**Master of Science**

# Enhancing Solubility and Purification Efficiency of Vitronectin in *Escherichia coli* and Application in Human Pluripotent Stem Cell Culture

# 원핵생물에서 Vitronectin 의 수용성 발현 및 정제효율의 증가와 인간 만능 줄기 세포 배양에 대한 응용

**The Graduate School**
**of the University of Ulsan**

**Department of Medical Science**
**Ryu, Dayoung**

# Enhancing Solubility and Purification Efficiency of Vitronectin in *Escherichia coli* and Application in Human Pluripotent Stem Cell Culture

Supervisor Han Choe

Master's thesis

Submitted to
the Graduated School of the University of Ulsan
in partial Fulfillment of the Requirements
for the Degree of

Master of Science

by

Ryu, Dayoung

Department of Medical Science
Ulsan, Korea
August 2023

# Enhancing Solubility and Purification Efficiency of Vitronectin in *Escherichia coli* and Application in Human Pluripotent Stem Cell Culture

This certifies that the dissertation
of Ryu, Dayoung is approved

Committee Vice-Chair Dr. Leem, Chae-Hun

Committee member Dr. Han Choe

Committee member Dr. Chang, Suhwan

Department of Medical Science
Ulsan, Korea
August 2023

**Contents**

**LIST OF TABLES**



**LIST OF FIGURES**



**LIST OF SUPPLEMENTARY TABLE**



**LIST OF SUPPLEMENTARY FIGURE**

## LIST OF ABBREVIATIONS

| | |
|---|---|
| BSA | Bovine serum albumin |
| CV | Column volume |
| ECM | Extracellular matrix |
| *E. coli* | *Escherichia coli* |
| ESCs | Embryonic stem cells |
| GST | Glutathione S-transferase |
| His6 | Hexahistidine |
| His8 | Octahistidine |
| hPSCs | human pluripotent stem cells |
| IPTG | Isopropyl 1-thio-β-d-galactopyranoside |
| iPSCs | induced Pluripotent stem cells |
| LB medium | Luria-Bertani medium |
| LC | Liquid chromatography |
| MBP | Maltose binding protein |
| NusA | N-utilisation substance protein A |
| OD | Optical density |
| PBS | Phosphate buffered saline |
| PCR | Polymerase Chain Reaction |
| PDI | protein disulfide bond isomerase |
| PDI b'a' | b'a' domain of protein disulfide bond isomerase |
| PSCs | Pluripotent stem cells |
| SEM | Standard Error of the Mean |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| TEV | Tobacco etch virus |
| TEVrs | Tobacco etch virus protease recognition site |
| Trx | Thioredoxin |
| VTN | Vitronectin |

**Abstract**

## Enhancing Solubility and Purification Efficiency of Vitronectin in Escherichia coli and Application in Human Pluripotent Stem Cell Culture

**Ryu, Dayoung**
**The Graduate School**
**University of Ulsan**
**College of Medical Science**

Vitronectin (VTN) is a crucial protein substrate in human pluripotent stem cell (hPSC) culture, although challenges have been encountered in its bacterial expression and solubility. This study aimed to enhance the solubility and purification efficiency of VTN and its truncated forms, VTN(N) and VTN(NC), in the Escherichia coli expression system by fusion with tag proteins. Seven tags were evaluated, with MBP emerging as the most effective, significantly increasing the water solubility of VTN, VTN(N), and VTN(NC) from 5-60% to over 95%. The increased solubility of the fusion proteins, MBP-VTN(N) and MBP-VTN(NC), simplified the purification processes by removing solubilization and refolding steps of VTN. The purified fusion proteins also demonstrated superior stability during long-term storage. In a biological activity test, MBP-VTN(N) and MBP-VTN(NC) allowed for hPSC maintenance at the single cell level, a persistent challenge with VTN alone. Notably, the MBP-VTN(N) and MBP-VTN(NC) preserved cell morphology and pluripotency after several passages, outperforming VTN, which led to cell detachment after a few days. These findings suggest that the MBP-VTN(N) and MBP-VTN(NC) offer significant advantages for hPSC culture, paving the way for cost-effective and efficient stem cell research. Further studies are needed to optimize this approach and explore its full potential in stem cell biology.

Keywords: Vitronectin (VTN), Fusion Proteins, *E. coli* Expression, Maltose-Binding Protein (MBP), Pluripotent Stem Cells (hPSC)

## Introduction

Vitronectin (VTN), a glycoprotein consisting of 459 amino acids, plays crucial roles in diverse biological processes, such as hemostasis, cell adhesion, tissue regeneration, tumor metastasis, and immunity [1, 2]. Primarily produced by the liver, VTN is present in serum, the extracellular matrix (ECM), and bone [1, 3, 4]. In blood circulation, VTN exists as a monomer [4], while the ECM and platelet releasate contain higher molecular weight oligomers of VTN [5]. This multifunctional glycoprotein interacts with glycosaminoglycans, collagen, heparin, plasminogen, and the urokinase-receptor [5]. Furthermore, it interacts with integrin, a membrane protein, initiating integrin signaling which promotes cell adhesion and migration [6, 7]. Given its broad binding activities and integrin-stimulating properties, VTN is a preferred substrate for in vitro cell culture, specifically for pluripotent stem cells (PSCs) such as embryonic stem cells (ESCs) and induced PSCs (iPSCs) [8, 9]. Human PSCs are self-renewing and pluripotent cells with enormous clinical potential. Their ability to differentiate into all cell types present in the adult body makes them valuable for cell therapy, tissue repair, and drug development [8]. Notably, two truncated forms of VTN, VTN(N) and VTN(NC), exhibit a high efficacy in PSC maintenance [10]. These applications require an efficient production of VTN.

Although VTN can be extracted from human plasma [11, 12], the recombinant technology employed in Escherichia coli (*E. coli*) is a popular method for its production [13, 14]. The *E. coli* expression system is favored for the production of heterologous proteins due to its advantages like ease of handling, rapid doubling time, and potential for high-density cultivation [15-19]. However, this system can also contribute to challenges such as low solubility, incorrect protein folding, and formation of inclusion bodies [20]. These issues are particularly pronounced with VTN, VTN(N), and VTN(NC), which have a propensity to misfold and aggregate into inclusion bodies when expressed in *E. coli*, thus requiring an extensive solubilization and refolding process [13]. A myriad of fusion tags have been developed to address the solubility issues associated with the *E. coli* expression system [21, 22]. Nevertheless, the effectiveness of each tag is protein-dependent, and no universal fusion tag exists to ensure soluble overexpression of heterologous proteins in *E. coli*.

The aim of this study was to enhance the solubility and purification efficiency of VTN in the *E. coli* expression system by fusion with a tag. To achieve this, seven commonly used tags such as histidines (His), thioredoxin (Trx), glutathione S-transferase (GST), maltose-binding protein (MBP), N-utilisation substance protein A (NusA), protein disulphide isomerase (PDI), and b'a' domain of PDI (PDIb'a') were evaluated and MBP was ultimately chosen due to its performance. Subsequently, we fused MBP with the two truncated forms of VTN, VTN(N) and VTN(NC), resulting in the production of fusion proteins with high purity. To verify the biological activity of these purified fusion proteins, we examined their effectiveness in maintaining human PSCs.

## Material and Methods

### 1. Materials

All utilized chemicals were of analytical grade. Reagents were acquired from various suppliers: 1-thio-β-d-galactopyranoside (IPTG) from Anaspec (Fremont, CA, USA). Ampicillin from Duchefa Biochemie (Haarlem, Netherlands); Sodium chloride, hydrochloric acid, acetic acid glacial, yeast extract, urea and glycerol from Samchun Chemical (Pyeongtaek, Korea); Coomassie brilliant blue R-250 and Tris from Amresco (Solon, OH, USA). *E. coli* strains, BL21(DE3), SHuffle T7 Express, Rosetta-gami B (DE3) and DH5α cells were obtained from Novagen (Madison, WI, USA).

For purification, all chromatography columns and ÄKTA series (Prime, Start and Pure) were procured from GE Healthcare (Piscataway, NJ, USA). The 0.2 and 0.45 µm pore-size filters were from Hyundai Micron (Seoul, Korea). Dialysis membranes and Amicon Ultra concentrators were from Viskase (Darien, IL, USA) and Merck Millipore (Billerica, MA, USA), respectively. Acrodisc syringe filters were sourced from Pall Korea (Seoul, Korea), BCA kits were from Abbkine (Wuhan, China). Ultrasonic cell disruptor JY99-IIDN was obtained from Ningbo Scientz Biotechnology (Guangdong, China). The Primers were sourced from Bionics (Gangwon, Korea) and Macrogen (Seoul, Korea).

For cell culture, materials were procured from various suppliers: Activin A, and epidermal growth factor (EGF) from Peprotech (Rocky Hill, NJ); holo-transferrin and human insulin from Prospec (Rehovot, Israel); RNase inhibitor from Roche (Basel, Switzerland). L-ascorbic acid-2-phosphate, sodium selenite, polyvinyl alcohol (PVA), and ethylene glycol tetraacetic acid (EGTA) sourced from Sigma-Aldrich (St. Louis, MO). Cell culture plates and 15 ml, 50 ml conical tubes were from SPL Life Sciences (Pocheon, Korea). DMEM/F12 HEPES, penicillin-streptomycin, and TrypLE Express enzyme were from Thermo Fisher Scientific (Waltham, MA). ROCK inhibitor Y27632 was from Carbosynth (Berkshire, UK). The H9 hESCs from WiCell (Madison, WI) under material transfer agreement. Reverse Transcriptase master premix (5X), oligo d(T) and dNTP mix (2.5 mM each, 10 mM) were sourced from ELPIS (Daejon, Korea). The plasmid for VTN was a gift from James Thomson (Addgene plasmid #30223).

### 2. Plasmid construction of expression vectors

In order to generate VTN expression plasmid, we employed gateway cloning systems, which utilize BP and LR recombination reactions [42]. A DNA sequence encoding 459 amino acids of mature human VTN (based on NCBI Reference Sequence: NM_000638.3) was codon-optimized for *E. coli* expression and synthesized by GenScript (Piscataway, NJ). We positioned the tobacco etch virus recognition site (TEVrs), ENLYFQ/G, at the N-terminal of the hVTN nucleotide. This particular DNA fragment was then subcloned into the pDONOR207 vector using a BP reaction, resulting in an entry clone named pENTR-hVTN. Subsequently, LR reactions were used to create expression vectors by integrating the pENTR-hVTN with seven different destination vectors: pDest-H, pDest-H-Trx, pDest-H-GST, pDest-H-PDIb'a', pDest-H-MBP, pDest-H-NusA, and pDest-H-PDI. The accuracy of all the sequences was verified by sequencing (Macrogen, Deajeon, Korea). The VTN(N) and VTN(NC) expression plasmids were engineered by Bionics (Gangwon, Korea), using a VTN sequence that was codon-optimized for expression in *E. coli*. The target genes were precisely inserted into the pET-3C vector at the NdeI and BamHI restriction sites.

