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RESEARCH PAPER

Improvement of Tol2 Transposon System by Modification of Tol2 Transposase

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Abstract Tol2 transposon-based vector platform has been developed as a useful strategy to improve protein yield. Tol2 transposase identifies inverted terminal repeats of transposons and enhances transgene integration efficiency in the genome. However, Tol2 transposon system does not reach a level of efficiency for use in commercial production, limiting its usefulness for mass production of recombinant proteins. Here, we proposed a novel strategy to improve the existing Tol2 transposase by adding a nuclear localization signal of histone H2B next to the Tol2 transposase (*i.e.*, NLS-H2B-Tol2 transposase). Comparing the NLS-H2B-Tol2 transposase with the traditional Tol2 transposase, a significant increase in protein production was measured. Concurrently, a significant increase in nuclear transport of NLS-H2B-Tol2 transposase was observed, suggesting a causal relationship between increased nuclear transport and increased protein production. Furthermore, importin beta maximized NLS-H2B-Tol2-mediated protein productivity. Taken together, our findings offer useful ways for improving conventional Tol2 transposon-based vector platform. This new platform will be a breakthrough in the field of biopharmaceuticals to produce therapeutic proteins.

Keywords: Tol2 transposase, transposon, importin beta

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1. Introduction

Transposon technology has been widely used to enhance transgene integration [1]. Transposon system is driven using a cut-and-paste mechanism after the transposase identifies the inverted terminal repeats (ITRs) of transposon [2]. Tol2, Sleeping Beauty (SB) and PiggyBac (PB) are three types of transposons with diverse evolutionary origins and protein production profiles [3]. Transgenes encoding therapeutic antibodies are typically larger than 6 kb in size, so transposon systems capable of delivering large transgenes are desirable [4]. SB transposon exhibits a reduced translocation efficiency of up to 50% when harboring a 6 kb transgene [3]. Additionally, PB transposon can deliver up to 14 kb but are less efficient [5]. However, a 10 kb transgene can be carried by Tol2 transposon without significantly reducing translocation activity [3].

Tol2 transposon is a member of the Tc1/mariner transposon superfamily, which includes the Tol2 transposase and two ITRs. Tol2 transposase induces transgene translocation by binding to the ITR at each end of the transposon [6]. An increase in translocation activity was observed concurrently with an increase in Tol2 transposase concentration within the test range [4]. Given these results, strategies to increase the concentration of Tol2 transposase within certain limits are worth exploring to build more effective Tol2 transposon systems.

Nuclear localization signals, mostly viral origin, have

been characterized and used to increase the efficiency of transporting target proteins to the nucleus [7]. However, the use of viral origin signals in biopharmaceutical production can lead to unexpected results. Viral contamination, including the use of sequences of viral origin, can affect parts of the biopharmaceutical production process [8]. In particular, the biopharmaceutical production process should additionally include processes such as virus testing and virus removal, which increases the production cost and time of the biopharmaceutical.

Histones are a type of basic protein that binds to DNA in the nucleus and aids in its condensation into chromatin [9]. Histones comprise of five major families: H2A, H2B, H3, H4, and H1. Histone H2B is one of the 5 main histone proteins and functions as a core histone protein. Histone H2B consists of an N-terminal domain, a major globular domain and a C-terminal tail. The N-terminal domain shares the characteristics of classical nuclear localization signaling (NLS) and aids in transporting proteins to the nucleus [10,11]. Therefore, the N-terminal domain of histone H2B has been used to attach in front of the protein of interest to enhance the efficiency of transport to the nucleus.

The nuclear membrane, which consists of a double membrane, separates the nucleus and the cytoplasm. Through the nuclear pore complex (NPC) that penetrates the nuclear membrane, proteins are delivered nucleocytoplasmically [12]. Small proteins with molecular weights less than 30-40 kDa can pass through NPCs by diffusion, but most macromolecules, including Tol2 transposase, are transported through NPCs through selective mechanisms mediated by certain transport factors. Importin is a protein transporter that moves protein molecules from a cell's cytoplasm to its nucleus [13]. Importin is made up of two subunits: alpha and beta. Importin beta, in particular, can bind and carry cargo alone or in heterodimers with importin alpha. Importin beta mediates interactions with the pore complex, allowing the transport of protein to nucleus.

