Indian Journal of Biotechnology
Vol. 17, October 2018, pp 553-560

# CRISPR/Cas9 knock-in of GST-tagged human Noggin in the β-casein gene locus of bovine ear fibroblast cells

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*Received 13 February 2018; revised 25 April 2018; accepted 29 June 2018*

We developed knock-in vector system of human Noggin mature sequence with glutathione S-transferase (GST) containing factor Xa protease linker to facilitate the subsequent purification of recombinant protein. To achieve this, bovine ear fibroblast cells were isolated and transfection conditions were optimized by electroporation. To generate knock-in vector, human Noggin lacking its native signal peptide is fused to GST and foot and month disease virus 2A (F2A), and then inserted into bovine β-casein gene exon 3. We also generated enhanced green fluorescent protein (EGFP) expression vector of GST-human Noggin mature fused to β-casein signal peptide and F2A, and successfully detected recombinant human Noggin protein secreted into culture media, followed by factor Xa cleavage. Then, we co-transfected human Noggin knock-in vector with single-guided RNA and Cas9 expression vectors into bovine ear fibroblasts and obtained the stably-integrated colonies by antibiotic selection. PCR screening analysis revealed that 26 out of 35 colonies positively integrated human Noggin knock-in vector into bovine β-casein locus. One of positive clones was subjected to chromosome analysis, presenting normal karyotypes. Our data may provide the additional purification guideline of recombinant proteins by tagging GST with a protease linker sequence in the upstream of target genes and a high efficiency of integration ratio into bovine β-casein locus.

**Keywords**: hNoggin, CRISPR/Cas9, bovine ear fibroblast cells, bovine β-casein locus, sgRNA, factor Xa

## Introduction

The production of recombinant proteins by transgenesis in livestock species has considerable potential for application in a variety of research and clinical sectors ranging from animal productivity to biomedical uses and ultimately aims to improve human health. Nevertheless, the application of transgenic processes is limited due to random transgene integration and low copy number[1,2]. Recently, targeted genome editing by site-specific endonucleases has been developed to overcome the technical limitations of random gene targeting and low integration efficiency due to the reliance of homologous recombination on the DNA repair system. To date, three kinds of endonucleases have been successfully used to produce genetically modified animals: zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), and clustered regularly interspaced short palindromic repeats (CRISPR)[3-6]. CRISPR technology utilizes Cas9 protein (CRISPR-associated nuclease) and a single guide RNA (sgRNA), which is composed of 20 nucleotides recognized by the protospacer adjacent molif (PAM) sequence (5'-NGG-3'). The sgRNA directs Cas9 to introduce a double-stranded break (DSB) in the target site[7]. As CRISPR is an RNA-guided system rather than protein-guided, it is easier to engineer and more flexible than ZFN and TALEN[2,7-9].

Noggin is a secreted homodimeric glycoprotein with a signal peptide (SP) and structurally resembles bone morphogenetic proteins (BMPs). Binding of some of the BMPs to Noggin inhibits the activation of BMP receptors, thus blocking SMAD-dependent and SMAD-independent signaling[10-11]. Noggin, a pleiotropic factor, is essential in both early and late developmental stages. In Noggin null mice increased BMP activity results in severe developmental abnormalities such as neural tube formation failure, hair-follicle retardation, dysmorphogenesis of the axial

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skeleton, and joint lesions[12-13]. In contrast, the combinational treatment of exogenous Noggin and basic fibroblast growth factor (FGF) is sufficient to maintain the pluripotency of human embryonic stem cells in the absence of feeder cells[14]. Moreover, Noggin is involved in the regulation of the stem cell niche during neurogenesis by antagonizing BMP signaling[15].

It has been reported that β-casein is not essential for viability, fertility, or nursing capability of homozygous mutant mice, even though β-casein is a major milk protein component. This suggests that the caseins are viable candidates for genetic manipulation to improve milk composition qualitatively[16]. Therefore, it is possible that a specific exogenous gene could be inserted into the β-casein gene locus by knock-in, and its expression could be controlled by endogenous β-casein gene-regulatory elements[8]. To improve the production and purification efficacy of recombinant human Noggin (hNoggin) in the mammary gland, we developed a knock-in gene targeting system wherein hNoggin fusion gene expression is controlled by the endogenous bovine β-casein promoter and enhancers. To this end, the hNoggin open reading frame lacking the SP sequence was fused to glutathione S-transferase (GST) containing a factor Xa linker sequence (IEGR) to facilitate the subsequent purification of recombinant protein. Factor Xa, a trypsin-like serine protease that cleaves peptide bonds of basic amino acid residues, was located downstream of the GST gene. To induce the effective secretion of Noggin protein through the mammary gland and into milk, the β-casein SP was inserted upstream of GST. GST is the most common fusion tag used in pull-down assays and can contain protease cleavage sites, such as thrombin and factor Xa, which provide a convenient method for separating GST from the protein of interest by protease cleavage[17]. Finally, a self-cleaving F2A peptide, first identified in foot-and-mouth disease[18], was inserted into the sequence between the bovine β-casein SP and GST to separate the upstream bovine β-casein SP from the GST fusion gene. This study provides insight for the efficient production and purification of recombinant proteins in the bovine mammary gland.