For the production of recombinant MBP-VTN(NC) plasmid, the VTN(NC) fragment, attB1-TEVrs-VTN(NC)-attB2, was amplified by PCR and then a BP reaction with pDONR207 resulted in pENTR-VTN(NC). The subsequent LR reaction with pDEST-H-MBP resulted in an expression vector, MBP-VTN(NC) (Supplementary figure 1). For the production of truncated MBP-VTN(N) construct, the VTN(N) fragment and the vector pDEST-H-MBP fragment were amplified by PCR, and in vivo *E. coli* cloning (iVEC) [43] of the two PCR fragments successfully generated the expression vector for MBP-VTN(N).

**3. Expression VTNs from *E. coli***

The VTN plasmid was transformed into the *E. coli* BL21 (DE3) strain. The transformed BL21 (DE3) were inoculated in a 1:100 ratio into antibiotic-containing LB media from an overnight culture. These cultures were incubated at 37°C with shaking at 200 rpm until they reached an OD of 0.8 - 1.2. Protein expression was then induced with 0.5 mM IPTG, and the cultures were incubated under the same conditions overnight. The bacterial cells were subsequently harvested via centrifugation at 2,700 × g and 4°C for 30 min.

The VTN(N) and VTN(NC) plasmids were introduced into *E. coli* BL21 (DE3) and Rosetta pLys strains. High-protein expressing colonies were identified by cultivating the transformed *E. coli* on LB agar plates, which were supplemented with 50 μg/mL of ampicillin. Colonies of interest were then grown overnight in LB media containing the same concentration of antibiotic at 37°C with shaking at 180 rpm. The overnight culture was used to inoculate 1 L of fresh LB medium at a 1:100 ratio, which was then incubated at 37°C with shaking at 180 rpm. When the OD600 reached a value between 0.5 and 0.6, IPTG was introduced to a final concentration of 0.5 mM to trigger the expression of the recombinant protein. The bacterial culture was further incubated for 4 hours at 37°C with shaking at 180 rpm, after which cells were collected by centrifugation at 2,700 × g and 4°C for 30 minutes.

The MBP-VTN(N) plasmid was similarly transformed into the *E. coli* BL21 (DE3) strain. Colonies expressing high protein levels were screened by growing transformed *E. coli* on LB agar plates supplemented with 50 μg/mL of ampicillin. Selected colonies were then cultured overnight in antibiotic-containing LB media at 37°C with shaking at 180 rpm. The overnight culture was inoculated at a 1:100 ratio into 1 L of fresh LB medium and incubated at 37°C with shaking at 180 rpm. Once the OD600 reached 0.5 - 0.6, IPTG was added to a final concentration of 0.5 mM to induce recombinant protein expression. Cells were then cultured overnight at 18°C with shaking at 180 rpm and harvested by centrifugation at 2,700 × g and 4°C for 30 min.

For the MBP-VTN(NC) plasmid, transformation was carried out into the *E. coli* SHuffle T7 express strain. After screening for high protein expression as described above, selected colonies were cultured overnight in antibiotic-containing LB media at 37°C with shaking at 180 rpm. The overnight culture was then inoculated at a 1:100 ratio into 1 L of fresh LB medium and incubated at 37°C with shaking at 180 rpm. When the OD600 reached 0.5 - 0.6, IPTG was added to a final concentration of 0.5 mM to induce recombinant protein expression. Unlike the previous procedure, cells were cultured for 4 hours at 37°C with shaking at 180 rpm and then harvested by centrifugation at 2,700 × g and 4°C for 30 min.

**4. Purification of VTNs from *E. coli***

For VTN, the collected bacterial pellet was resuspended in sonication buffer (20 mM Tris-HCl(pH 8.0), 5% glycerol) and disrupted using an ultrasonic cell disruptor, with a cycle pattern of 3 sec burst and 27 sec rest, repeated 60 times on ice. The disrupted cells were then centrifuged at 25,000 × g for 20 min at 4°C, and the pellet was washed three times with the sonication buffer. After washing, the pellet was solubilized in buffer A (20 mM Tris-HCl (pH 8.0), 5% glycerol, 150 mM NaCl, 8 M Urea, and 5 mM DTT) while stirring at room temperature for 2 hours. The solubilization product was then centrifuged at 25,000 × g for 20 min at 4°C, and the supernatant was filtered through a 0.2 μm PES hydrophilic membrane filter for column application. The HiTrap Heparin HP column was prepared with 5 column volumes (CV) of buffer A at a flow rate of 2.5 mL/min. After equilibration, the sample was applied at a flow rate of 1 mL/min. The column was washed with buffer A for 10 CVs to remove unbound proteins, and the target protein was eluted by a gradient of 0-1 M NaCl in Buffer A over 10 CVs. The eluted protein was verified through electrophoresis in 10% tricine SDS-PAGE. The verified fractions were dialyzed against PBS and treated with 0.1% Triton X-114(v/v) to remove endotoxin. The final product was supplemented with glycerol to a final concentration of 50%, filtered through a 0.2 μm membrane filter, and stored at -20°C.

For VTN(N) and VTN(NC), the cell pellet was lysed on ice using an ultrasonic cell disruptor, adhering to a cycle of 3-second bursts and 27-second rests for 60 cycles, in buffer A (20 mM Tris-HCl (pH 8.0), 5% glycerol (v/v)). The lysed cells were then subjected to centrifugation at 25,000 × g for 20 min at 4°C, separating soluble components from the insoluble ones. The resulting supernatant was filtered through a 0.2 μm PES hydrophilic membrane filter prior to column application. The HiTrap Q HP column was prepared by running 5 CVs of buffer A at a flow rate of 2.5 mL/min. After equilibration, the sample was loaded at a flow rate of 1 mL/min. Subsequently, the column was washed with 10 CVs of buffer A to remove non-specifically bound proteins. Weakly binding proteins were washed off with a gradient of 5, 10, and 20% buffer B (20 mM Tris-HCl (pH 8.0), 1 M NaCl, 5% glycerol (v/v)) across 5 CVs. The target protein was eluted using 30% of buffer B across 5 CVs, and the eluted protein was dialyzed in buffer A. Subsequently, the HiTrap SP HP column was prepared similarly, using 5 CV of buffer A at a flow rate of 2.5 mL/min. The sample from the HiTrap Q HP column was loaded and the target protein was eluted with 10 CVs of buffer A. In the final step, the HiTrap Heparin HP column was prepared using the same procedure as the previous columns. The column was washed with buffer A for 10 CVs to remove any unbound proteins, and the target protein was eluted with 5% of buffer B over 5 CVs. For each step of column purification, the eluted proteins were examined using 10% tricine SDS-PAGE. The validated fractions were dialyzed against PBS. The final protein preparation was enhanced with glycerol to a final concentration of 50%, filtered through a 0.2 μm membrane filter, and preserved at -20°C.

For MBP-VTN(N) and MBP-VTN(NC), the cell pellet was lysed on ice using the same ultrasonic cell disruptor and a cycle pattern of 3 sec burst and 27 sec rest for 60 cycles in buffer A (20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 5% glycerol (v/v)). The lysed cells were then centrifuged at 25,000 × g for 20 min at 4°C to separate the soluble from the insoluble components. The supernatant was filtered through a 0.2 μm PES hydrophilic membrane filter for column application. Two linked MBPTrap™ HP columns were prepared with 5 CV of buffer A at a flow rate of 2.5 mL/min. After equilibration, the sample was applied at a flow rate of 2.5 mL/min. The column was washed with buffer A for 15 CVs to remove unbound proteins, and the target protein was eluted with elution buffer (20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 30 mM Maltose, 5% glycerol (v/v)) over 5 CVs. The eluted protein was confirmed through electrophoresis in 10% tricine SDS-PAGE. The verified fractions were dialyzed

against PBS. The final product was supplemented with glycerol to a final concentration of 50%, filtered through a 0.2 µm membrane filter, and stored at -20°C.

**5. Analysis of SDS-PAGE gels**

Protein expression and purification steps were assessed using 10% Tricine SDS-PAGE gel. The protein fractions were denatured by boiling for 5 minutes at 95°C with 5X sample buffer (250 mM Tris-HCl, pH 6.8, 30% glycerol, 10% SDS, 0.01% bromophenol blue, 300 mM DTT). The SDS PAGE gels were then stained with Coomassie brilliant blue R-250 solution for protein band detection. The expression and solubility levels of the fusion proteins, as well as the purity of the target proteins, were analyzed using Gel Analyzer software (http://www.gelanalyzer.com) and calculated with Microsoft Excel following the equations:

$$\text{Expression level} = F/T$$
$$\text{Solubility} = S/(S+ P)$$

where, F stands for the amount of fusion protein, T for the total cellular protein amount after induction with IPTG, S for the amount of fusion protein in the supernatant, and P for the amount of fusion protein in the pellet.

**6. Culture of hESC**

Human embryonic stem cells (H9 hESCs) were cultured on Vitronectin in E8 media. This media consisted of DMEM/F12 supplemented with 64 µg/ml L-ascorbic acid-2-phosphate, 14 ng/ml sodium selenite, 10 µg/ml holo-transferrin, 20 µg/ml insulin, 1 mg/ml NaCl, 1% penicillin-streptomycin, 2 ng/ml TGF-β, and 100 ng/ml FGF-2. Cells were maintained at 37°C and 5% CO2, and the culture medium was refreshed daily. All experimental procedures involving hESCs were conducted following the approved guidelines of the Institutional Ethical Committee, University of Ulsan College of Medicine, Seoul, Korea.

When passaging, we utilized two methods: mechanical and enzymatic dissociation. The mechanical dissociation approach involved manually scraping the cells with a plastic pipette tip, then transferring the resulting cell clumps to a 60x15 mm cell culture dish. On the other hand, the enzymatic dissociation method involved treating hESCs with TrypLE Express to dissociate the cells [44]. Following dissociation, cell counts were determined using a standard hemocytometer and cells were resuspended in PBS. Cells were then transferred to a 60x15 mm cell culture dish at a density of 1 x 105 cells per plate for further culture.

**7. Colony formation and adhesion test of single hESCs**

Cells were plated at a density of 500 cells/cm2 (approximately 1,900 cells per well) onto a 12-well plate to test colony formation. Each well was filled with 1 ml of media, which was refreshed daily. After 8 days, colonies were stained using 500 µl/well of 1-Step NBT/BCIP Substrate Solution (Thermo Scientific, USA). Colony quantification was carried out using the ImageJ software (NIH, USA, and LOCI, University of Wisconsin).

**8. Maintaining of single hESCs**

Cells were plated at a density of 500 cells/cm2 (approximately 1,900 cells per well) in a 12-well plate for cell maintenance assays. After eight days of cultivation, hESCs were transferred to the subsequent generation.

