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"All-in-one" *in vitro* selection of collagen-binding vascular endothelial growth factor

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ABSTRACT

To enhance the therapeutic effect of growth factors, a powerful strategy is to direct their localization to damaged sites. To treat skin wounds and myocardial infarction, we selected vascular endothelial growth factor (VEGF) carrying binding affinity to collagen. A simple conjugation of a reported collagen-binding sequence and VEGF did not increase the collagen-binding affinity, indicating that the molecular interaction between the two proteins abolished collagen binding activity. Here, we present a new molecular evolution strategy, "all-in-one" *in vitro* selection, in which a collagen-binding VEGF (CB-VEGF) was directly identified from a random library consisting of random and VEGF sequences. As expected, the selected CB-VEGFs exhibited high binding affinity to collagen and maintained the same growth enhancement activity for endothelial cells as unmodified VEGF in solution. Furthermore, the selected CB-VEGF enhanced angiogenesis at skin wounds and infarcted myocardium. This study demonstrates that "all-in-one" *in vitro* selection is a novel strategy for the design of functional proteins for regenerative medicine.

1. Introduction

Tissue repair and regeneration are regulated by key signaling molecules called growth factors. The therapeutic application of growth factors has been studied using numerous strategies including releasing devices [1–4]. A crucial point for the effective action of growth factors is the ability to deliver and retain molecules at a site of damage because a key initial step in the healing process or in tissue regeneration is to promote angiogenesis at the damaged site [5]. Type I and type III collagen are abundant at damaged sites where they form a matrix. The incorporation of collagen binding affinity to growth factors is, therefore, a reasonable strategy for maintaining growth factor activity at damaged sites for sustained periods [6–12].

Collagen-binding growth factors have therefore been developed using growth factors conjugated with sequences from natural collagen-binding proteins, such as von-Willebrand Factor (vWF), collagenase, and fibronectin [13–16], or with peptide sequences selected by the phage display method [17,18]. Recently, Martino et al. discovered that a domain in placenta growth factor-2 binds promiscuously and with exceptional affinity to extracellular matrix (ECM) proteins, and growth factors (GFs) fused with this domain had super-affinity to the ECM [6]. However, the simple combination of a binding peptide with a desired protein may alter the conformation of the binding peptide, leading to decreased binding affinity, as shown in Fig. 1(A). Therefore, we devised a "tailor-made" strategy to prepare growth factors with collagen binding affinity by *in vitro* selection from an "all-in-one" library of random sequences conjugated with a growth factor [19]. The main advantage of this procedure is that the binding affinity after selection is not affected

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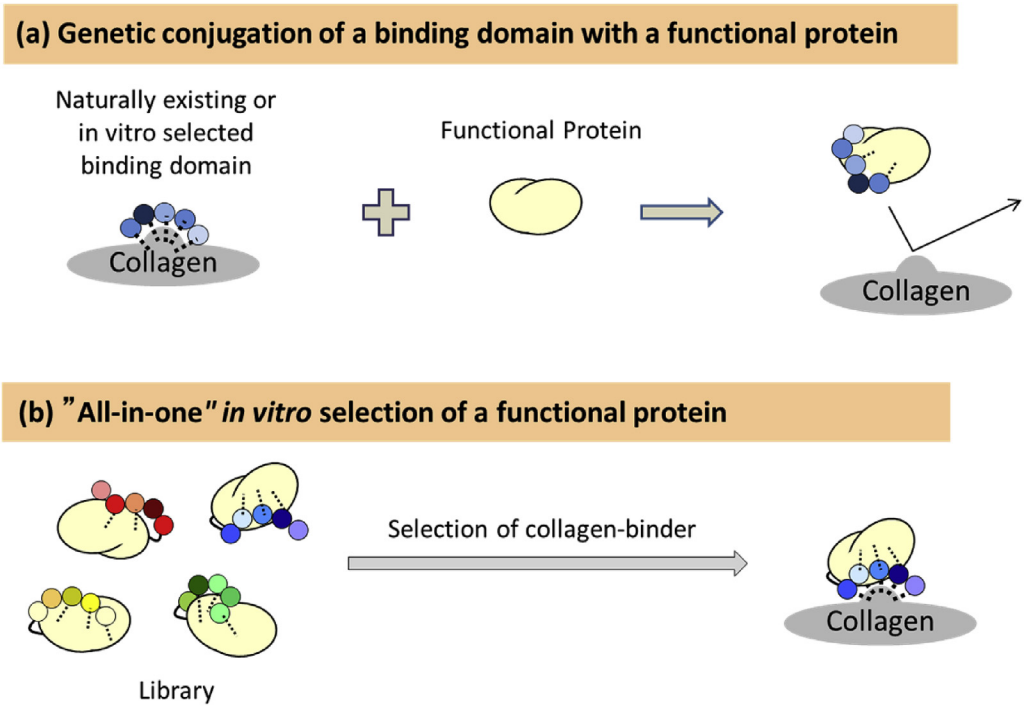

Fig. 1. Strategies for the preparation of a binding growth factor. (A) Growth factor connected via an existing binding domain. In this case there is a possibility of conformational changes induced by the connection. (B) "all-in-one" *in vitro* selection from a random sequence-connected growth factor library. Considering the length of the random sequence, sufficient affinity but a small effect on growth factor conformation is expected.

by the conjugation, as shown in Fig. 1(B). The growth factor region is considered to work as a scaffold.