## Materials and Methods

### Cell Cultures

Bovine fibroblast cells were isolated from postmortem ear tissues of fifteen-day old male Holstein cattle according to the method that has been previously reported[8,19], with a minor modification. The fibroblast cells were grown in culture medium including Dulbecco's Modified Eagle's Medium (DMEM, Hyclone Laboratories, Inc., Logan, UT, USA), 15% defined fetal bovine serum (FBS, Hyclone), 0.1 mM non-essential amino acids (Welgene, Daegu, South Korea), 1 mM sodium pyruvate (Welgene), 143 mM β–mercaptoethanol (Sigma, St. Louis, MO, USA), 1% penicillin/streptomycin (Life Technologies, Grand Island, NY, USA) in the presence of 5% $CO_2$ at 37°C. HEK293T cells (American Type Cell Collection) were cultured in DMEM (Hyclone) supplemented with 10% FBS (Life Technologies) and 1% penicillin/streptomycin.

### Plasmid Constructs

pBluescript SK(+)-RG1_bβCE3_F2A-hFGF2 neo knock-in vector as a template and pMCDT-A (Gibco BRL, Grand Island, NY, USA)[8] were used to generate knock-in vector of bovine β-casein homologous 5'- and 3'-arm fragments with GST-tagged hNoggin mature. To achieve this, hNoggin mature cDNAs lacking its own SP was amplified by PCR using the primers specific to hNoggin flanked by *Kpn*I and *Sma*I sites (underlined): (forward, 5'-GGTACCCTGCGGGCG ACACCGGCCGGCGGCC-3'; reverse, 5'-CCCGGG CTAGCACGAGCACTTGCACTCGGAA-3'). GST with factor X linker was obtained from pGEX-5X-1 vector (GE Healthcare Life Sciences, Little Chalfont, UK) by PCR using the specific primers (forward, 5'-GGTACCATGTCCCCTATACTAGGTTATTGG-3'; reverse, 5' GGTACCGGATCCACGACCTTCGAT CAGATC-3'). To observe the secretion capacities of endogenous bovine β-casein SP with GST-tagged hNoggin mature, bovine β-casein SP-F2A-GST-hNoggin mature sequences were ligated into EF1α promoter-driven EGFP expression vector to produce pEF1α-bovine β-casein SP-F2A-GST-hNoggin-EGFP expression vector. All cloned plasmids were verified by sequencing.

### Transient Transfection and Western Blot

HEK293T cells were transfected with pEF1α-bovine β-casein SP-F2A-GST-hNoggin-EGFP expression vector using 25 kDa L-polyethylenimine transfection reagent (Polysciences, Warrington, PA, USA), as previously reported[20]. pEF1α-EGFP plasmid was used as a control. EGFP expression was visualized by the fluorescence microscope (Nikon, Tokyo, Japan). The transfected cells were harvested 2 days after transfection, lysed with RIPA lysis buffer [150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate,

0.1% SDS, 50 mM Tris (pH 8.0), 1% protease inhibitor cocktail (GenDEPOT, Barker, Texas, USA)] on ice for 20 minutes, and then centrifuged with 15000 rpm at 4°C for 20 minutes. The culture media were collected and concentrated using concentrators (Pierce Biotechnology, Inc., Rockford, IL, USA) with 2500 rpm at 4°C for 90 minutes. Whole cell lysates and concentrated culture media were pull-downed with glutathione sepharose 4B (GE Healthcare Life Sciences) at 4°C for 2 days and then eluted in GST-bead elution buffer (100 mM Tris-HCl, 15 mM reduced glutathione, 120 mM NaCl, 10% glycerol). The post-eluted supernatants were treated with factor Xa protease (New England Biolabs, Ipswich, MA, USA) in factor Xa buffer (20 mM Tris-HCl, 100 mM NaCl, 2 mM $CaCl_2$) at 23°C for 6 hours to cleave the linker between GST and hNoggin mature. Western blotting was performed using primary antibodies (anti-GST monoclonal-1:2500, Santa Cruz Biotechnology, Dallas, TX, USA; anti-GFP monoclonal 1:2500, Santa Cruz Biotechnology; anti-Noggin monoclonal-1:2500, BD Pharmingen, San Diego, CA, USA; anti-β-actin monoclonal: 1:2500, Santa Cruz Biotechnology) and followed by incubation with a horseradish peroxidase-conjugated secondary anti-mouse antibody (1:5000, Santa Cruz Biotechnology). Immunoreactive proteins were detected using an enhanced ECL Western blotting substrate (BIOFECT, Daejeon, South Korea).