**9. Quantification by RT-PCR**

Total RNAs were isolated from cells using Trisure reagent, as per the manufacturer's guidelines. All reactions were conducted on three independent samples and each reaction was performed in triplicate using one-step qPCR. The primer sequences are presented in the Table. All PCR data were analyzed using the delta–delta Ct method and normalized to the housekeeping gene, GAPDH.

**10. Statistical Analysis**

Data are represented as the mean ± standard error of the mean (SEM) for $n \leq 3$ samples. Each experiment was performed independently and in triplicate. A $p$-value of less than 0.05 was considered statistically significant.

## Results

**1. Fusion of VTN with Tags**

In an effort to augment the soluble overexpression of Vitronectin (VTN) in *E. coli*, we fused seven distinct N-terminal tags—His, Trx, GST, PDIb'a', MBP, PDI, and NusA—to the N-terminus of VTN, employing molecular recombinant techniques (Fig. 1A). Subsequently, we evaluated the solubility-enhancing impacts of these tags at three varying temperatures (37℃, 30℃, and 20℃) (Fig. 1B and Table 1). At 37℃ and 30℃, the VTN with His tag was insoluble; however, reducing the temperature to 20℃ improved its solubility. Three tags, specifically PDIb'a', MBP, and PDI, enabled soluble VTN expression across all three temperatures (Table 1). Given its relatively compact size and available MBP affinity chromatography, we selected MBP as the preferred tag.

**2. Effects of the MBP Tag on the Expression and Solubility**

Earlier studies have demonstrated that two truncated variants of VTN, namely VTN(N) and VTN(NC), exhibit strong biological activity in the maintenance of hPSCs. However, when expressed in *E. coli* for their production, these proteins were insoluble, necessitating a complex and laborious purification process involving solubilization and refolding steps [10]. Therefore, we attached the MBP tag to the N-terminus of VTN(N) and VTN(NC), creating MBP-VTN(N) and MBP-VTN(NC) (Fig. 2A). Initially, we evaluated the expression and solubility of the two newly synthesized fusion proteins alongside VTN, MBP-VTN, VTN(N), and VTN(NC). These tests were conducted using *E. coli* BL21, a strain frequently employed as a host for heterologous protein expression. All constructs, with the exception of MBP-VTN(NC), demonstrated expression levels exceeding 20% at both 37℃ and 18℃ (Table 2). Consequently, to augment the expression of MBP-VTN(NC), two other *E. coli* strains, SHuffle

and Rosetta, were tried. The SHuffle strain manifested a significantly improved expression level. The expression of three other constructs, namely MBP-VTN(N), VTN(N), and VTN(NC), was also tested employing SHuffle and Rosetta for comparison. Based on these outcomes, SHuffle was selected as the expression host for MBP-VTN(NC), while BL21 was chosen for the other constructs. Both MBP-VTN(N) and MBP-VTN(NC) exhibited high solubility, particularly at 18°C. Interestingly, both VTN(N) and VTN(NC) also demonstrated high solubility across all three *E. coli* strains (Table 2).

**3. Purification of VTN, MBP-VTN(N), and MBP-VTN(NC)**

Purifications of the five proteins, VTN, VTN(N), VTN(NC), MBP-VTN(N), and MBP-VTN(NC) were attempted. First, for the purification of VTN, a high concentration (8 M) of urea for solubilization and refolding method was used (Figures 3A, 3C) as reported previously 10). Because VTN(N) and VTN(NC) were expressed as soluble forms, they did not require the cumbersome solubilization and refolding. Instead, normal chromatograpical processes were tried (Figures 3A). For the purification of VTN(N), three chromatograpical methods, i.e. anion exchange chromatography, cation exchange chromatography and heparin exchange chromatography, were used. Similarly, in the case of VTN(NC), two chromatographical methods, i.e. anion exchange chromatography and heparin affinity chromatography, were employed. However, in the end the protein yield was very low because several chromatograpical steps were applied and the purified proteins were aggregated (Data not shown), so the purification of VTN(N) and VTN(NC) were not pursued.

Because MBP-VTN(N) and MBP-VTN(NC) were expressed in *E. coli* as a soluble form and have MBP proteins, a maltose-binding protein affinity chromatography was applied for the purification of the proteins and the single chromatography step was enough for the purification of the proteins (Figures 3A, 3D, E). From 500 mL flask culture, we obtained 6.6 ± 0.1 mg (n=3) and 10.4 ± 3.7 mg (n=3) of MBP-VTN(N) and MBP-VTN(NC), respectively. Because there is the TEV protease cleavage site between the MBP tag and the target proteins, we further tried to remove the MBP tag from the fusion proteins using TEV protease treatments. However, the cleaved VTN(N) and VTN(NC) were aggregated substantially resulting in very low purification efficiency (data not shown). Therefore, we did not pursue the purification of VTN(N) and VTN(NC).

**4. Activity of VTN with the MBP tag**

To test the biological activities of the two purified fusion proteins, MBP-VTN(N) and MBP-VTN(NC), we used the proteins as a substrate for maintaining H9 hESCs. Two passaging methods, mechanical dissociation and enzymatic dissociation, were tried. In the mechanical dissociation method, both fusion proteins successfully formed colonies, which was comparable to the effect of VTN (Figure 4A). When the MBP tag alone was used, no colony was formed (Figure 4A). When the enzymatic dissociation method was used, the two fusion proteins also formed colonies like VTN did (Figure 4B). Alkaline phosphatase, one of the pluripotency markers, were stained, all of the proteins supported good expression of alkaline phosphatase (Figure 4C). Even though 20 μg/ml of VTN showed more colonies than 20 μg/ml of MBP-VTN(N) and MBP-VTN(NC), when the concentration was converted to molar concentration and the colony areas were plotted, they showed similar effects (Figure 4D). Binding efficiency of the proteins also showed similar results (Table 4).

It was reported that VTN only permits cell clump passaging, not single-cell passaging, in chemically defined media [23]. Therefore, we conducted five passages of continuous subculture in a single-cell passaging with the enzyme-dissociation method (Figure 5A). Five

days after seeding the single cell in passage 5, VTN induced several protrusions at the boundary of colonies and fewer cells were attached compared to the MBP fusion proteins. By day 8, MBP-VTN(N) and MBP-VTN(NC) facilitated the formation of a larger number and size of colonies. Additionally, pluripotency markers such as OCT4, NANOG, and SOX2 were evaluated by RT-PCR (Figure 5B). Compared to VTN, the MBP fusion proteins demonstrated significantly enhanced pluripotency.

## Discussion

Several extracellular proteins, such as VTN, laminin, and matrigel, have been widely used as substrates for in vitro cell culture of hPSCs. Laminin can't be produced from *E. coli* but requires eukaryotic cells for production due to its multiple disulfide bonds and glycosylations with multimeric architecture [23, 24]. Matrigel, a complex extracellular matrix (ECM) secreted by mouse sarcoma cells [25] is limited for clinical application due to its xenogeic nature. An advantage of VTN is that it can be produced from *E. coli* with relatively low cost. However, because VTN was expressed as an unfolded form resulting in aggregation to form inclusion bodies in *E. coli*, complicated urea solubilization and refolding were necessary for its purification [10]. In this study, of all the fusion tags examined, the MBP tag mitigates many of the expression- and purification-related problems. Proteins tagged with MBP showed a dramatic increase in water solubility, from 5-60% to over 95%, significantly enhancing the purification efficiency in prokaryotic biological systems. The MBP tag proved to be effective at improving both the solubility and productivity of various fusion partners and facilitating the folding of proteins with disulfide bonds [26-38]. Studies suggest that MBP operates as a "chaperone magnet," drawing chaperones that normally associate with MBP towards the target protein [39] or forming large micelle-like aggregates with incompletely folded passenger proteins held inside [40, 41].

Two passaging methods were used for the maintenance of hPSCs. One is clump passaging which is usually done by mechanical dissociation method and the other is single-cell passaging which can be done one by enzymatic dissociation method. Clinical application of hPSCs would require a large amount of cells which can be obtained by enzymatic dissociation method. The single cell passaging of hPSCs using enzymatic dissociation method was supported only by laminin and matrigel, not by VTN which supports only mechanical dissociation method [23]. The previously challenging task of maintaining single cells with VTN was feasible with the MBP fusion in this study. Our results suggest that MBP-VTN(N) and MBP-VTN(NC) preserved the cell's morphology even after five continuous single-cell passages. In contrast VTN led to cell detachment and a loss of shape after just two to three days of subculture. The cells remained intact with MBP-VTN(t), retaining their colony shape over multiple generations. Pluripotency was also preserved, with an average increase of 2.6-fold for MBP-VTN(N) and 4.5-fold for MBP-VTN(NC), highlighting its potential for use in hESC research.

In conclusion, the production of MBP-VTN(N) and MBP-VTN(NC) is a cost-effective method and could significantly enhance hESC research in the future. As such, it represents a valuable tool in the advancement of stem cell research, potentially overcoming the previous limitations associated with the use of VTN. Further research is warranted to optimize this approach and explore its full potential in the field of stem cell biology.

## Table

**Table 1. The solubility of VTN fused with tags when expressed in the cytoplasm of *E. coli.***

| | | His6 | His6 -Trx | His6 -GST | His8 -PDIb'a' | His6 -MBP | His8 -PDI | His6 -NusA |
|---|---|---|---|---|---|---|---|---|
| 37℃ | 2hr | - | N/E | - | + | + | + | - |
| | 4hr | - | - | - | + | + | + | - |
| 30℃ | 3hr | - | - | - | + | + | + | - |
| | 6hr | - | - | - | + | + | + | - |
| 20℃ | 6hr | + | - | + | + | + | + | + |
| | O/N | + | + | + | + | + | + | + |

