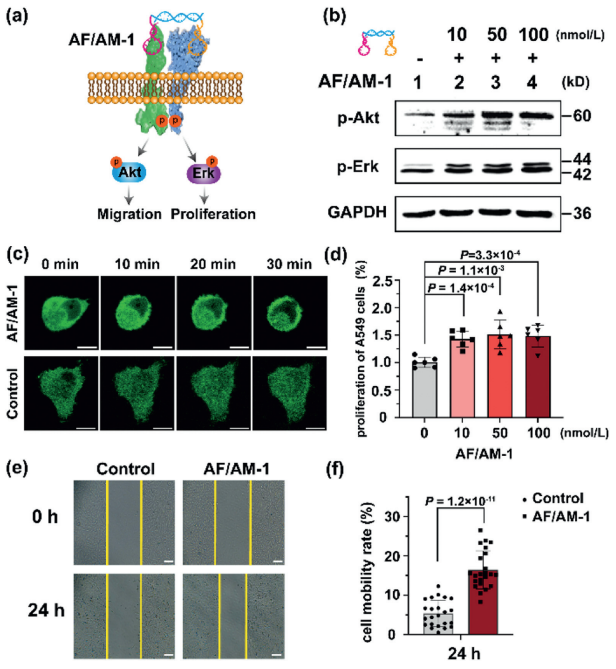

Fig. 3. (a) Schematic representation of signaling pathways with AF/AM-1. (b) Western blot analysis of p-Akt and p-Erk expression in A549 cells with different concentration AF/AM-1 treatment. (c) Bivalent aptamers-triggered cellular actin remodeling was observed by CLSM. A549 cells were treated with or without AF/AM-1 for 30 min and photographed every 10 min. Scale bars=10 μm. (d) Viability of AF/AM-1 treated with different concentration for 24 h. Mean ± S.D. (three technical replicates, $n=6$). *P* values were calculated using t tests on the averages of replicates. (e) Cell mobility regulated by AF/AM-1 in A549 cells was determined by wound healing assay. The images were taken at 0 h and 24 h. The yellow lines indicate boundaries between cells in the monolayer and the scratched areas uncovered by cells. Scale bars = 200 μm. (f) Quantification of relative cell mobility rates of indicated experiment settings. The cell mobility rates were analyzed using ImageJ software. Data are presented as mean ± S.D. ($n=24$). *P* values were calculated using *t* tests on the averages of replicates.