**Transfection of Knock-in Vector into Bovine Fibroblasts**

Primary bovine fibroblast cells ($4 \times 10^5$) were suspended in 500 µl R buffer of neon transfection system kit (Invitrogen, Carlsbad, CA, USA) and mixed with 15 µg hNoggin knock-in donor vector linearized by *Not*I restriction enzyme, 7.5 µg U6 promoter-sgRNA expression vector (pRGEN_Bovine CSN2_U6_SG plasmid, ToolGen Inc., Seoul, Korea), and 7.5 µg CMV promoter-Cas9 (pCMV-Cas9) nuclease expression vector (ToolGen, Inc.). The mixture was electroporated in a 100 µl gold-tip using a microporator (MP100; Digital Bio Tech., South Korea) under the condition of a single 30 ms pulse using 1350 V capacitive discharges. After electroporation, the cells were resuspended in 10 ml antibiotic-free culture medium, dispensed in a 0.1% gelatin coated 48-well plate (1500 cells per well, respectively), and then replaced with the complete culture medium containing antibiotics on next day. After additional 2 day culture, the transfected cells were treated with 400 µg/ml G418 (Gibco/Life Technologies, Gaithersburg, MD, USA) for 14 days. After selection, each single colony was picked using a colony cylinder and independently further sub-cultured for screening transgenic positive clones, on the basis of previously reported studies[8,21-22]. Prior to the transfection of hNoggin knock-in vector into bovine fibroblast cells, transfection condition was optimized by delivering pEGFP-C1 plasmid (Clontech, Mountain View, CA, USA) and EGFP expression was observed using fluorescence microscope (Nikon).

**PCR Screening Analysis of Knock-in Colonies**

The genomic DNAs of G418-resistant colonies were extracted using genomic DNA extraction kit (Bioneer, Daejeon, South Korea) and subjected to PCR screening analyses. To simultaneously amplify both outside and inside recombinant regions, the following primers were used: the 5' region - β-E2 UP 5' S1 (5'-GTCAGTCCAAGAAAACATGTGGATC AATGG-3') and hNoggin 3' AS (5'-CATG AAGCCTGGGTCGTAGTG-3'); the 3' region- Neo 3-4-S (5' CGATCAGGATGATCTGGACGAAGAG CATCA-3') and KI Sc AS (5' GCTGATAAATGAT CAGATGAGGATTAGTGC-3'), 1X PCR buffer, 1 U Top *Taq* DNA polymerase (Bioneer), and 250 µM of each dNTP. PCR amplification was run for 40 cycles of 30 seconds at 94°C for denaturation, 30 seconds at 66°C or 60°C for annealing, and 3 minutes at 72°C for extension. Then, to identify both targeted and non-targeted alleles in the knock-in cells, PCR was performed with 50 ng of genomic DNA using the primers β-E2 5' S1 (5'-GATCAAACCTGGGCTCC CCTGGAAGCA-3') located in Exon 2 and KI Sc AS (5'-GCTGATAAATGATCAGATGAGGATTAG TG C-3') located outside the 3' recombinant region spanning the targeting site, 1X PCR buffer, 1 U KOD FX Neo DNA polymerase (Toyobo Co., Osaka, Japan), and 200 µM of the dNTP mixture in a total volume of 50 µl. PCR amplification was performed using step-down cycles[8].

**Chromosome Analysis of hNoggin Knock-in Bovine Fibroblast Cell Clones**

The chromosome analysis was performed by Korea Research of Animal Chromosomes (Seoul, South Korea). Briefly, karyotypes were analyzed by inducing metaphase spreads of knock-in stable cells. After arresting the metaphase by treatment with colcemid (Gibco, 10 ug/ml), those cells were harvested and treated with a hypotonic solution at

37°C for 25 min. After centrifugation at 15,000 rpm for 10 min, supernatant was discarded and fixative solution (methan : acetic acid = 3:1) was added onto those cells. After fixative solution was changed with additional two more times, cell pellet was suspended in a small volume, dropped on cold wet slide and air-dried. The slide was treated with trpsin, stained with Giemsa to make G-banding pattern of chromosome, and then observed under light microscope (x 1,000).

