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Keywords: IL-21, codon optimization, signal peptide, NK cells, CHO cells

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1 **Efficient interleukin-21 production by optimization of codon and signal peptide in Chinese**
2 **hamster ovarian cells**

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23 Running title: Improved IL-21 production in CHO-K1 cells

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Abstract

Interleukin-21 is a common γ-chain cytokine that controls the immune responses of B cells, T cells, and natural killer cells. Targeting IL-21 to strengthen the immune system is promising for the development of vaccines as well as anti-infection and anti-tumor therapies. However, the practical application of IL-21 is limited by the high production cost. In this study, we improved IL-21 production by codon optimization and selection of appropriate signal peptide in CHO-K1 cells. Codon-optimized or non-optimized human IL-21 was stably transfected into CHO-K1 cells. IL-21 expression was 10-fold higher for codon-optimized than non-optimized IL-21. We fused five different signal peptides to codon-optimized mature IL-21 and evaluated their effect on IL-21 production. The best result (a 3-fold increase) was obtained using a signal peptide derived from human azurocidin. Furthermore, codon-optimized IL-21 containing the azurocidin signal peptide promoted IFN-γ secretion and STAT3 phosphorylation in NK-92 cells similar to codon-optimized IL-21 containing original signal peptide. Collectively, these results indicate that codon optimization and azurocidin signal peptides provide an efficient approach for the high-level production of IL-21 as a biopharmaceutical.

Key words: IL-21, codon optimization, signal peptide, NK cells, CHO cells

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Introduction

Interleukin-21 (IL-21) is a pleiotropic cytokine which regulates various immune responses [1]. IL-21 is predominantly produced in $CD4^+$ T cells and natural killer T (NKT) cells. However, its receptor (IL-21R) is expressed in multiple cellular components of the innate and adaptive immune systems [2]. Therefore, it has potent immune regulatory properties in the context of infection, autoimmune diseases, and cancer [3-5]. IL-21 enhances the proliferation of $CD4^+$ T cells and regulates the development of IL-17 producing T-helper T cells, which are associated with autoimmune diseases and inflammatory conditions [6, 7]. IL-21 also enhances the proliferation and cytotoxicity of natural killer (NK) cells and promotes their interferon-γ secretion [8, 9]. Although recombinant IL-21 (rhIL-21) therapy has been ongoing in preclinical studies and in Phase I and II clinical trials to treat patients with metastatic melanoma and renal cell cancer [10-12], the therapeutic applications of rhIL-21 are limited by the relatively high production cost.

There are several expression systems producing recombinant protein, such as bacteria, fungi, and mammalian cells. Mammalian cells are proper hosts for the production of recombinant proteins, because they are beneficial for post-translational modifications (PTMs). Most marketed recombinant protein pharmaceuticals were produced by Chinese hamster ovary (CHO) cells [13, 14]. Gene optimization and selection of appropriate signal peptide can enhance the production of recombinant proteins in mammalian cells. A number of parameters, including codon usage, mRNA stability and the GC content, and RNA instability motifs and splicing sites, are considered for gene optimization to improve transcription, translation, and folding of recombinant protein [15, 16]. The selection of proper signal peptides is also critical for establishing a manufacturing process for protein production to improve the correct processing and secretion of recombinant

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65 proteins through the transport of the translated proteins into the endoplasmic reticulum [17, 18].
66 Recent studies have demonstrated the effectiveness of different signal peptides on the production
67 of recombinant protein in CHO cells [19-22].

68 The aim of the current study was to improve the production of IL-21 in the CHO-K1 cell
69 line. IL-21 codon optimization and the selection of a proper signal peptide improved productivity
70 and maintained the biological properties of IL-21.

71

72 Materials and Methods

73 Gene optimization, synthesis, and vector construction

74 Codon optimization was performed using the Gene Optimization algorithm of Bioneer
75 (Daejeon, Korea). Human IL-21 cDNA (BC066260) and all codon-optimized DNAs were
76 synthesized and cloned into the pGH cloning vector (Bioneer). All genes encoding IL-21 were
77 cloned into pcDNA3.1/zeo(+)-GS (24) at the *Afl*II/*BamH*I site.