In this study, we investigated whether increasing nuclear translocation by modifying conventional Tol2 transposon-based vector platform is a useful strategy to increase protein productivity. Here, we report that modification of traditional Tol2 transferase leads to high-yield protein synthesis.

2. Materials and Methods

2.1. Cell culture

This work made use of Chinese hamster ovary (CHO) DG44 cells (A1100001; Thermo Fisher Scientific, Waltham, MA, USA). Cells were cultured according to previously described methods [1]. The Cedex HiRes Analyzer (05650216001; Roche, Basel, Switzerland) was used to measure cell density and vitality.

2.2. Plasmid design and construction

The recombinant plasmids were created using conventional cloning techniques. Transposon vector (TP) and non-transposon vector (non-TP) were designed as previously described [1]. By introducing a cDNA encoding Tol2 transposase into the pcDNA6 vector (V22020; Invitrogen, Carlsbad, CA, USA), a Tol2 transposase vector was created. Nuclear localization signal of histone H2B next to the Tol2 transposase (NLS-H2B-Tol2 transposase) vector was constructed by inserting the N-terminal region of histone H2B in front of the Tol2 transposase. By introducing a cDNA encoding importin alpha or importin beta into a pcDNA3.1 vector (V79020; Invitrogen), vectors expressing importin alpha or importin beta were created.

2.3. Transfections

CHO DG44 cells were transfected using the SG Cell Line 4D-Nucleofector™ X Kit L (V4XC-3024; Lonza, Basel, Switzerland) according to the manufacturer's instructions.

2.4. Measurement of luciferase activity

Using a luciferase assay kit (E1500; Promega, Madison, WI, USA), the activity of luciferase was determined. All procedures were performed according to the methods described in our previous study [14].

2.5. Immunofluorescence

A culture slide well (30114; SPL, Pocheon, Korea) was used for cell plating. Then, cells were treated with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature. Cells were permeabilized in PBS containing 0.1% Triton X-100 for 15 min. Cells were blocked with 2.5% fetal bovine serum (FBS) in PBS for 1 h at room temperature. Anti-myc-tag antibody (2276s, 1:200 dilution; Cell Signaling Technology, Danvers, MA, USA) was diluted in PBS containing 2.5% FBS. Overnight at 4°C, cells were incubated with diluted primary antibody. Following that, cells were washed three times with ice-cold PBS and treated for 1 h with Alexa Fluor 594-conjugated anti-mouse antibody (A11005; 1:200 dilution; Invitrogen). Hoechst 33342 (H3570; Invitrogen) was used to stain the nuclei, and samples were mounted using Dako Fluorescence Mounting Medium (S3023; Agilent, Santa Clara, CA, USA). Images were taken using a Carl Zeiss Axio Imager Z1 microscope (Carl Zeiss, Jena, Germany).

2.6. Statistical analyses

The statistical program (SigmaPlot 12.5; Systat Software,

San Jose, CA, USA) was used for statistical analysis. The significance of the differences was assessed using Student's *t*-test (two-tailed).

3. Results

3.1. Effect of NLS-H2B-Tol2 transposase on protein production

Tol2 transposon system consists of two parts: a transposon vector and a helper vector [1]. The transposon vector includes transgene surrounded by two ITRs. The helper vector includes Tol2 transposase, which binds to the ITR and promotes transgene integration [15]. A non-TP was generated to carry a cytomegalovirus (CMV) promoter-driven luciferase gene (Fig. 1A), whereas a TP harbored a CMV promoter-driven luciferase surrounded by two ITRs (Fig. 1B). Moreover, a CMV promoter-driven Tol2 transposase was included in the helper vector (Tol2 transposase, Fig. 1C). Since Tol2 transposase must be translocated to the nucleus to carry out translocation, we hypothesized that facilitating nuclear transport of Tol2 transposase would increase translocation and consequently increase protein productivity. Therefore, we integrated the N-terminal domain of histone H2B containing the NLS in front of the Tol2 transposase (NLS-H2B-Tol2 transposase, Fig. 1D).