## Results and Discussion

### Optimization of Transfection Efficiency

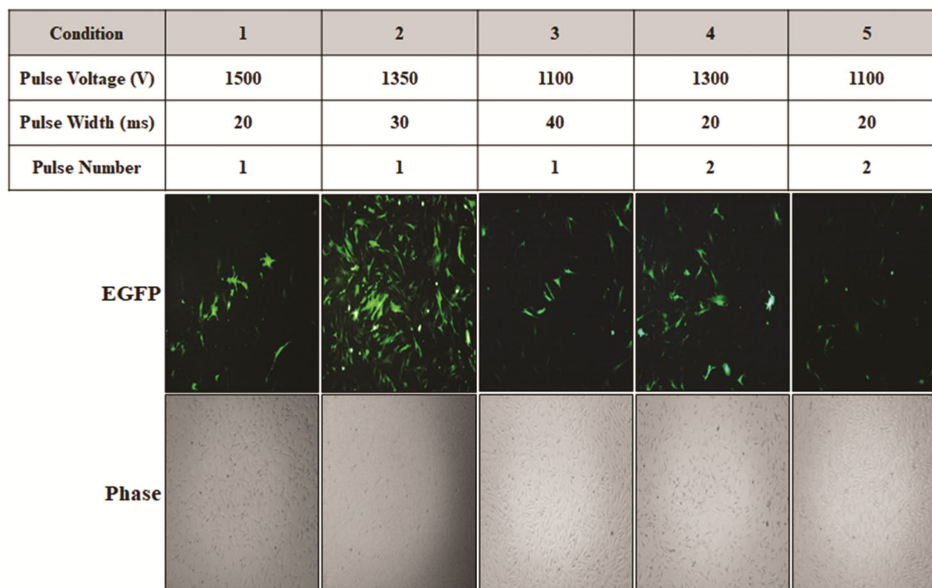
Prior to delivering the hNoggin knock-in vector into bovine fibroblast cells, transfection efficiency was optimized by testing various electroporation conditions. Primary cells were transfected with 1.5 μg pEGFP-C1 plasmid using a microporator. After 72 hours, EGFP expression was detected to be the highest with a single 30-ms pulse and 1350 V capacitive discharges. Therefore, these conditions were used for subsequent transfections (Fig. 1).

### Construction of a hNoggin Knock-in Vector in the Bovine β-casein Gene Locus

To develop stably-integrated hNoggin knock-in bovine fibroblasts, we used the CRISPR/Cas9 nuclease system. The sgRNA target sequence (CTGGAAGAACTCAATGTACC) was located in β-casein exon 3 region, recognized by the PAM sequence (TGG), and the sgRNA-Cas9 protein complex was expected to cause a DSB in the target site (Fig. 2A). A diagram of the hNoggin knock-in vector for expression of GST-tagged hNoggin in the bovine β-casein locus is shown in Fig. 2B. In detail, the knock-in vector consisted of the 5'-homologous arm, F2A peptide sequence, GST (factor Xa linker), hNoggin cDNA lacking its own SP, BGH polyA signal, PGK promoter-neomycin gene (PGK-neo, a positive selection marker), 3'-homologous arm, and diphtheria toxin A fragment gene (DT-A, a negative selection marker). The selection markers lacking poly A signal were only expressed if they were inserted at a gene locus ahead of the poly A signal. The mature F2A-fused GST (factor Xa linker)-hNoggin was introduced into the exon 3 locus of the bovine β-casein gene (Fig. 2C). The result of this construction allowed mature GST (factor Xa linker)-hNoggin to be expressed together with the bovine β-casein SP through the F2A sequence.

### Expression and Secretion of hNoggin Fusion Protein Produced by EF1α promoter-Driven hNoggin Knock-in System

To examine the secretion capability of bovine β-casein SP with mature GST-tagged functional hNoggin, mature bovine β-casein SP-F2A-GST-hNoggin sequences were ligated into an EF1α promoter-driven EGFP expression vector to produce a pEF1α-bovine β-casein-SP-F2A-GST-hNoggin-EGFP expression vector (Fig. 3A). HEK293T cells were transfected with a control pEF1α-EGFP or target



| Condition | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Pulse Voltage (V) | 1500 | 1350 | 1100 | 1300 | 1100 |
| Pulse Width (ms) | 20 | 30 | 40 | 20 | 20 |
| Pulse Number | 1 | 1 | 1 | 2 | 2 |



Fig. 1 — Optimization of transfection efficiency in bovine fibroblast cells: Primary cells were transfected with 1.5 μg pEGFP-C1 plasmid by electroporation under various pulse voltage (V), pulse width (ms), and pulse number conditions. After 72 hours, EGFP expression was observed under a fluorescence microscope.