N/E: No expression
-: insoluble
+: soluble

**Table 2. Expression and solubility level of VTNs**

| | *E. coli* host | Expression level (%) | | Solubility (%) | |
|---|---|---|---|---|---|
| | | 37°C | 18°C | 37°C | 18°C |
| **VTN** | BL21 | 40.7 ± 3.3 | 21.8 ± 1.0 | 38.3 ± 5.9 | 73.0 ± 1.1 |
| **MBP-VTN** | BL21 | 25.2 ± 0.9 | 31.2 ± 5.3 | 66.9 ± 9.4 | 99.4 ± 0.6 |
| **VTN(N)** | BL21 | 20.3 ±2.8 | 30.6 ± 2.3 | 74.3 ± 0.3 | 67.3 ± 6.7 |
| | SHuffle | 12.2 ± 0.9 | 20.2 ± 0.4 | 95.3 ± 0.3 | 92.2 ± 0.6 |
| | Rosetta | 11.1 ± 0.3 | 15.2 ± 2.4 | 93.6 ± 1.1 | 84.9 ± 3.2 |
| **VTN(NC)** | BL21 | 25.9 ± 3.2 | 38.4 ± 1.5 | 78.3 ± 1.5 | 74.0 ± 13.0 |
| | SHuffle | 40.3 ± 5.3 | 23.3 ± 5.5 | 91.8 ± 4.5 | 95.7 ± 2.8 |
| | Rosetta | 47.6 ± 4.8 | 10.9 ± 0.1 | 96.5 ± 0.1 | 93.3 ± 6.8 |
| **MBP-VTN(N)** | BL21 | 38.1 ± 7.3 | 34.7 ± 7.2 | 63.1 ± 0.8 | 96.3 ± 2.7 |
| | SHuffle | 45.0 ± 3.3 | 24.4 ± 5.4 | 81.0 ± 1.5 | 95.6 ± 2.2 |
| | Rosetta | 34.2 ± 5.0 | 36.6 ± 5.5 | 51.3 ± 5.0 | 92.2 ± 6.1 |
| **MBP-VTN(NC)** | BL21 | 17.8 ± 2.2 | 10.8 ± 4.3 | 79.9 ± 9.6 | 91.7 ± 4.7 |
| | SHuffle | 55.9 ± 2.8 | 21.3 ± 1.8 | 82.2 ± 1.3 | 87.9 ± 6.1 |
| | Rosetta | 14.0 ± 0.8 | 19.5 ± 1.4 | 73.1 ± 4.8 | 93.6 ± 1.8 |

**Table 3. Purification yield of MBP-VTN(N) and MBP-VTN(NC)**

| | Total Soluble Protein (mg) | Total Soluble Target Protein (mg) | Purified Target Protein (mg) | Purity (%) | Yield (%) |
|---|---|---|---|---|---|
| VTN | - | - | 9.1 | 95.8 | - |
| MBP-VTN(N) | 127.3 ± 11.2 | 44.7 ± 3.9 | 6.6 ± 0.1 | 96.2 | 14.3 ± 0.6 |
| MBP-VTN(NC) | 184.7 ± 16.8 | 103.2 ± 9.4 | 10.4 ± 3.7 | 88.0 | 8.8 ± 0.6 |

**Table 4. Number of colonies counting by alkaline phosphatase staining with enzymatic method**

| Area of colonies (%, mean ± SD) | | | |
|---|---|---|---|
| **Concentration** | **VTN** | **MBP-VTN(N)** | **MBP-VTN(NC)** |
| **10 µg/mL** | 100 ± 27.2 | 80.0 ± 11.6 | 90.9 ± 22.8 |
| **20 µg/mL** | 228.0 ± 24.6 | 125.2 ± 15.4 | 143.0 ± 23.4 |

**Figure**

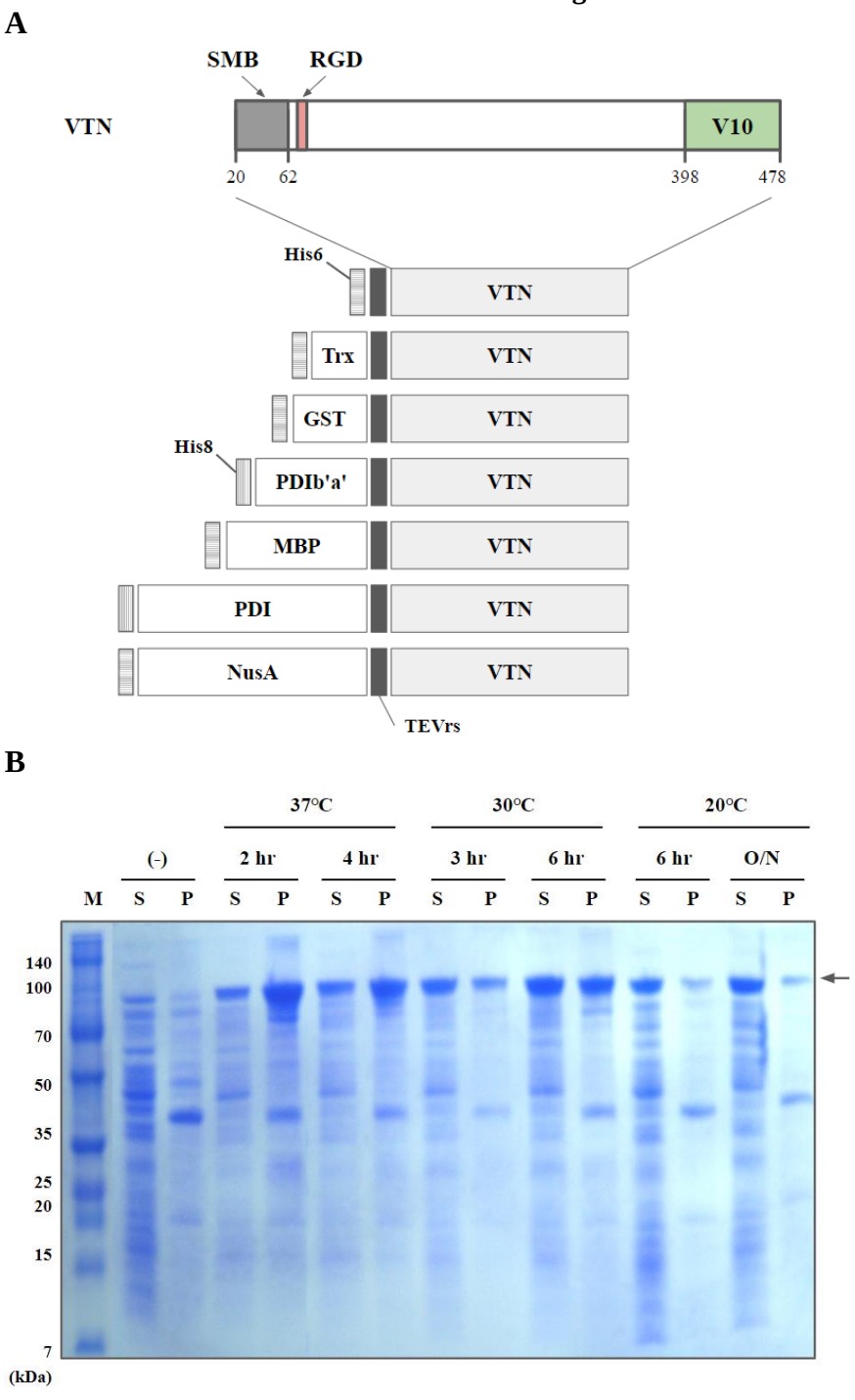
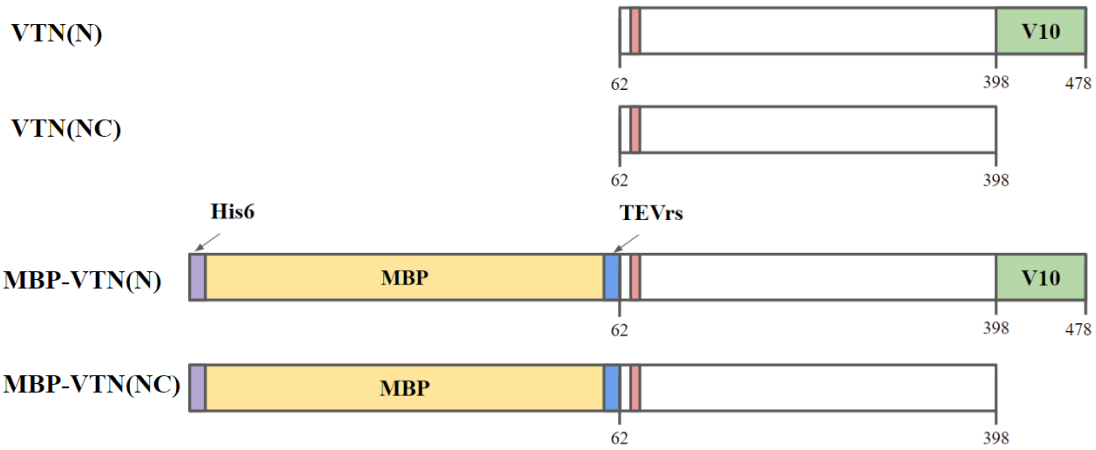
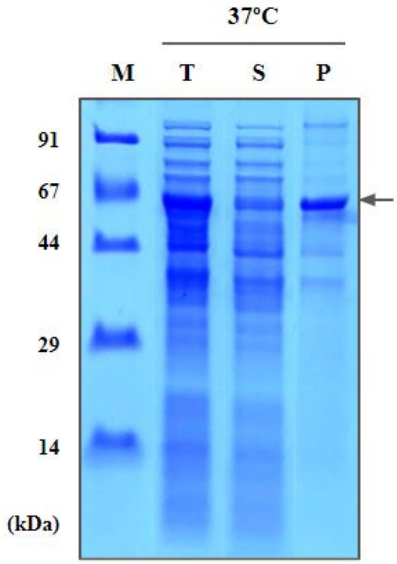



**Figure 1. VTN with various tags.**
(A) Diagram depicting VTN constructs with seven N-terminal tags. (B) An SDS-PAGE image illustrating MBP-VTN expression at three distinct temperatures. (SMB, Somatomedin B-like domains; RGD, Integrin-binding site; M, Molecular weight size marker; S, Soluble supernatant after cell lysis; P, Insoluble portion after cell lysis; (-), Total proteins before induction)

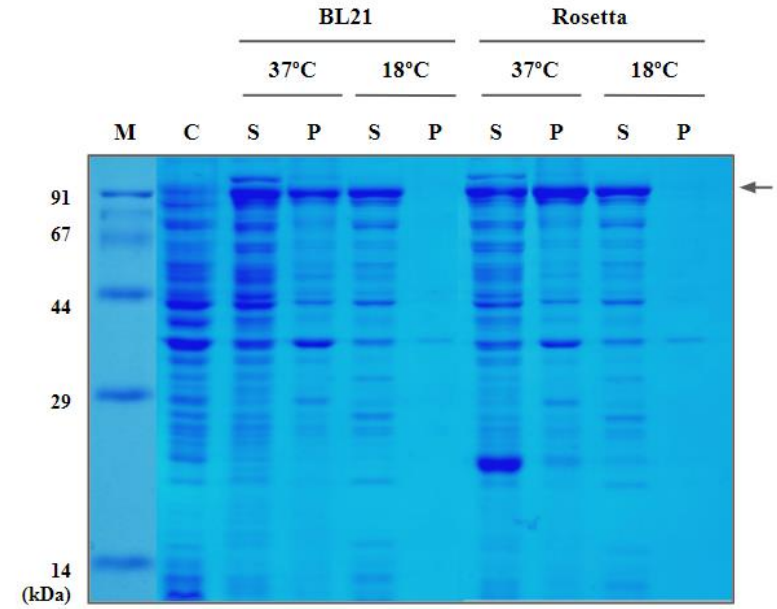
A


VTN(N)
V10
62
398
478
VTN(NC)
62
398
His6
TEVrs
MBP-VTN(N)
MBP
V10
62
398
478
MBP-VTN(NC)
MBP
62
398

B


37°C
M
T
S
P
91
67
44
29
14
(kDa)