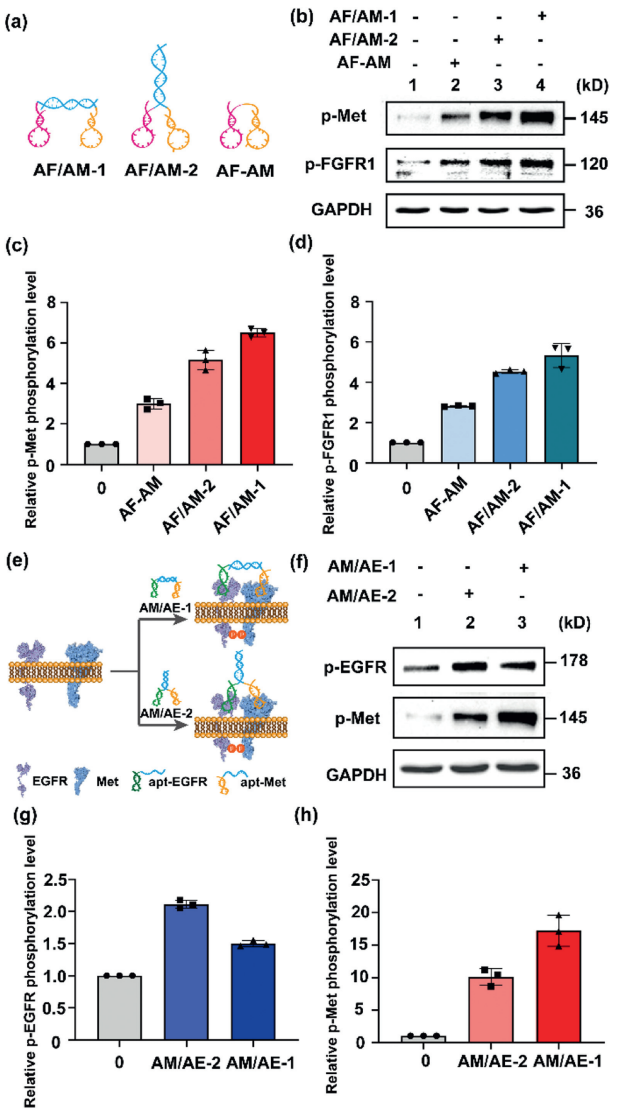

Fig. 4. (a) Schematic representation of AF/AM-1, AF/AM-2 and AF-AM. (b) Western blot analysis of p-Met and p-FGFR1 expression in A549 cells with different bivalent aptamers (100 nmol/L) treatment. (c) The level of p-Met phosphorylation in A549 cell lysates treated with different bivalent aptamers. (d) The level of p-FGFR1 phosphorylation in A549 cell lysates treated with different bivalent aptamers. (e) Schematic illustration of AM/AE-1 and AM/AE-2 bivalent aptamers induced protein-pairing on the cell membrane. (f) Western blot analysis of p-Met and p-FGFR1 expression in A549 cells with AM/AE-1 (100 nmol/L) and AM/AE-2 (100 nmol/L) treatment. (g) The level of p-EGFR phosphorylation in A549 cell lysates treated with AM/AE-1and AM/AE-2. (h) The level of p-Met phosphorylation in A549 cell lysates treated with AM/AE-1 and AM/AE-2.

To validate the DNA agonist system universality for application, we designed three different DNA agonists, AF-AM, AF/AM-1 and AF/AM-2 (Fig. 4a and Fig. S1). All the three agonists exhibited distinctly phosphorylation of c-Met and FGFR1 (Fig. 4b). As shown in Figs. 4c and d, the AF/AM-1 exhibited the best phosphorylation level of c-Met and FGFR1. It is mainly due to the distance of two aptamers in the agonist AF/AM-1, compared with the AF-AM and AF/AM-2. In AF/AM-1, the distance about c-Met and FGFR1 aptamer is 23 bp, however, in AF-AM and AF/AM-2, the distance is 0 bp, as shown in Fig. 4a. The distance between two aptamers is of great importance in the activation of c-Met and FGFR1, as the results in Fig. S8 (Supporting information). The distance between c-Met and FGFR1 aptamer in AF/AM-1 may be more suit to anchor c-Met and FGFR1 simultaneously, resulting in the best phosphorylation of c-Met and FGFR1. Moreover, all the three agonists resulted in the obviously proliferation of A549 cells (Fig. S13 in Supporting information). Then, we evaluated the feasibility of our agonists in the other cells (Figs. S14–S18 in Supporting information). These results demonstrated that the design of our DNA agonists exhibited good generality. Next, we evaluate the feasibility of our agonists in the other heterodimerization receptor pairs, for instance, EGFR/c-Met receptor heterodimerization and EGFR/FGFR1 (Fig. 4e and Fig. S20 in Supporting information). The two agonists AE/AM-1 and AE/AM-2 both exhibited the enhanced phosphorylation level of EGFR and c-Met (Figs. 4f–h). As shown in Fig. S19 (Supporting information), simply mixing EGFR and c-Met aptamers without complementary sequence could hardly activate EGFR and c-Met phosphorylation. In addition, the two agonists AF/AE-1 and AF/AE-2 both exhibited the enhanced phosphorylation level of FGFR1 and EGFR (Fig. S20).

In summary, we have developed a universal method to construct DNA agonist for the activation of the heterodimerization RTK receptors between different families. To the best of our knowledge, this is the first example of using DNA agonist for the activation of heterodimerization RTK receptors and signaling pathways. The DNA agonist shows excellent versatility in potential applications owing to (1) the flexibility of the recognition module of the agonist, which may include various aptamers binding for different receptors, allowing for a wide range of receptor signaling pathways to be used as specific applications, and (2) the customizability of the linking modules with other hybridization manner or strand displacement reaction approach, establishing the possibility to expand available DNA nanotechnology and DNA nanodevices. Notably, we have simultaneously controlled target receptors het-

erodimerization and activated the receptor signaling pathways by using DNA agonists, potentially offering a facile and practical non-genetic approach for customized cell behaviors *in vivo* by receptors heterodimerization in the future. This strategy represents a new avenue for the smart regulation of cell behavior in cell-based therapy and regenerative medicine.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Kun Liu: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Yulin Cong:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Xiongfeng Luo:** Data curation, Formal analysis, Investigation, Software, Validation, Visualization. **Meicun Yao:** Conceptualization, Project administration, Resources, Software, Supervision, Writing – review & editing. **Zhiyong Xie:** Conceptualization, Funding acquisition, Resources, Software, Supervision, Validation, Writing – review & editing. **Hao Li:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2024.109839.

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