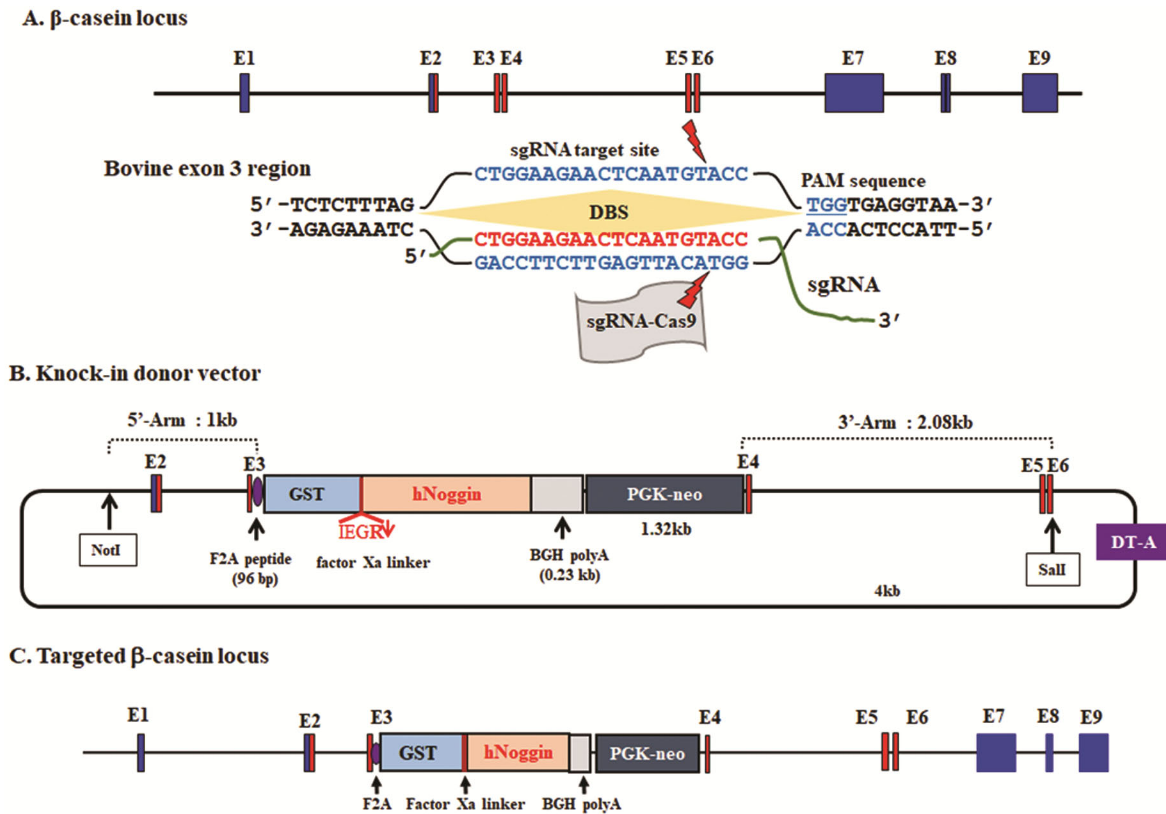



Fig. 2 — Construction of hNoggin knock-in vector in bovine β-casein gene gene locus: (A) Partial sequence of the β-casein exon 3 region. The 20-nucleotide sgRNA target site and PAM recognition sequence (TGG) are indicated. DSB indicates a double-stranded break introduced by the sgRNA-Cas9 protein complex. (B) A diagram of the knock-in vector for expression of GST-tagged hNoggin in the bovine β-casein locus. The 5'-homologous arm, F2A peptide sequence, GST (factor Xa linker, IEGR), hNoggin cDNA lacking its own SP, BGH polyA signal, PGK promoter-neomycin gene (PGK-neo), 3'-homologous arm, and diphtheria toxin A (DT-A) fragment gene are indicated. PGK-neo and DT-A were used as positive and negative selection markers, respectively. (C) F2A-fused GST (factor Xa linker)-hNoggin-BGH polyA-PGK-neo was introduced into the exon 3 locus of the bovine β-casein gene.