C




D

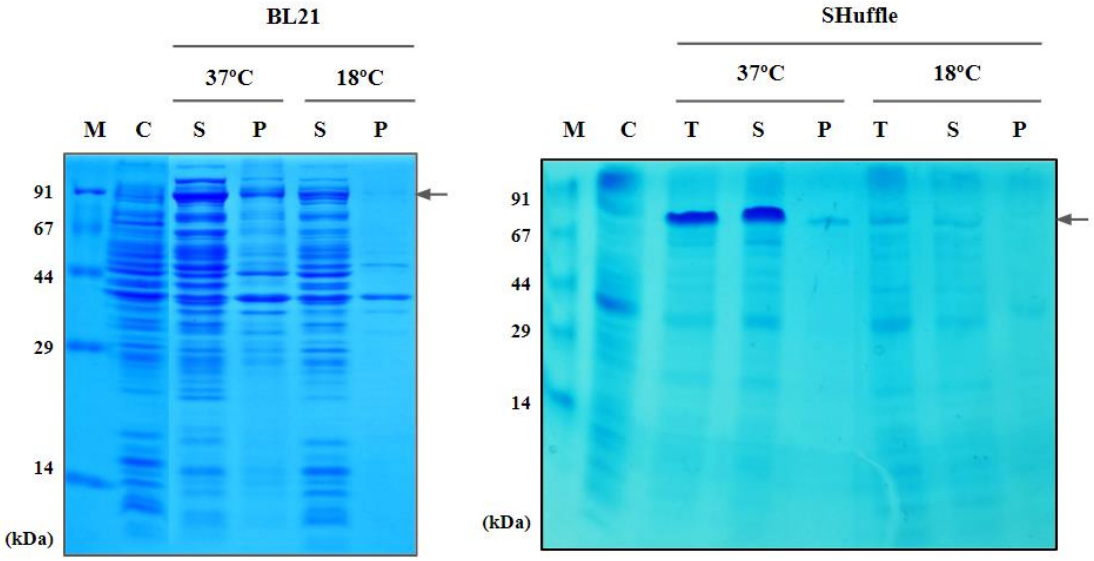
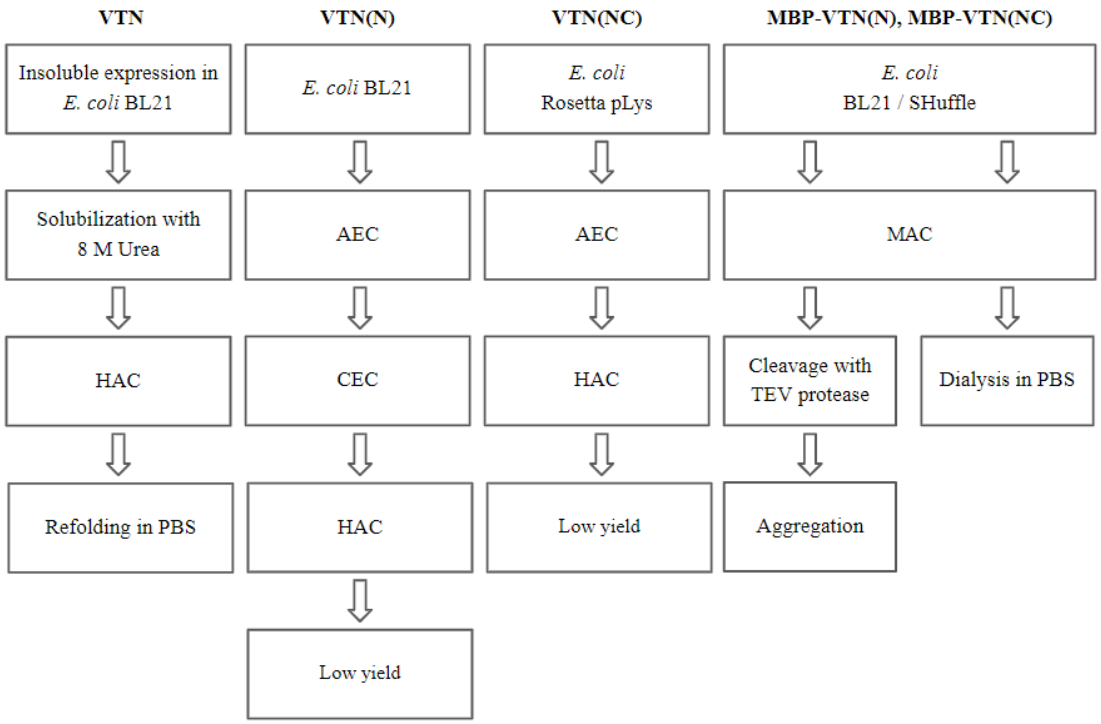
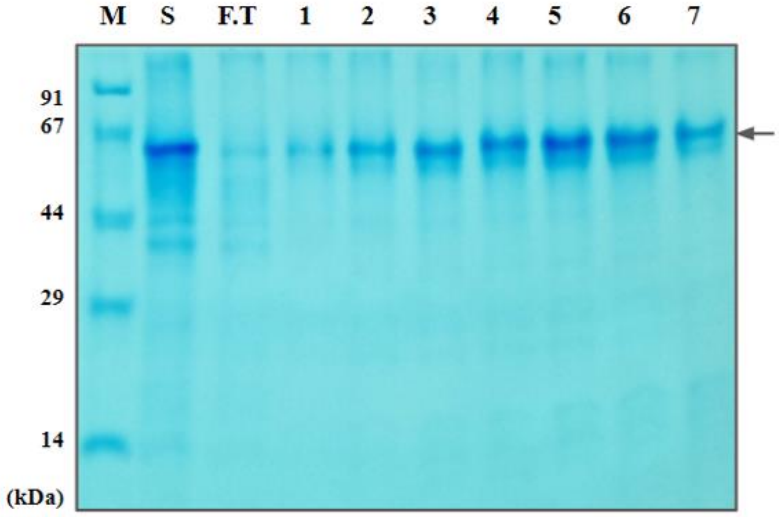



**Figure 2. Expression and Solubility of MBP Tag Fusion Protein.**
(A) Depiction of constructs of VTN(N), VTN(NC), MBP-VTN(N), and MBP-VTN(NC). (B) VTN in BL21, (C) MBP-VTN(N) in BL21 and Rosetta, (C) MBP-VTN(NC) in BL21 and SHuffle. The arrows mark MBP-VTN(N) (91.5 kDa), and MBP-VTN(NC) (82.2 kDa). (SMB, Somatomedin B -like domains; TEVrs, Tobacco etch virus recognition site; RGD, Integrin-binding site; M, Molecular weight size marker; C, Total proteins before induction; T, Total proteins after induction; S, Soluble supernatant after cell lysis; P, Insoluble portion after cell lysis.)

**A**


VTN
VTN(N)
VTN(NC)
MBP-VTN(N), MBP-VTN(NC)
Insoluble expression in E. coli BL21
E. coli BL21
E. coli Rosetta pLys
E. coli BL21 / SHuffle
Solubilization with 8 M Urea
AEC
AEC
MAC
HAC
CEC
HAC
Cleavage with TEV protease
Dialysis in PBS
Refolding in PBS
HAC
Low yield
Aggregation
Low yield

**B**


M
S
F.T
1
2
3
4
5
6
7
91
67
44
29
14
(kDa)