The luciferase activity was measured after transient transfection of cells with the designated vectors. Compared to non-TP, TP with Tol2 transposase significantly enhanced luciferase activity, confirming the previous observation that TP with Tol2 transposase is an effective vector platform to increase protein productivity (Fig. 1E; green asterisks) [1]. We then tested whether TP with NLS-H2B-Tol2 transposase improved Tol2 transposon system-mediated protein production. In comparison to the non-TP and "TP with Tol2 transposase" groups, TP with varied concentrations of NLS-H2B-Tol2 transposase significantly boosted luciferase activity (Fig. 1E; red asterisks and blue asterisks, respectively). These results indicate that NLS-H2B-Tol2 transposase can function as an effective helper vector for increasing protein productivity. However, NLS-H2B-Tol2 transposase did not show a dose-dependent increase in luciferase activity (Fig. 1E). The discrepancy between the amount of luciferase activity and the amount of the helper vector encoding the NLS-H2B-Tol2 transposase can be explained by the observation that the ideal ratio of transposase to transposon was found only within a small concentration range [16]. Since 2 μg TP with 0.2 μg NLS-H2B-Tol2 transposase had the maximum luciferase activity, this ratio (TP:NLS-H2B Tol2 transposase = 10:1) was selected as the most suitable for future studies (Fig. 1E).

Creating a single clone from a pool is essential to meet

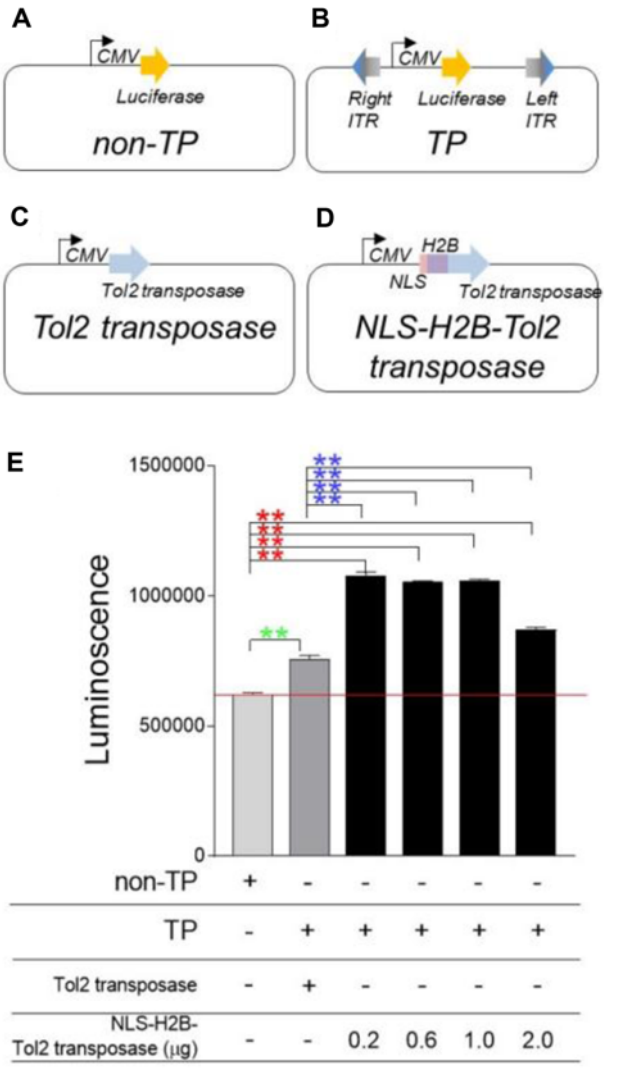

Fig. 1. Effect of NLS-H2B-Tol2 transposase on protein production. (A) A non-transposon vector (non-TP) was generated to carry a CMV promoter-driven luciferase gene. (B) A transposon vector (TP) harbored a CMV promoter-driven luciferase surrounded by two ITRs. (C) A CMV promoter-driven Tol2 transposase was included in the helper vector (Tol2 transposase). (D) N-terminal region of histone H2B was incorporated in front of the Tol2 transposase (NLS-H2B-Tol2 transposase). (E) Protein productivity among cells transfected with non-TP, "TP with Tol2 transposase", or "TP with NLS-H2B-Tol2 transposase". **$p < 0.01$, Student's *t*-test. Means ± S.D., N = 3. NLS-H2B-Tol2 transposase: nuclear localization signal of histone H2B next to the Tol2 transposase, CMV: cytomegalovirus, ITR: inverted terminal repeat.