78

79 Cell culture and transfections

80 CHO-K1 cells were cultured in DMEM (-)Gln (11960-069; Thermo, MA, USA) containing
81 10% dialyzed fetal bovine serum (dFBS, 26400-044; Thermo) and GS expression medium
82 supplement (GSEM, G9785; Sigma-Aldrich, St. Louis, MO, USA). Cells were transfected with a
83 vector containing mock, non-optimized, and optimized human IL-21 genes using X-tremeGENE
84 9 DNA Transfection Reagent (06 365 787 001; Roche, Basel, Switzerland) as described
85 previously [23-25]. After transfection, 2×10^3 cells were seeded in 96-well plates and selected
86 with DMEM (-)Gln containing 10% dFBS, GSEM, 300 μg/mL zeocin (R250-01; Thermo), and
87 25 μM GS System L-Methionine Sulfoximine (Sigma-Aldrich). IL-21 concentrations in the

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88 supernatants were measured when the cell pools reached confluence. To construct single cell
89 clones, cell pools high-expressing non-optimized IL-21 or codon-optimized IL-21 were gradually
90 expanded and subjected to limited dilution. IL-21 production in the respective clonal lines was
91 measured by ELISA. The top three high-producing cell clones were used for further analyses.
92

93 **Signal peptide optimization**

94 Five different signal peptides were identified by literature search and fused with the codon-
95 optimized mature IL-21 [16-19].The origin and amino acid sequence of signal peptides are shown
96 in Table 1.
97

98 **Quantification of IL-21 and IFN-γ by ELISA**

99 IL-21 and IFN-γ cytokines were evaluated in the culture supernatant using specific ELISA kits
100 from Invitrogen [26-28]. NK92 cells were seeded in 5×10^5/mL with IL-2-free media for 24 h.
101 Following starvation, NK92 cells were stimulated with the commercial IL-21 expressed in
102 bacteria (rhIL-21), original signal peptide fused-IL-21 (WT/IL-21), or Azurocidin signal peptide-
103 fused IL-21 (Az/IL-21) for 24 h. IFN-γ concentration was measured using specific ELISA kits
104 (Invitrogen) according to the manufacturer's instructions.
105

106 **Western blotting**

107 Western blot analysis was performed as described previously, with minor modifications [29,
108 30]. In brief, cells were lysed in ice-cold lysis buffer (20 mmol/L Tris (pH 8.0), 137 mM NaCl,
109 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture
110 (Roche), 1 mM sodium vanadate). Cell lysates were separated by SDS-PAGE and transferred to a
111 polyvinylidene difluoride membrane (Amersham Biosciences, Amersham, UK). Subsequently,

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112 membranes were incubated in TBST supplemented with 5% non-fat dry milk and probed with
113 STAT3 (Cell Signalling Technology, Danvers, MA, USA) and phosphor-STAT3 (Cell Signalling
114 Technology) antibodies at 4°C overnight. The bound antibodies were visualized with a suitable
115 secondary antibody conjugated to horseradish peroxidase using enhanced chemiluminescence
116 reagent (AB Frontier, Seoul, Korea) [31-32].

117

118 **Statistical analysis**

119 Data were obtained from at least three independent experiments. All quantitative data are
120 presented as means ± standard deviations and were analyzed using Student's *t*-tests. $P < 0.05$ was
121 considered statistically significant.