As the growth factor we chose vascular endothelial growth factor (VEGF) which induces the formation of blood vessels during embryonic development and promotes endothelial cell growth [20–24]. The selected collagen-binding (CB)-VEGF was used for treatment of skin wounds and infarcted myocardium.

2. Materials and methods

2.1. Construction of DNA template for "all-in-one" in vitro selection

To immobilize a functional form of VEGF on collagen, we used tandem VEGF for *in vitro* selection. Because two VEGFs have to be dimerized to function against a VEGF receptor [35], the effective concentration of a dimeric form of VEGF on the collagen surface would be lower than expected compared with individual CB-VEGFs selected separately. Because we previously confirmed that a tandem VEGF functions similarly to dimerized VEGF, we used tandem VEGF for the *in vitro* selection of a CB-VEGF; we simply call this tandem form 'VEGF' in this study.

We prepared a template dsDNA library for *in vitro* selection. We used the plasmid, pTolA5, which contains a T7 promotor, a Shine-Dalgarno sequence, two *Sfi*I restriction sites, a VEGF sequence, an α-helix linker sequence (TolA) and a ribosome arrest sequence (SecM) as shown in Fig. S2. To insert a library in between the two *Sfi*I sites, we designed the ssDNA library sequence; 5′-ATCAGGCCTGAGTGGCCATG-$(NNB)_{15}$-GGCCAGCATGGCCAGAA-3′, where N represents T, A, C, or G and B represents T, C, or G. The template and all primers used below were purchased from Eurofins Genomics (Tokyo, Japan). The ssDNA library was converted to double-stranded by single-cycle PCR using a reverse primer [Rev_No1 (Rev-sfiI3-library), 5′-CCTGAGTGGCCAGAA-3′] and PrimeSTAR GXL DNA polymerase (Takara Bio, Japan). The PCR product was purified using a NucleoSpin® Gel and a PCR Clean-up kit (Takara Bio, Japan) and subsequently digested with *Sfi*I (New England Biolabs, Beverly, MA, USA). We ligated the library in between the two *Sfi*I sites of similarly digested plasmids using T4 DNA Ligase (Mighty Mix, Takara Bio). The ligated DNAs were amplified by PCR using forward and reverse primers [Fwd_No1 (P3FwdT7USb111214), 5′-GAGTCAGTGAGCGAGGAAGC-3′ and Rev_No2 (RevM13), 5′-CAGGAAACAGCTATGAC-3′]. The PCR product was size-selected and used as a DNA template for aptamer selection.

2.2. In vitro selection using ribosome display

We transcribed the DNA templates using the RiboMAX Large Scale RNA production system (Promega, Madison, WI, USA). After purification using RNA Clean & Concentration™ −25 (Zymo Research, CA, USA), we mixed 60–100 pmol of RNA with 50 μl of PURESYSTEM Classic II cell-free translation kit solution (Wako Pure Chemical Ind., Osaka, Japan) for *in vitro* translation. After translation at 37 °C for 15 min, we added 200 μl ice-cold WBT buffer (WBT; 50 mM Tris/acetate, 150 mM NaCl, 50 mM magnesium acetate and 0.05% Tween 20, pH 7.3) and a piece of collagen membrane (type 1 Atelocollagen, approximately 1 mm × 5 mm; Koken, Japan). The mixture was gently shaken for 30 min at 4 °C. To remove any unbound components, we washed the collagen membrane eight times with the ice-cold WBT buffer. Then we eluted mRNA bound to the collagen membrane by shaking in elution buffer (50 mM Tris-acetate buffer, 150 mM NaCl, 50 mM EDTA, pH 7.5) for 30 min at 4 °C. After purification, mRNA was reverse-transcribed to cDNA (PrimeScript™ Reverse Transcriptase, Takara Bio). We amplified the cDNA using PrimeSTAR GXL DNA polymerase and primers [Fwd_No2 (Fwd_T7TolAlong_120719) 5′-TAATACGACTCACTA-TAGGGCAGAAGCAAGGGCGGCACTTTAAG-3′ and Rev_No3 (Rev_tolA110617) 5′-TTAGCTCACCGAAAATATCATCTG-3′]. After