vector. EGFP expression was confirmed by fluorescence microscopy. As EGFP lacks a nuclear localization signal, its expression was expected to be limited to the cytoplasm. However, because of the relatively small molecular weight (26 kDa), control EGFP was diffusely localized to both the cytoplasm and nucleus. In contrast, hNoggin-fused EGFP with a larger molecular weight (~52 kDa) was observed clearly and predominantly in the cytoplasm (Fig. 3B). Next, we tested whether the EGFP-fused mature bovine β-casein-SP-F2A-GST-hNoggin expression vector was normally secreted into the culture media by SP and cleaved by factor Xa protease. Following transient transfection, whole cell lysates and concentrated culture media were pulled-down with glutathione sepharose 4B. The eluted supernatants were treated with factor Xa protease and subsequently subjected to Western blotting. EGFP-fused hNoggin expression (~52 kDa) in the media and the whole cell lysates was detected with α-Noggin and α-GFP antibodies (Fig. 3C).

GST tagging is a useful tool to purify recombinant proteins and can involve different protease cleavage sites. Factor Xa, a trypsin-like serine protease, recognizes the linker sequence IEGR and cleaves peptide bonds of basic amino acid residues[23]. In our knock-in system, the GST gene with a factor Xa linker was fused upstream of the hNoggin mature sequence lacking its own SP, and then F2A was inserted upstream of GST-hNoggin. Following transient transfection, recombinant hNoggin protein fused to EGFP was successfully secreted by bovine β-casein SP and separated from the GST gene by factor Xa protease cleavage, suggesting that the *in vitro* vector system was functional.

**PCR Screening for Stable hNoggin Knock-in Bovine Fibroblast Cell Clones and Chromosome Analysis**

Following the confirmation of transfection, expression, and secretion of the knock-in vector,