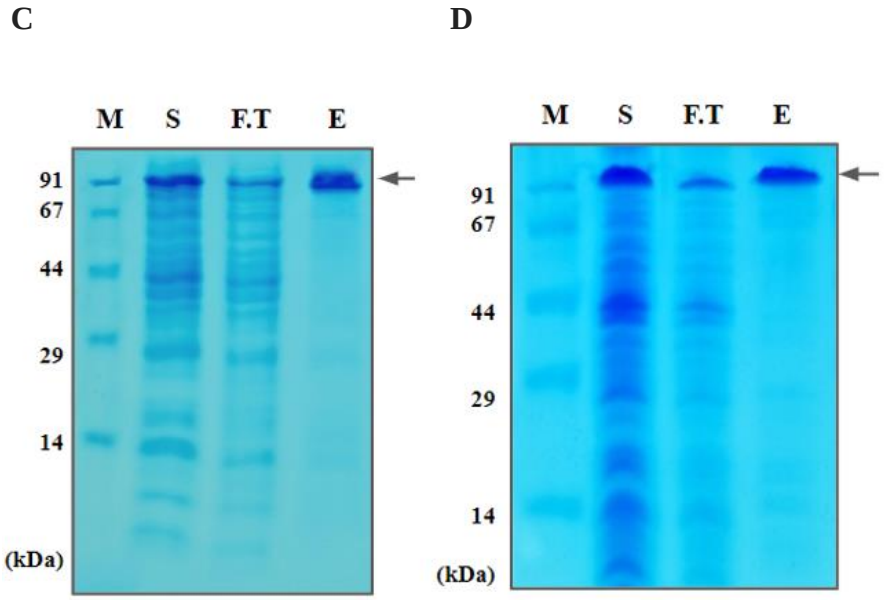
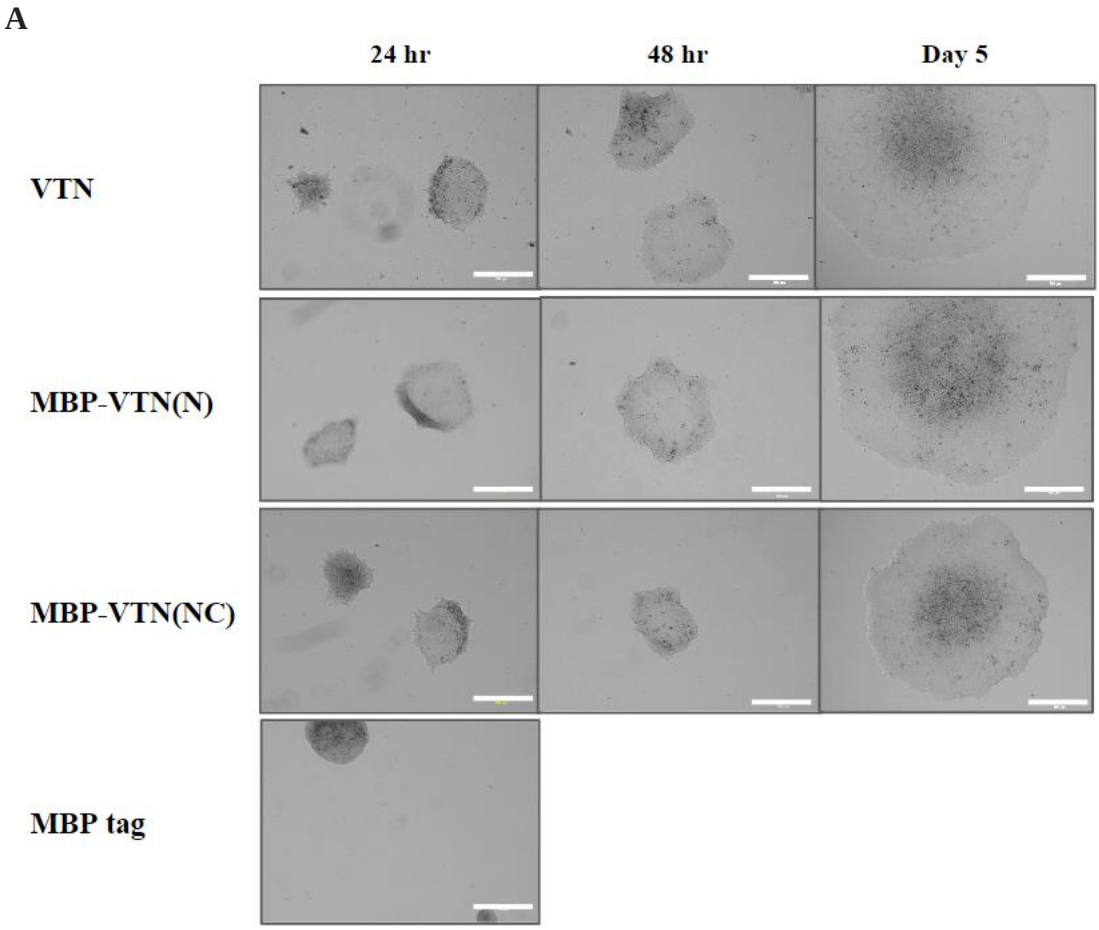
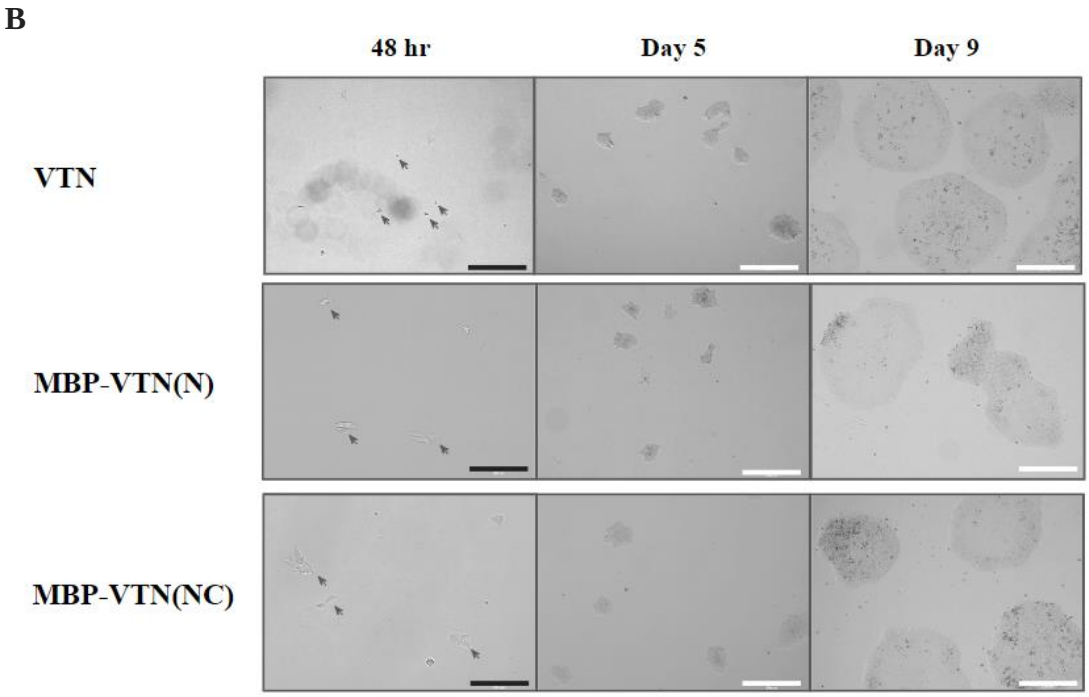



**Figure 3. Purification of VTN, MBP-VTN(N), and MBP-VTN(NC).**
(A) Flow chart illustrating the purification steps for VTN, VTN(N), VTN(NC), MBP-VTN(N), and MBP-VTN(NC). (H.A.C, Heparin affinity chromatography; A.E.C, Anion exchange chromatography; C.E.C, Cation exchange chromatography; M.A.C, MBP affinity chromatography) (B) Depiction of VTN purification and expression in *E. coli* BL21 utilizing affinity chromatography. (M, Molecular weight size marker; S, Soluble proteins after cell sonication from total cell proteins; F.T, Flow through after affinity chromatography; 1 - 8, VTN (54.6 kDa) purified with affinity chromatography eluted by 0 - 1 M NaCl.) (C) Representation of MBP-VTN(N) purification and expression in *E. coli* BL21 using affinity chromatography. (M, Molecular weight size marker; S, Soluble proteins after cell sonication from total cell proteins; F.T, Flow through after affinity chromatography; E, MBP-VTN(N) fusion protein (91.5 kDa) purified with affinity chromatography.) (D) Illustration of MBP-VTN(NC) purification and expression in *E. coli* BL21 using affinity chromatography. (M, Molecular weight size marker; S, Soluble proteins after cell sonication from total cell proteins; F.T, Flow through after affinity chromatography; E, MBP-VTN(NC) fusion protein (82.2 kDa) purified with affinity chromatography.)


A
24 hr
48 hr
Day 5
VTN
MBP-VTN(N)
MBP-VTN(NC)
MBP tag


B
48 hr
Day 5
Day 9
VTN
MBP-VTN(N)
MBP-VTN(NC)

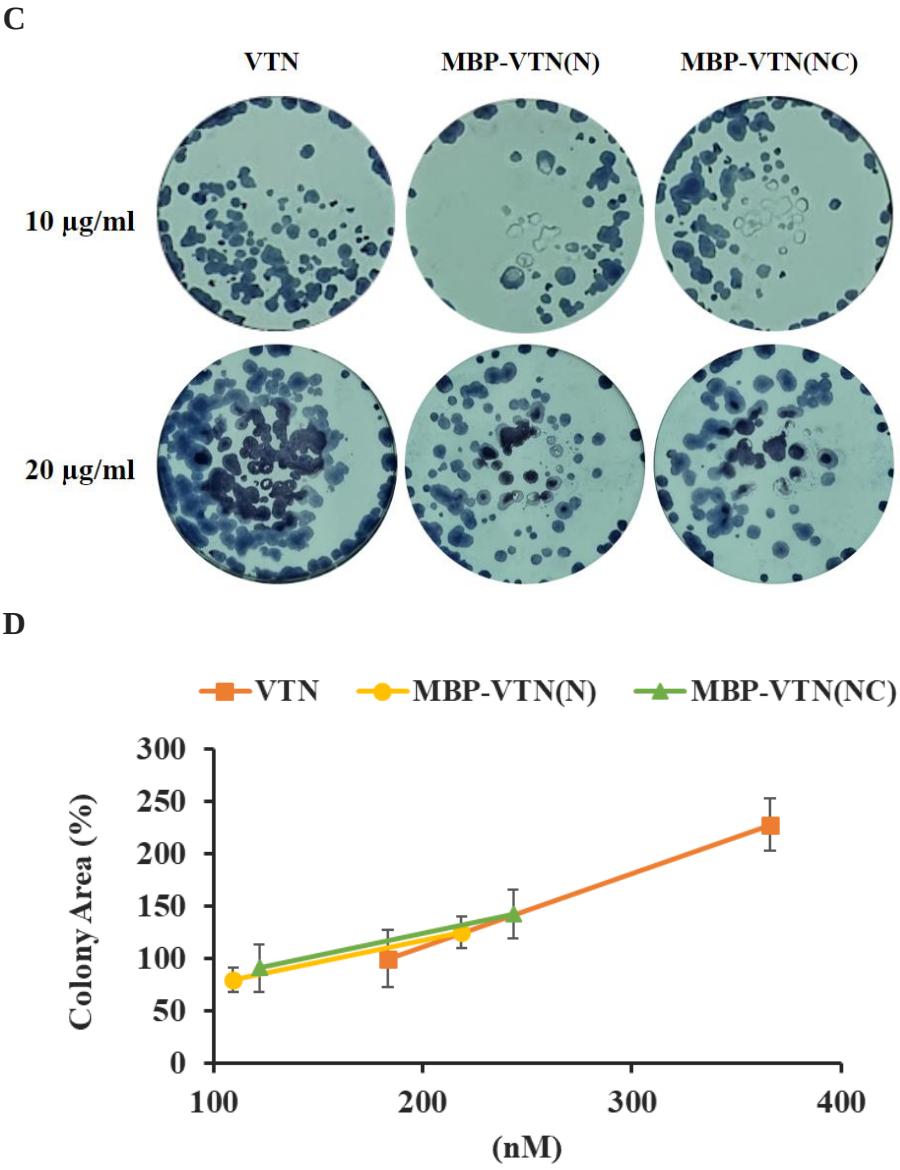



**Figure 4. Colony formation and attachment of H9 hESCs on VTN, MBP-VTN(N), MBP-VTN(NC).**

(A) Microscopic images showcasing H9 hESCs colony formation through the mechanical dissociation method at 24 hr, 48 hr, and 5 day time points. The coating densities of VTN, MBP-VTN(N), and MBP-VTN(NC) were 10 µg/mL, respectively (White scale bar, 500 µm) (B) Microscopic images displaying single cell H9 hESCs colony formation via the enzymatic dissociation method at 48 hr, 5 day, and 9 day time points. The coating density of VTN MBP-VTN(N), and MBP-VTN(NC) were 10 µg/mL, respectively. (Black scale bar, 200 µm; White scale bar, 500 µm; Arrow indicates Single cells) (C) Microscopic images of alkaline phosphatase staining applied to H9 hESCs data. (D) Statistical representation of the colony area (n=3). Note that concentration of proteins was converted to molar concentration.