regulatory requirements, as single clones ensure monoclonality and reliable protein synthesis [17]. Then, for each group, single cell clones were isolated and produced (Fig. 2). Each group exhibited a heterogeneous expression pattern consistent with previous findings that cell pools consist of populations with variable and unpredictable expression due to random genomic integration of transgenes and uncontrolled interference of integrated chromosomal regions [10,11]

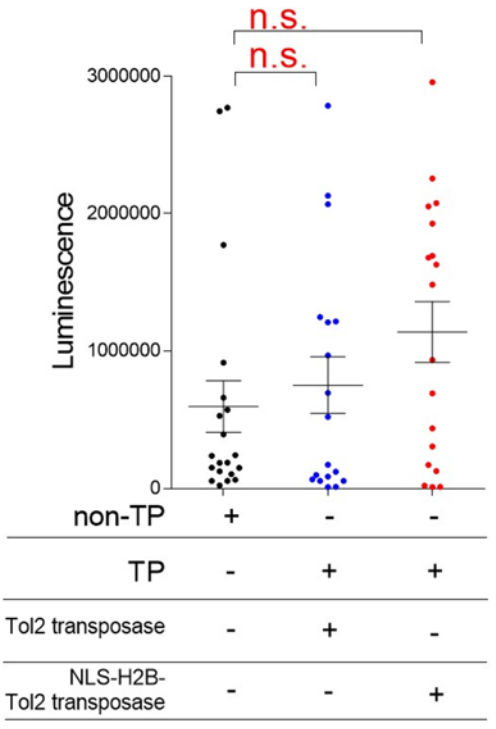

Fig. 2. Maintaining improved productivity of NLS-H2B-Tol2 transposase in single cell clones. The luciferase activity of single cell clones is represented by each dot. The horizontal bar shows the average of luciferase activity under each condition. Student's *t*-test. Means ± S.D., N = 20, N = 18, N = 18, respectively. NLS-H2B-Tol2 transposase: nuclear localization signal of histone H2B next to the Tol2 transposase, TP: transposon vector, non-TP: non-transposon vector, n.s.: not significant.

(Fig. 2). Therefore, single cell clones built under "TP with NLS-H2B-Tol2 transposase" did not show a significant increase in luminescence compared to non-TP and "TP with Tol2 transposase" groups (Fig. 2). However, when considering the average of each luciferase activity for comparison, single cell clones constructed from "TP with NLS-H2B-Tol2 transposase" showed significant increases on average compared to the non-TP and "TP with Tol2 transposase" groups (Fig. 2). These findings indicate that the improved productivity of NLS-H2B-Tol2 transposase persisted even in single cell clones, validating the use of NLS-H2B-Tol2 transposase in cell line development.

3.2. Identification of the mechanism by which NLS-H2B-Tol2 transposase enhances protein production

We then investigated the mechanism by which NLS-H2B-Tol2 transposase promotes protein production. As a proof-of-concept, the nuclear localization of transposase was investigated to determine whether increased protein production was due to enhanced nuclear localization. When compared to control, the nuclear localization of the Tol2 transposase was significantly increased, confirming the previous finding that Tol2 transposase was transported to the nucleus, where it binds to the ITRs of the transposon vector and causes transposition (Fig. 3) [1]. Furthermore, as compared to that of Tol2 transposase, the nuclear localization of NLS-H2B-Tol2 transposase was significantly increased (Fig. 3). These results indicate that integration of