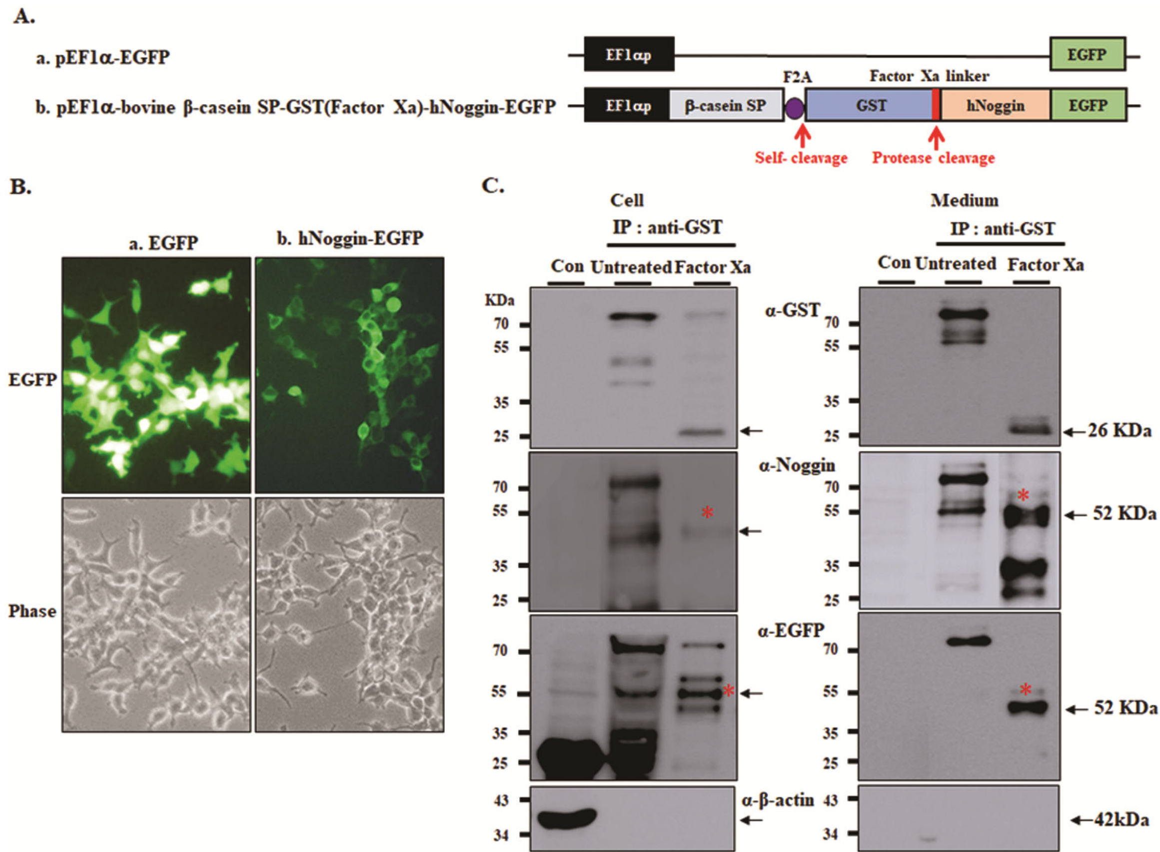



Fig. 3 — Expression and secretion of hNoggin protein produced by EF1α promoter-driven knock-in system. (A) Schematic diagram of pEF1 α-EGFP (a, control) and pEF1 α-bovine β-casein SP-GST (factor Xa linker)-hNoggin-EGFP (b) expression vectors. (B) HEK293T cells were transfected with these vectors, and EGFP expression was confirmed by fluorescence microscopy. (C) Whole cell lysates and concentrated culture media were pulled-down with glutathione sepharose 4B. The post-eluted supernatants were treated with factor Xa protease and then subjected to western blotting using α-GST (~26 kDa), α-Noggin (~26 kDa), α-GFP (~26 kDa), and α-β-actin (~42 kDa) antibodies. Control (Con) indicates the pEF1 α-EGFP-transfected HEK2T cell lysate and culture media. Asterisk (*) indicates the expression of the hNoggin-EGFP fusion protein (~52 kDa).

Table 1 — Frequencies of knock in the β-casein gene locus in bovine fibroblast using RGE

| Knock-in vector | Source of cells | CRISPR/Cas9 | No. of cells transfected | No. of G418-resistant colonies | No. of colonies analyzed by PCR | No. of positive colonies |
|---|---|---|---|---|---|---|
| hNoggin | Male | + | 5 x $10^6$ | 66 | 35 | 26/35 (74.2%) |

*Not*I-linearized hNoggin knock-in vector was co-transfected with sgRNA and Cas9 expression vectors in primary bovine fibroblast cells. After 14 days of G418 antibiotic selection, 66 resistant colonies were obtained and 35 were subjected to PCR analysis. Of the 35 PCR-tested colonies, 26 were positive for the knock-in gene, resulting in targeting with 74% frequency (Table 1). In Figure 4A, the location of primers used for PCR screening to identify positive clones is indicated in the bovine β-casein gene locus. PCR analysis was performed using β-E2 UP 5'-S1 and target-specific hNoggin 3'-AS primers to detect the 5'-recombination region (a, 2.6 kb). The 3'-recombination region (b, 3 kb) was detected using primers within the knock-in vector (Neo 3-4-S) and outside the recombination target site (KI Sc AS). Simultaneous detection of the targeted (c-*upper*, 6.7 kb) and non-targeted (c-*lower*, 4 kb) sites in the knock-in clones was performed using β-E2 5'-S1 and KI Sc AS primers (Fig. 4B). One representative clone (No. 25) was subjected to chromosome analysis. The cells were induced to metaphase spreads, exposed to G-band patterning, and exhibited the normal karyotype arrangement of 29 pairs of autosomes and one pair of XY sex chromosomes (Fig. 4C).

Production of recombinant proteins has been of interest in diverse fields, and various systems have been developed to optimize functional protein

expression and production. The bovine mammary gland provides an optimal system for the efficient production of recombinant proteins due to large quantities of milk production over the long period of lactation. Nevertheless, the generation of transgenic cattle is limited and problematic due to the low efficiency of transgenesis and long period of evaluation and production of recombinant protein[24-25]. To reduce those limitations, site-specific CRISPR/Cas9 endonuclease technology has been developed and successfully applied in livestock animals[2]. Previously, it was demonstrated that bovine knock-in fibroblasts could be generated by introducing a CRISPR/Cas9-mediated knock-in vector, containing homologous 5'- and 3'-arm fragments and F2A-fused human FGF2 fusion gene, into the bovine β-casein exon 3 locus[8]. Furthermore, knock-in blastocysts were produced, suggesting that CRISPR/Cas9-mediated homologous recombination can be efficiently accomplished in bovine fibroblasts[8]. Therefore, we employed this system with the additional insertion of GST protein containing a factor Xa linker upstream of the mature hNoggin sequence to facilitate the purification of the recombinant fusion protein. It has been reported that the CRISPR/Cas9 system has high DNA cleavage activity when two components of sgRNA and Cas9 are introduced into cultured cells, mice, rats, or domestic animals[3,26-29]. This suggests that it is possible to achieve CRISPR/Cas9-mediated homologous recombination for knock-in of an exogenous transgene into a specific gene locus to ensure stable expression of the transgene[8]. Moreover, in the previous study[8], CRISPR/Cas9-induced homologous recombination occurred in bovine primary fibroblasts with high efficiency (75.4-80%). Here, we also demonstrated high rates of knock-in efficiency in fibroblasts and confirmed that the exogenous gene targeting knock-in