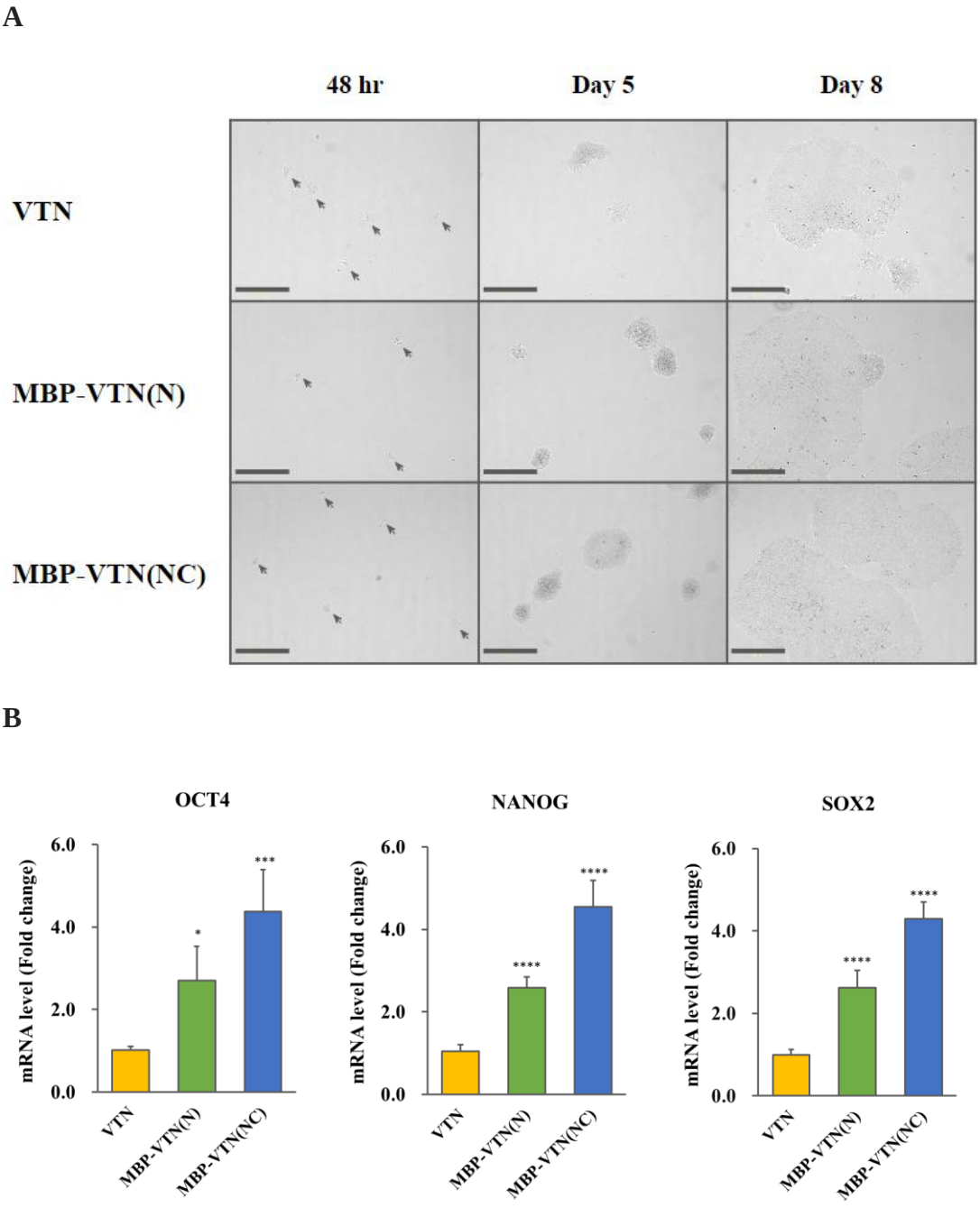



**Figure 5. Single cell maintenance of hESCs on VTN, MBP-VTN(N), and MBP-VTN(NC).** (A) Images captured at 4X, demonstrating the morphology of hESCs over time under different coating conditions. (Scale bar, 500 µm; Arrow points to Single cells) (B) RT-PCR data exhibiting pluripotent gene expression. (*p< 0.05, ***p< 0.005, ****p< 0.001, Student's T-test, n=6)

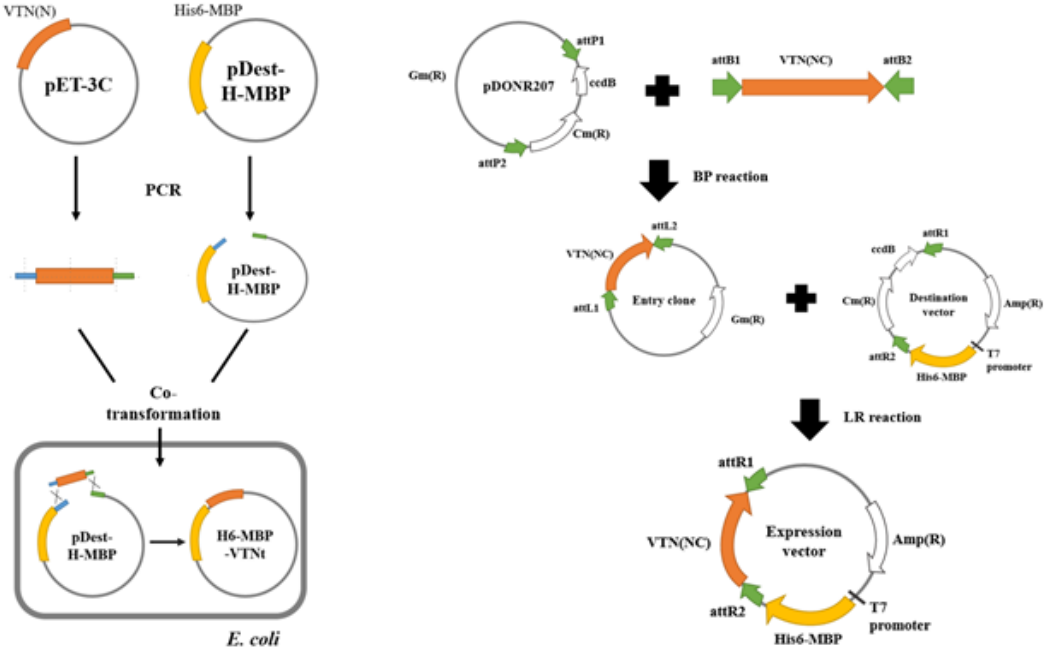
## Supplementary Table

**Supplementary table 1.** Primer sequences for plasmid constructions

| **Primer** | **Sequence** |
|---|---|
| PCR BP forward | 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CGA GAA CCT GTA CTT CCA GGG CGT GAC TCG CGG GGA TGT G-3' |
| PCR BP reverse | 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT TAG CGG GAT GGC CGG CG-3' |
| Truncated VTN forward | 5'-ATG GTG ACT CGC GGG GAT G-3' |
| Truncated VTN reverse | 5'-CAG ATG GCC AGG AGC TGG-3' |
| Vector forward | 5'-CTG GCT GGG CTG CCC AGC TCC TGG CCA TCT GTA AAC CCA GCT TTC TTG TAC AAA GTG G-3' |
| Vector reverse | 5'-GCA TAG TGA ACA CAT CCC CGC GAG TCA CCA TGC CCT GGA AGT ACA GGT CTC-3' |

**Supplementary Figure**

**A**




**B**

```
1    MDQESCKGRCTEGFNVDKKCQCDELCSYYQSCCTDYTAECKPQVTRGDVFTMPEDEYTVY
61   DDGEEKNNATVHEQVGGPSLTSDLQAQSKGNPEQTPVLKPEEEAPAPEVGASKPEGIDSR
121  PETLHPGRPQPPAEEELCSGKPFDAFTDLKNGSLFAFRGQYCYELDEKAVRPGYPKLIRD
181  VWGIEGPIDAAFTRINCQGKTYLFKGSQYWRFEDGVLDPDYPRNISDGFDGIPDNVDAAL
241  ALPAHSYSGRERVYFFKGKQYWEYQFQHQPSQEECEGSSLSAVFEHFAMMQRDSWEDIFE
301  LLFWGRTSAGTRQPQFISRDWHGVPGQVDAAMAGRIYISGMAPRPSLAKKQRFRHRNRKG
361  YRSQRGHSRGRNQNSRRPSRAMWLSLFSSEESNLGANNYDDYRMDWLVPATCEPIQSVFF
421  FSGDKYYRVNLRTRRVDTVDPPYPRSIAQYWLGCPAPGHL
```

**C**

```
1    MGSSHHHHHHGTKTEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLE
61   EKFPQVAATGDGPDIIFWAHDRFGGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYN
121  GKLIAYPIAVEALSLIYNKDLLPNPPKTWEEIPALDKELKAKGKSALMFNLQEPYF
181  TWPLIAADGGYAFKYENGKYDIKDVGVDNAGAKAGLTFLVDLIKNKHMNADTDYSI
241  AEAAFNKGETAMTINGPWAWSNIDTSKVNYGVTVLPTFKGQPSKPFVGVLSAGINA
301  ASPNKELAKEFLENYLLTDEGLEAVNKDKPLGAVALKSYEEELAKDPRIAATMENA
361  QKGEIMPNIPQMSAFWYAVRTAVINAASGRQTVDEALKDAQTGTGSYITSLYKKAG
421  FENLYFQGVTRGDVFTMPEDEYTVYDDGEEKNNATVHEQVGGPSLTSDLQAQSKGN
481  PEQTPVLKPEEEAPAPEVGASKPEGIDSRPETLHPGRPQPPAEEELCSGKPFDAFT
541  DLKNGSLFAFRGQYCYELDEKAVRPGYPKLIRDVWGIEGPIDAAFTRINCQGKTYL
601  FKGSQYWRFEDGVLDPDYPRNISDGFDGIPDNVDAALALPAHSYSGRERVYFFKGK
661  QYWEYQFQHQPSQEECEGSSLSAVFEHFAMMQRDSWEDIFELLFWGRTSAGTRQPQ
721  FISRDWHGVPGQVDAAMAGRIYISGMAPRPSLAKKQRFRHRNRKGYRSQRGHSRGR
781  NQNSRRPSRAMWLSLFSSEESNLGANNYDDYRMDWLVPATCEPIQSVFFFSGDKYY
841  RVNLRTRRVDTVDPPYPRSIAQYWLGCPAPGHL
```

**Supplement figure 1. Sequence of VTN, MBP-VTN(N) and MBP-VTN(NC)** (A) Schematic structure of the MBP-VTN(N) construct with In vivo cloning and the MBP-VTN(NC) construct with Gateway cloning. (B) Amino acid sequence of VTN. (C) Amino acid sequence of MBP-VTN(N) (underline: Somatomedin B -like domains, Red: RGD region, Green: V10).

