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Cardiac differentiation of embryonic stem cells by substrate immobilization of insulin-like growth factor binding protein 4 with elastin-like polypeptides

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ABSTRACT

The establishment of cardiomyocyte differentiation of embryonic stem cells (ESCs) is a useful strategy for cardiovascular regenerative medicine. Here, we report a strategy for cardiomyocyte differentiation of ESCs using substrate immobilization of insulin-like growth factor binding protein 4 (IGFBP4) with elastin-like polypeptides. Recently, IGFBP4 was reported to promote cardiomyocyte differentiation of ESCs through inhibition of the Wnt/β-catenin signaling. However, high amounts of IGFBP4 (approximately 1 μg/mL) were required to inhibit the Wnt/β-catenin signaling and induce differentiation to cardiomyocytes. We report herein induction of cardiomyocyte differentiation using IGFBP4-immobilized substrates. IGFBP4-immobilized substrates were created by fusion with elastin-like polypeptides. IGFBP4 was stably immobilized to polystyrene dishes through fusion of elastin-like polypeptides. Cardiomyocyte differentiation of ESCs was effectively promoted by strong and continuous inhibition of Wnt/β-catenin signaling with IGFBP4-immobilized substrates. These results demonstrated that IGFBP4 could be immobilized using fusion of elastin-like polypeptides. Our results also demonstrate that substrate immobilization of IGFBP4 is a powerful tool for differentiation of ESCs into cardiomyocytes. These findings suggest that substrate immobilization of soluble factors is a useful technique for differentiation of ESCs in regenerative medicine and tissue engineering.

1. Introduction

Damaged cardiac tissues do not normally regenerate because cardiomyocytes cannot proliferate in adults. To establish effective therapies for cardiac diseases, regenerative medicines using embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are promising candidates. Since ESCs and iPSCs have pluripotency, the cardiomyocytes into which ESCs and iPSCs differentiate may be a source of cells for transplantation. Previously, several studies have reported effective differentiation of ESCs and iPSCs into cardiomyocytes using bone morphogenetic proteins (BMPs), BMP inhibitors, Wnts, and Wnt inhibitors [1–3]. These factors promote cardiogenesis by regulating Wnt/β-catenin signaling. Regulation of Wnt/β-catenin signaling is considered important to promote differentiation of ESCs into cardiomyocytes. Recently, insulin-like growth factor binding protein 4 (IGFBP4) was reported to strongly promote cardiomyocyte differentiation of ESCs in the late phase after embryoid body (EB) formation through inhibition of Wnt/β-catenin signaling [4]. IGFBP4 was found to inhibit binding of Wnt3a to a Wnt receptor, Frizzled 8 (Frz8), and a Wnt co-receptor, low-density lipoprotein receptor-related protein 6 (LRP6) through interaction with these receptors [4]. Moreover, in chick and frog embryos, IGFBP4 is required for cardiogenesis through inhibition of Wntβ-catenin signaling [4]. Therefore, promotion of cardiomyocyte differentiation of ESCs using IGFBP4 is physiologically relevant. However, high concentrations of IGFBP4 are required for promotion of cardiomyocyte differentiation because this factor is unstable in medium. In addition, Wnts are regulated in an autocrine manner and bind to heparan sulfate proteoglycans on the cell surface [5]. Wnts molecules bind to the cell surface, so high concentrations of IGFBP4 are required to inhibit binding of Wnts to receptors.

Here, we induced cardiomyocyte differentiation using IGFBP4-immobilized substrates. Growth factor-immobilized substrates regulate maintenance of the undifferentiated state of ESCs and induce differentiation of ESCs [6–8]. E-cadherin-Fc, composed of the extracellular domain of E-cadherin and the Fc domain of immunoglobulin, was stably immobilized on polystyrene dishes and used to culture undifferentiated mouse and human ESCs and to

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induce hepatocyte differentiation of mouse ESCs [9,10]. The immobilization of various factors is beneficial for regulation of cell functions because the immobilized factors are stably condensed on the substrates and can stimulate cells locally and continuously.

To prepare IGFBP4-immobilized substrates, we designed chimeric proteins composed of the Wnt receptor-binding domain of IGFBP4 and elastin-like polypeptides. IGFBP4 is composed of an insulin-like growth factor binding domain and a thyroglobulin type 1 (Thy) domain. The Thy domain, C-terminus of IGFBP4, is a Wnt receptor-binding site [4]. Elastin-like polypeptides have a repetitive sequence, Gly-Val-Gly-Val-Pro (GVGVP), and possess a thermo-sensitivity by which hydrophobicity can be altered [11–13]. Above the phase transition temperature (Tg(t)), these polypeptides can stably adsorb to substrates such as polystyrene dishes through hydrophobic interactions. At lower temperatures, these polypeptides detach from the substrates due to increasing hydrophilicity. The Tg(t)s of elastin-like polypeptides depend on the number of GVGVP repeats [13]. Therefore, elastin-like polypeptides, $(GVGVP)_n$, are useful for immobilization of various factors. In this study, $(GVGVP)_n$-IGFBP4 was designed to link the N-terminus of Thy with $(GVGVP)_n$. We examined the effect of temperature on the adsorptive stability of $(GVGVP)_n$-IGFBP4 and the effect of $(GVGVP)_n$-IGFBP4 on cardiomyocyte differentiation of ESCs.