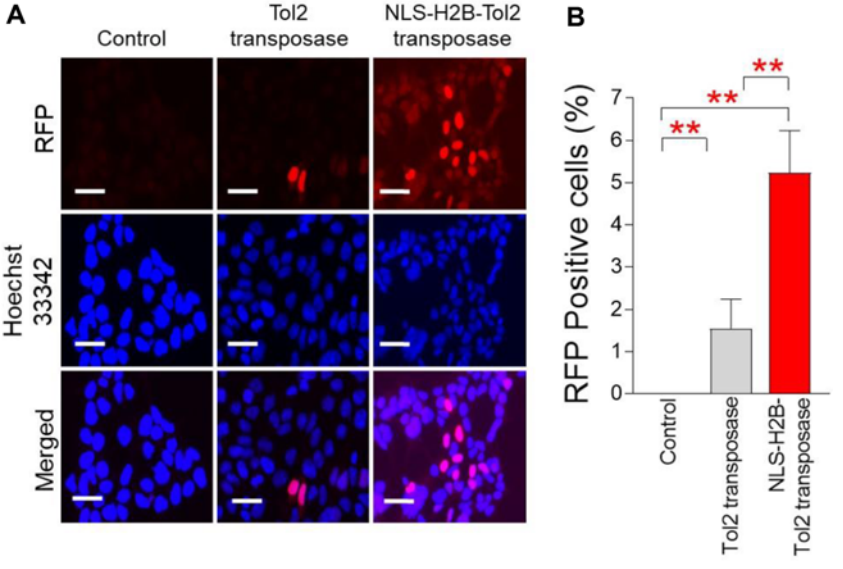

Fig. 3. Mechanism through which NLS-H2B-Tol2 transposase enhances protein synthesis. (A) Immunostaining for Tol2 transposase (red) and NLS-H2B-Tol2 transposase (red) (Scale bar = 10 μm). For nuclear counterstaining, Hoechst 33342 was used (blue). Helper vectors containing Tol2 transposase or NLS-H2B-Tol2 transposase were used. As a negative control, a helper vector without Tol2 transposase was used. (B) Comparison of the nuclear transport efficiency. The nuclear localization of NLS-H2B-Tol2 transposase was significantly higher than that of control and Tol2 transposase. **p < 0.01, Student's *t*-test. Means ± S.D., N = 3. NLS-H2B-Tol2 transposase: nuclear localization signal of histone H2B next to the Tol2 transposase, RFP: red fluorescent protein.

NLS-H2B in front of Tol2 transposase increased nuclear transport of Tol2 transposase, resulting in increased protein production.

3.3. Importin beta maximizes the effect of NLS-H2B-Tol2 transposase on protein production

Tol2 transposase has a size of 73 kDa and belongs to macromolecules. It is more likely to pass through NPCs through mechanisms mediated by importin alpha or importin beta rather than passing through NPCs by passive diffusion [18]. Thus, we hypothesized that the overexpression of importin alpha or importin beta would maximize the effect of NLS-H2B-Tol2 transposase on the protein production. To overexpress importin alpha or beta, a transgene encoding importin alpha or beta was cloned into a CMV promoter driven expression vector (Fig. 4A). We then overexpressed the corresponding expression vector in the NLS-H2B-Tol2 transposase-mediated protein productivity enhancement group. As a proof of concept, the expression of importin alpha or beta was examined. To amplify the introduced