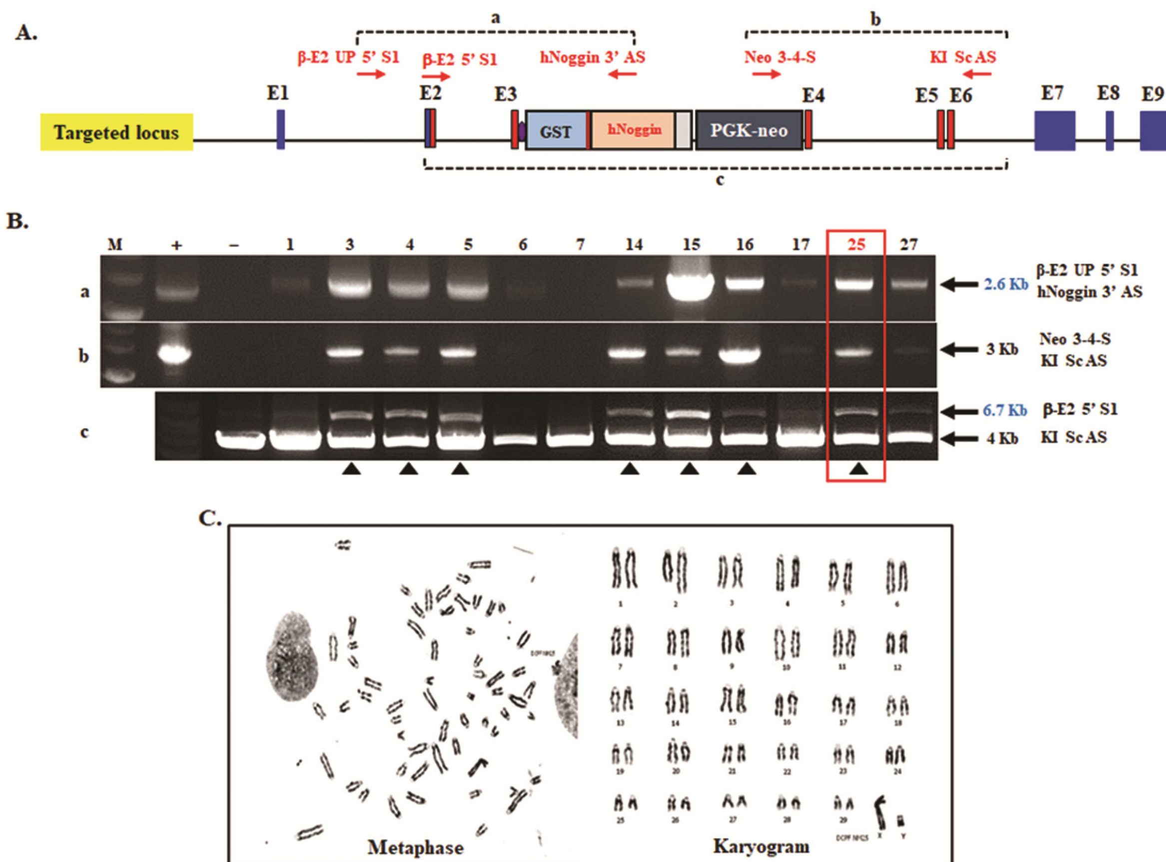



Fig. 4 — PCR screening for stable hNoggin knock-in bovine fibroblast cell clones and chromosome analysis. (A) Location of primers (a, b, c) used for PCR screening in the bovine β-casein gene locus. (B) PCR analysis to detect the 5'-recombination region (a), the 3'-recombination region (b), and the targeted site (c) in the knock-in clones. The red square box indicates the representative positive clone selected for further chromosomal analysis. (C) A representative clone was subjected to chromosome analysis. The cells were induced to metaphase spreads and exposed to G-band patterning.

system does not interrupt normal chromosome arrangement. Taken together, we have shown that the development of a transgene knock-in system successfully produced secretory hNoggin from bovine cells, and the resulting knock-in cells could serve as donor cells for the production of transgenic cattle expressing hNoggin within the bovine β-casein gene locus.

## Acknowledgment

This research was supported through the Next-Generation BioGreen21 Program (No. PJ01122202) funded by the Rural Development Administration, Republic of Korea.

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