## References


1. Preissner KT. Structure and biological role of vitronectin. Annu Rev Cell Biol 1991;7:275-310.
2. Preissner KT, Seiffert D. Role of vitronectin and its receptors in haemostasis and vascular remodeling. Thromb Res 1998;89(1):1-21.
3. Holmes R. Preparation from human serum of an alpha-one protein which induces the immediate growth of unadapted cells in vitro. J Cell Biol 1967;32(2):297-308.
4. Tomasini BR, Mosher DF. Vitronectin. Prog Hemost Thromb 1991;10:269-305.
5. Schvartz I, Seger D, Shaltiel S. Vitronectin. Int J Biochem Cell Biol 1999;31(5):539-44.
6. Orlando RA, Cheresh DA. Arginine-glycine-aspartic acid binding leading to molecular stabilization between integrin alpha v beta 3 and its ligand. J Biol Chem 1991;266(29):19543-50.
7. Vogel BE, Lee SJ, Hildebrand A, Craig W, Pierschbacher MD, Wong-Staal F, et al. A novel integrin specificity exemplified by binding of the alpha v beta 5 integrin to the basic domain of the HIV Tat protein and vitronectin. J Cell Biol 1993;121(2):461-8.
8. Braam SR, Zeinstra L, Litjens S, Ward-van Oostwaard D, van den Brink S, van Laake L, et al. Recombinant vitronectin is a functionally defined substrate that supports human embryonic stem cell self-renewal via alphavbeta5 integrin. Stem Cells 2008;26(9):2257-65.
9. Prowse AB, Doran MR, Cooper-White JJ, Chong F, Munro TP, Fitzpatrick J, et al. Long term culture of human embryonic stem cells on recombinant vitronectin in ascorbate free media. Biomaterials 2010;31(32):8281-8.
10. Chen G, Gulbranson DR, Hou Z, Bolin JM, Ruotti V, Probasco MD, et al. Chemically defined conditions for human iPSC derivation and culture. Nat Methods 2011;8(5):424-9.
11. Yatohgo T, Izumi M, Kashiwagi H, Hayashi M. Novel purification of vitronectin from human plasma by heparin affinity chromatography. Cell Struct Funct 1988;13(4):281-92.
12. Akiyama SK. Purification of vitronectin. Curr Protoc Cell Biol 2013;60:10 6 1- 6 7.
13. Wojciechowski K, Chang CH, Hocking DC. Expression, production, and characterization of full-length vitronectin in Escherichia coli. Protein Expr Purif 2004;36(1):131-8.
14. Halford MM, He YC, Stacker SA. Expression and purification of bioactive, low-endotoxin recombinant human vitronectin. Biotechniques 2014;56(6):331-3.
15. Fossum S, Crooke E, Skarstad K. Organization of sister origins and replisomes during multifork DNA replication in Escherichia coli. EMBO J 2007;26(21):4514-22.
16. Khalilzadeh R, Shojaosadati SA, Maghsoudi N, Mohammadian-Mosaabadi J, Mohammadi MR, Bahrami A, et al. Process development for production of recombinant human interferon-gamma expressed in Escherichia coli. J Ind Microbiol Biotechnol 2004;31(2):63-9.
17. Knorre WA, Deckwer WD, Korz D, Pohl HD, Riesenberg D, Ross A, et al. High cell density fermentation of recombinant Escherichia coli with computer-controlled optimal growth rate. Ann N Y Acad Sci 1991;646:300-6.
18. Rosano GL, Ceccarelli EA. Recombinant protein expression in Escherichia coli: advances and challenges. Front Microbiol 2014;5:172.
19. Sorensen HP, Mortensen KK. Advanced genetic strategies for recombinant protein expression in Escherichia coli. J Biotechnol 2005;115(2):113-28.

20. Baeshen NA, Baeshen MN, Sheikh A, Bora RS, Ahmed MM, Ramadan HA, et al. Cell factories for insulin production. Microb Cell Fact 2014;13:141.
21. Loughran ST, Walls D. Tagging Recombinant Proteins to Enhance Solubility and Aid Purification. Methods Mol Biol 2017;1485:131-56.
22. Ki MR, Pack SP. Fusion tags to enhance heterologous protein expression. Appl Microbiol Biotechnol 2020;104(6):2411-25.
23. Miyazaki T, Futaki S, Suemori H, Taniguchi Y, Yamada M, Kawasaki M, et al. Laminin E8 fragments support efficient adhesion and expansion of dissociated human pluripotent stem cells. Nat Commun 2012;3:1236.
24. Rodin S, Domogatskaya A, Strom S, Hansson EM, Chien KR, Inzunza J, et al. Long-term self-renewal of human pluripotent stem cells on human recombinant laminin-511. Nat Biotechnol 2010;28(6):611-5.
25. Talbot NC, Caperna TJ. Proteome array identification of bioactive soluble proteins/peptides in Matrigel: relevance to stem cell responses. Cytotechnology 2015;67(5):873-83.
26. Baneyx F, Mujacic M. Recombinant protein folding and misfolding in Escherichia coli. Nat Biotechnol 2004;22(11):1399-408.
27. Lebendiker M, Danieli T. Purification of proteins fused to maltose-binding protein. Methods Mol Biol 2011;681:281-93.
28. Do BH, Ryu HB, Hoang P, Koo BK, Choe H. Soluble prokaryotic overexpression and purification of bioactive human granulocyte colony-stimulating factor by maltose binding protein and protein disulfide isomerase. PLoS One 2014;9(3):e89906.
29. Nguyen MT, Koo BK, Thi Vu TT, Song JA, Chong SH, Jeong B, et al. Prokaryotic soluble overexpression and purification of bioactive human growth hormone by fusion to thioredoxin, maltose binding protein, and protein disulfide isomerase. PLoS One 2014;9(3):e89038.
30. Vu TT, Koo BK, Song JA, Chong SH, Park CR, Nguyen MT, et al. Soluble overexpression and purification of bioactive human CCL2 in *E. coli* by maltose-binding protein. Mol Biol Rep 2015;42(3):651-63.
31. Nguyen MT, Krupa M, Koo BK, Song JA, Vu TT, Do BH, et al. Prokaryotic Soluble Overexpression and Purification of Human VEGF165 by Fusion to a Maltose Binding Protein Tag. PLoS One 2016;11(5):e0156296.
32. Vu TT, Jeong B, Krupa M, Kwon U, Song JA, Do BH, et al. Soluble Prokaryotic Expression and Purification of Human Interferon Alpha-2b Using a Maltose-Binding Protein Tag. J Mol Microbiol Biotechnol 2016;26(6):359-68.
33. Do BH, Nguyen MT, Song JA, Park S, Yoo J, Jang J, et al. Soluble Prokaryotic Expression and Purification of Bioactive Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand. J Microbiol Biotechnol 2017;27(12):2156-64.
34. Nguyen AN, Song JA, Nguyen MT, Do BH, Kwon GG, Park SS, et al. Prokaryotic soluble expression and purification of bioactive human fibroblast growth factor 21 using maltose-binding protein. Sci Rep 2017;7(1):16139.
35. Nguyen MT, Prima MJ, Song JA, Kim J, Do BH, Yoo J, et al. Prokaryotic soluble overexpression and purification of oncostatin M using a fusion approach and genetically engineered *E. coli* strains. Sci Rep 2019;9(1):13706.
36. Nguyen MT, Heo Y, Do BH, Baek S, Kim CJ, Jang YJ, et al. Bacterial overexpression and purification of soluble recombinant human serum albumin using maltose-binding protein and protein disulphide isomerase. Protein Expr Purif 2020;167:105530.
37. Nguyen TKO, Vu TL, Nguyen MQ, Ta HKK, Park KS, Kim SH, et al. Soluble Prokaryotic Overexpression and Purification of Human GM-CSF Using the Protein Disulfide Isomerase b'a' Domain. Int J Mol Sci 2021;22(10).

38. Lee E, de Paula MN, Baek S, Ta HKK, Nguyen MT, Jeong TH, et al. Novel Bacterial Production of Two Different Bioactive Forms of Human Stem-Cell Factor. Int J Mol Sci 2021;22(12).
39. Randall LL, Topping TB, Smith VF, Diamond DL, Hardy SJ. SecB: a chaperone from Escherichia coli. Methods Enzymol 1998;290:444-59.
40. Nomine Y, Ristriani T, Laurent C, Lefevre JF, Weiss E, Trave G. A strategy for optimizing the monodispersity of fusion proteins: application to purification of recombinant HPV E6 oncoprotein. Protein Eng 2001;14(4):297-305.
41. Sachdev D, Chirgwin JM. Order of fusions between bacterial and mammalian proteins can determine solubility in Escherichia coli. Biochem Biophys Res Commun 1998;244(3):933-7.
42. Todorova S, Diankova L, Andreeva M, Khristova D, Tsachev K, Kamenova P. [Treatment of patients with postmenopausal and steroid-induced osteoporosis using sodium fluoride combined with vitamin D2 and calcium gluconate]. Vutr Boles 1990;29(1):77-81.
43. Nozaki S, Niki H. Exonuclease III (XthA) Enforces In Vivo DNA Cloning of Escherichia coli To Create Cohesive Ends. J Bacteriol 2019;201(5).
44. Prowse A, Wolvetang E, Gray P. A rapid, cost-effective method for counting human embryonic stem cell numbers as clumps. Biotechniques 2009;47(1):599-606.

**Acknowledgement**

I deeply thank all members of our laboratory for their technical support or constructive discussion.

## 국문요약

### 원핵생물에서 Vitronectin 의 수용성 발현 및 정제효율의 증가와 인간 만능 줄기 세포 배양에 대한 응용

Vitronectin(VTN)은 인간 만능 줄기 세포(hPSC) 배양에서 중요한 단백질 기질로 알려져 있으나, 이의 대장균 발현과 수용성이 낮다는 단점이 있다. 본 연구는 말토스 결합 단백질(MBP)를 VTN 및 이의 Truncated 형태인 VTN(N)과 VTN(NC)에 융합 시켜, 대장균(Escherichia coli) 발현 시스템에서의 수용성과 정제 효율을 향상시켰다. 7 개의 태그를 평가한 결과, MBP 가 가장 효과적으로 나타났으며, VTN, VTN(N), VTN(NC)의 수용성과 정제 효율을 5-60%에서 95% 이상으로 크게 증가시켰다. 게다가 MBP 에 태그 된 VTN 은 VTN 단독보다 단일 세포 수준에서 hPSC 의 유지를 가능하게 했으며, 장기 저장 동안 우수한 안정성을 보였다. 특히, truncated MBP-VTN 은 여러 번의 계대배양 후에도 세포 형태와 만능성을 보존하여, 수일 후에 세포 이탈을 유발하는 VTN 보다 뛰어난 성능을 보였다. 이러한 결과는 truncated MBP-VTN 융합 단백질이 hPSC 배양에 상당한 장점을 제공하며, 경제적이고 효율적인 줄기세포 연구의 가능성을 열어준다는 것을 시사한다. 이 접근법을 최적화하고 줄기세포 생물학에서의 전체적인 잠재력을 탐구하기 위해 추가 연구가 필요하다.

중심단어: 비트로넥틴, 융합단백질, 대장균 발현, 말토스 결합 단백질, 만능 줄기 세포