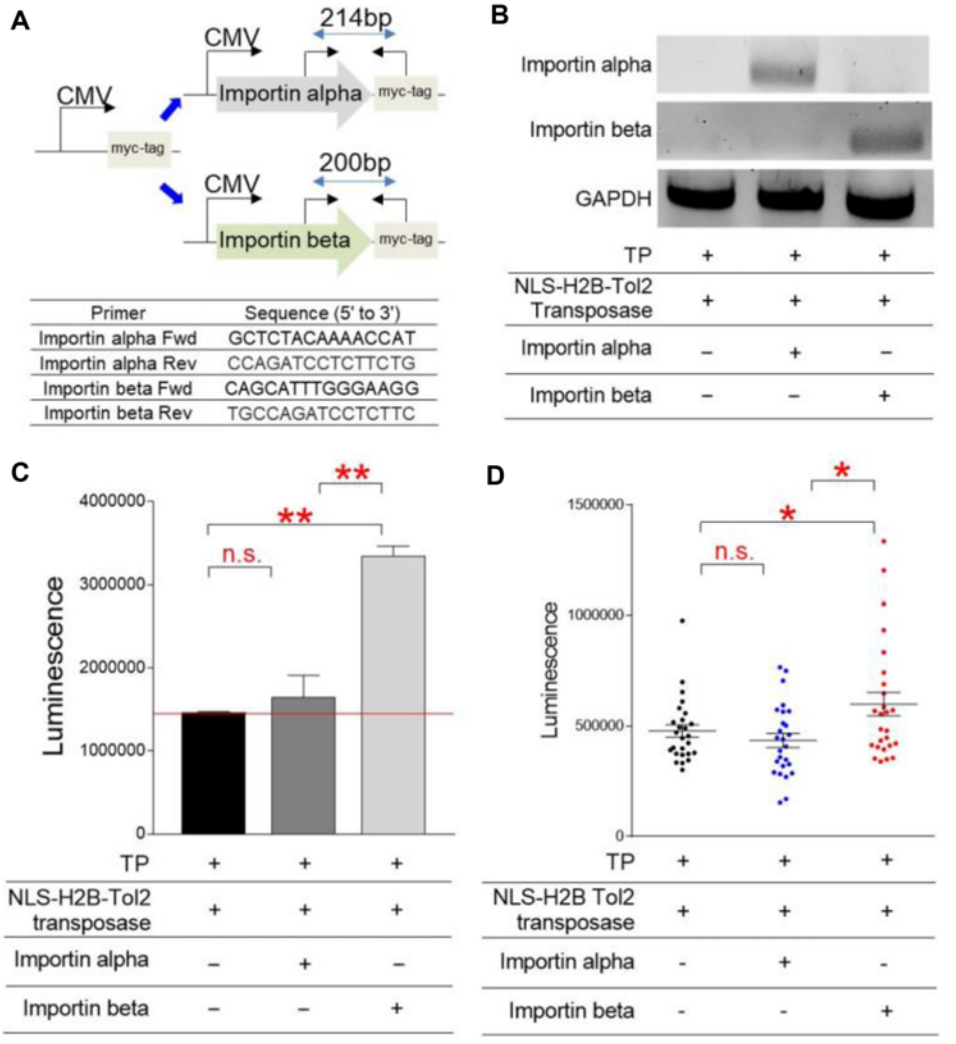

Fig. 4. Importin beta maximizes the effect of NLS-H2B-Tol2 transposase on protein production. (A) The importin alpha and importin beta were cloned to the CMV promoter-driven expression vector. (B) The PCR amplification using the designated set of primers only generated a 214 bp amplicon in importin alpha-overexpression group and a 200 bp amplicon in importin beta-overexpression group. The PCR amplification using the designated set of primers confirmed the overexpression of importin alpha in only importin alpha-overexpression group and the overexpression of importin beta in only importin beta-overexpression group. (C) The overexpression of importin alpha subtly increased the luciferase activity in the NLS-H2B-Tol2 transposase-mediated protein productivity enhancement group, whereas the overexpression of importin beta significantly increased it. **$p < 0.01$, Student's *t*-test. Means ± S.D., N = 3. (D) Protein production in stable cell clones built after overexpression of importin alpha or importin beta in the NLS-H2B-Tol2 transposase-mediated productivity enhancement group. The luciferase activity of single cell clones is represented by each dot. The horizontal bar shows the average of luciferase activity under each condition. *$p < 0.05$, Student's *t*-test. Means ± S.D., N = 26, N = 26, N = 26, respectively. NLS-H2B-Tol2 transposase: nuclear localization signal of histone H2B next to the Tol2 transposase, CMV: cytomegalovirus, PCR: polymerase chain reaction, TP: transposon vector, n.s.: not significant.

importin alpha or beta, polymerase chain reaction (PCR) was performed with primers designed to bind to the introduced gene and myc-tagged region (Fig. 4A). In the importin alpha-overexpression group, PCR amplification yielded only a 214 bp amplicon, whereas there was no amplification in the control and importin beta-overexpression groups (Fig. 4B). In the importin beta-overexpression group, PCR amplification yielded only a 200 bp amplicon, whereas there was no amplification in the control and importin beta-overexpression groups (Fig. 4B).

We then investigated whether importin alpha or beta maximizes the effect of NLS-H2B-Tol2 transposase on protein production. The overexpression of importin alpha subtly increased the luciferase activity in the NLS-H2B-Tol2 transposase-mediated protein productivity enhancement group, whereas the overexpression of importin beta significantly increased it (Fig. 4C).

In order to check whether the improved productivity persisted even in single cell clones, single cell clones for each group were produced. Single cell clones built after overexpression of importin alpha in the NLS-H2B-Tol2 transposase-mediated productivity enhancement group did not increase the luciferase activity, whereas single cell clones built after overexpression of importin beta significantly increased it (Fig. 4D). These results suggest that importin beta efficiently maximized protein production in the NLS-H2B-Tol2 transposase-mediated productivity enhancement group.