2. Materials and methods

2.1. Materials

Plasmid pMD20-T, restriction enzymes, and ligase were purchased from Takara BIO INC (Shiga, Japan). The restriction endonuclease *Pfl*MI was purchased from FERMENTAS INC. (MD, USA) and New England Biolabs Inc. (MA, USA). The His-tag expression vector pET14b was purchased from Novagen, (Merck K. GaA, Darmstadt, Germany). *Escherichia coli* JM109 for cloning was purchased from NIPPON GENE (Tokyo, Japan). *E. coli* HST04 dam^-/dcm^- for cloning with *Pfl*MI was a generous gift from New England Biolabs Inc. *E. coli* KRX for protein expression was purchased from Promega Corporation (WI, USA). P19CL6 cells (mouse cell line derived from embryonic carcinoma, C3H strain) were obtained from RIKEN Cell Bank (Ibaraki, Japan). Embryo Max Embryonic Stem Cell Line—Strain 129/SVEV, passage 11, were purchased from Millipore (MA, USA).

2.2. Construction of $(GVGVP)_n$ plasmids

These plasmids were prepared according to previously published methods [11,12]. In brief, oligonucleotides encoding *Xho*I-*Pfl*MI-*Not*I-*Bam*HI and *Pfl*MI-$(GVGVP)_{11}$-*Pfl*MI sites were purchased from Operon Biotechnologies (Tokyo, Japan) (Supplementary Fig. 1A). These oligonucleotides were subcloned into the pMD20-T vector of a Mighty TA-cloning Kit for PrimeSTAR (Takara). These vectors were digested by restriction endonuclease *Pfl*MI, and the *Pfl*MI-digested fragments of $(GVGVP)_{11}$ and *Pfl*MI-digested pMD20 vector including *Xho*I, *Pfl*MI, *Not*I, and *Bam*HI sites were obtained. The *Pfl*MI-digested pMD20 vector was dephosphorylated to avoid self-ligation of vector. The fragments and vector were ligated using T4 ligase (Nippon gene), and pMD20 vectors, including $(GVGVP)_{12}$, $(GVGVP)_{23}$, $(GVGVP)_{45}$, and $(GVGVP)_{67}$, could be obtained. Since the fragment and vector had *Pfl*MI sites at both termini and the vector was dephosphorylated, the concatemer of the fragments was constructed in the vector. The resulting plasmids were digested with *Xho*I and *Bam*HI. Recombinant plasmids expressing 6-His-tagged $(GVGVP)_{12}$, $(GVGVP)_{23}$, $(GVGVP)_{45}$, and $(GVGVP)_{67}$ were constructed by ligating the *Xho*I/*Bam*HI-digested fragments of $(GVGVP)_n$ with the *Xho*I/*Bam*HI-digested pET14b vector (Supplementary Fig. 1A).

2.3. Construction of $(GVGVP)_n$-IGFBP4

cDNA fragments encoding the Thy domain of IGFBP4, the Wnt receptor-binding domain, were amplified from cDNA of P19CL6 cells by polymerase chain reaction (PCR) with PrimeSTAR Max DNA Polymerase (Takara). PCR amplification of the Thy domain, encoded by amino acids (aa) 167–245, was performed using the following primers containing *Not*I, *Xho*I, and *Bam*HI digestion sites: IGFBP4 sense primers 5′– ATAAGAATGCGGCCGCTAAACTATCAGGGTTCCTGCCAGAGCGAGCTG –3′ and 5′– CGCTCGAGCGGCAGGGTTCCTGCCAGAGCGA –3′ and antisense primer 5′– CGGGATCCCGTCACTCTTGGAAGCTGTCAGCCAG –3′. cDNA fragments were subcloned into the pMD20-T vector of a Mighty TA-cloning Kit for PrimeSTAR (Takara). The resulting plasmids were digested with *Not*I or *Xho*I and *Bam*HI. The *Not*I/*Bam*HI-digested fragments, including the Thy domain, were integrated into *Not*I/*Bam*HI-digested pMD20 including $(GVGVP)_n$. The resulting plasmids that included $(GVGVP)_n$-IGFBP4 were digested with *Xho*I and *Bam*HI, producing $(GVGVP)_n$-IGFBP4 fragments. Recombinant plasmids expressing 6-His-tagged $(GVGVP)_{12}$-IGFBP4, $(GVGVP)_{23}$-IGFBP4, $(GVGVP)_{45}$-IGFBP4, $(GVGVP)_{67}$-IGFBP4, and IGFBP4 (Thy) were constructed by ligating the *Xho*I/*Bam*HI-digested fragments of $(GVGVP)_n$-IGFBP4 and *Xho*I/*Bam*HI-digested fragments of IGFBP4 (Thy) with the *Xho*I/*Bam*HI-digested pET14b vector (Supplementary Fig. 1B).