4. Discussion

The development of novel vector platforms is critical to becoming a breakthrough in the field of biopharmaceuticals, which is struggling with low protein productivity [19]. The Tol2-mediated transposon system has been approached for efficient protein production and can consequently increase protein yield [20]. Although this improvement is receiving a lot of attention, a consensus has begun to emerge that a strategy to increase Tol2 transposon-based protein production is needed to secure competitiveness in protein production. Recently, significant effort has been put into modifying ITRs to improve the Tol2 transposon system. Because of the importance of ITR size in transgene integration [21,22], a transposon vector containing minimum Tol2 ITRs (mini-TP) was developed [1]. Mini-TP boosted transgene integration compared to traditional Tol2 transposons, resulting in higher protein synthesis [1]. The success of improving protein productivity by size modification of the Tol2 ITR suggested the attempts to modify the Tol2 transposase, another component of the Tol2 transposon system, might also be worth testing. In line with this attempt, modification of transposase was conducted in SB transposon system to improve transposition. Generation of SB100X by a systemic mutagenesis approach in SB transposase resulted in effective germline translocation in several animal models [23,24]. In the present study, to improve the traditional Tol2 transposon-based vector platform, we added the NLS-H2B in front of Tol2 transposase that would be efficiently move to nucleus. NLS-H2B helped boost nucleus localization of Tol2 transposase, which in turn increased protein production. Furthermore, the effect of improved protein productivity was maintained up to single clones. To our knowledge, our study is the first to show that TP with NLS-H2B Tol2 transferase can be used as a novel strategy to enable better and more stable protein production.

Facilitation of protein transport to the nucleus by importin is one of the essential mechanisms of proteins that cannot pass through NPCs by diffusion because of their large protein size. Importin binds to the NLS of large-sized proteins, facilitating the transport of proteins through NPCs into the nucleocytoplasm [25]. Specifically, importin alpha binds to lysine-rich NLS and then induces a bridge between cargo and importin beta, forming an "NPC-targeted trimer complex" [26]. Once inside the nucleus, the small GTPase Ran binds directly with importin beta and initiates dissociation of the complex, allowing importin alpha and cargo protein to be released [27]. Alternatively to classical nuclear import, importin beta associates with cargo proteins directly and facilitate nuclear import. Furthermore, in addition to the nuclear import of cargo proteins, importin beta provides a potential new arena for acting as a chaperone for highly charged nuclear protein, emphasizing its role in the facilitation of protein transport. Although the basic mechanism of importins is well defined, the precise role of importin beta in the enhancement of nuclear protein transport to the nucleus remains unknown. Here, we uncovered a novel mechanism in which importin beta regulates protein productivity by enhancing the nucleus transport of NLS-H2B-transposase. Indeed, overexpression of importin beta significantly increased protein production in the NLS-H2B-Tol2 transposase-mediated protein productivity enhancement group. However, overexpression of importin alpha itself did not enhance the protein productivity. This phenomenon is partly explained by the finding that importin alpha functions to elevate transcription of *Serine/Threonine Kinase 35* and promotes non-apoptotic cell death [28]. Furthermore, this phenomenon can also be explained by the result that importin beta alone promotes nuclear import through direct binding to cargo protein in addition to the cooperative function of importin alpha and beta. However, we acknowledge that additional study is needed to support these ideas.

5. Conclusion

Our finding revealed that insertion of NLS-H2B next to the Tol2 transposase could be exploited to boost protein productivity. Furthermore, the use of NLS-H2B-Tol2 transposase in combination with importin beta would be effective for protein production. As a result of our findings, the novel technology that improves the existing Tol2 transposon system proposed here might be applied to the production of biopharmaceuticals.

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Author's Contributions

JYP, YB, HWK, and JTP conceived and designed the experiments. JYP, HL, ESS, YHL, MUK, and GK performed the experiments. JYP analyzed the data. JYP, YB, HWK, and JTP wrote and edited the paper.

Ethical Statements

The authors declare no conflict of interest.
Neither ethical approval nor informed consent was required for this study.

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