2.4. Expression and purification of recombinant proteins

Recombinant proteins were expressed in *E. coli* KRX cells according to the manufacturer's instructions (Promega). To avoid aggregation and insolubilization of these proteins in the *E. coli*, protein expression was performed at 20 °C, under the phase transition temperature. Proteins were purified using the COSMOGEL® His-Accept purification system, according to the manufacturer's instructions (Nacalai tesque, Kyoto, Japan). Proteins were dissolved in phosphate-buffered saline (PBS).

2.5. Determination of Tg(t) and temperature-induced aggregation profiles of the purified proteins $(GVGVP)_n$

Since the $(GVGVP)_n$ proteins had thermoactivity affecting hydrophobicity, aggregation of these proteins was observed above Tg(t). Tg(t) depends on the number of repeating GVGVP. Therefore, Tg(t)s of $(GVGVP)_{12}$, $(GVGVP)_{23}$, $(GVGVP)_{45}$, and $(GVGVP)_{67}$ were determined by turbidity assay. The temperature profiles for aggregation, which give rise to the Tg(t) values, were determined using

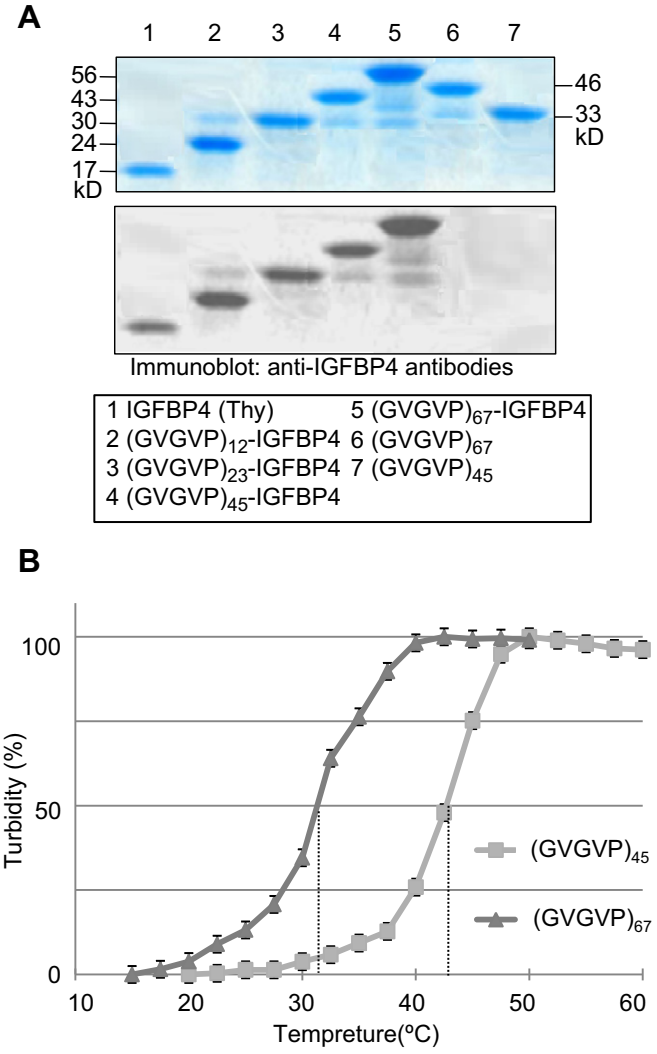

Fig. 1. The production and temperature-induced aggregation profiles of IGFBP4 (Thy), $(GVGVP)_{12}$-IGFBP4, $(GVGVP)_{23}$-IGFBP4, $(GVGVP)_{45}$-IGFBP4, and $(GVGVP)_{67}$-IGFBP4. (A) CBB staining of SDS-PAGE (upper) and immunoblotting with anti-IGFBP4 antibodies (lower) in these proteins. (B) Temperature-induced aggregation profiles of $(GVGVP)_{45}$-IGFBP4 and $(GVGVP)_{67}$-IGFBP4. Results are represented as average ± SD.