122

123 ## Results

124 **Enhanced production of IL-21 by gene optimization**

125 To investigate the effect of gene optimization on IL-21 expression in CHO-K1 cells, synthetic
126 IL-21 genes were assembled based on codon usage in Chinese hamster (*Cricetulus griseus*) as
127 described in Materials and Methods. Amino acid sequences for non-optimized IL-21 (WT IL-21)
128 and optimized IL-21 (opti IL-21) were identical. The sequence of WT IL-21 and opti IL-21 had
129 the identity of 82.4%. The codon optimization index (COI) increased from 0.71 to 0.84. The GC
130 content was also adjusted from 40% to 51%. To investigate the enhancement in productivity by
131 gene optimization, CHO-K1 cells were transfected with a vector containing WT or opti IL-21.
132 Transfected cells were seeded in 96-well plates and selected. IL-21 concentrations in the culture
133 supernatants were measured when the cell pools reached more than 90% confluence. Two cell
134 pools with high production of WT or opti IL-21 were gradually expanded. The concentration of

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135 IL-21 in the supernatant was analyzed by ELISA when cells reached at least 90% confluence.
136 Higher IL-21 concentrations were observed in two different cell pools transfected with optimized
137 IL-21 (38.8 and 35.7 ng/mL) than in two different cell pools transfected with non-optimized IL-
138 21 (13.7 and 13.5 ng/mL) (Fig. 1A). Next, cell pools were subjected to limiting dilution to
139 establish single cell clones. The top three high-producing clones were selected. IL-21 secretion in
140 respective clonal cell lines was analyzed by ELISA. The average yield of opti IL-21 (1495 ± 192
141 ng/mL) was 10-fold higher than that of WT IL-21 (159 ± 24 ng/mL) (Fig. 1B). These results
142 indicate that gene optimization enhances the production of IL-21 in CHO-K1 cells.
143

144 **Enhanced secretion of IL-21 by the azurocidin signal peptide**

145 Signal peptides play important roles in the secretion of proteins expressed in mammalian cells.
146 To enhance the secretion of IL-21 in CHO-K1 cells, five promising signal peptides were selected
147 by literature search [19-22]. Their origin and amino acid sequences are listed in Table 1. Five
148 different signal peptides were fused to codon-optimized mature IL-21 (Fig. 2A). To evaluate the
149 effects of signal peptides on IL-21 production, CHO-K1 cells were transfected with a vector
150 expressing IL-21 fused to WT or to five different signals. Cell pools were selected as described in
151 the Materials and Methods. IL-21 concentrations in the culture supernatants were analyzed by
152 ELISA when the cells reached confluence. Although IL-21 production was greater using the rat
153 serum albumin signal peptide (RSAP/mIL21) and tissue plasminogen activator signal peptide
154 (tPASP/mIL21) than using the original signal peptide, the greatest productivity was observed for
155 a cell pool expressing azurocidin signal peptide conjugated-IL-21 (AzSP/mIL21) (Fig. 2B).
156 Although the azurocidin signal peptide improved IL-21 productivity in cell pools, the
157 performance in stable cell lines can differ. Therefore, the AzSP/mIL21 cell pool was subjected to
158 limiting dilution to generate stable single cell clones. IL-21 concentrations in the supernatants of

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159 twenty clones were measured by ELISA (data not shown). The top two clones with respect to IL-
160 21 production (opti Az/IL21-5 and opti Az/IL21-12) were selected and subjected to ELISA with
161 two clones producing the optimized original signal peptide IL-21 (opti IL21-3 and opti IL21-11).
162 Based on ELISA, opti Az/IL21-5 and opti Az/IL21-12 cell clones resulted in a more than 2.5-fold
163 increase in the IL-21 concentration compared to the concentrations for opti IL21-3 and opti IL21-
164 11 cell clones (Fig. 2C). These data suggest that the azurocidin signal peptide enhances the
165 secretion of IL-21 in CHO-K1 cells.
166

167 **Biological activities of codon- and signal peptide-optimized IL-21**

168 IL-21 induces the phosphorylation of STAT3 and promotes the secretion of interferon-γ
169 (IFN-γ) in NK cells [2, 9]. To verify the biological activity of optimized IL-21, NK-92 cells were
170 stimulated with various concentrations of commercial IL-21 expressed in bacteria (rhIL-21),
171 original signal peptide IL-21 (WT/IL-21), or azurocidin signal peptide IL-21 (Az/IL-21). After 24
172 h, secreted IFN-γ in the culture supernatant was measured by ELISA. WT/IL-21 and Az/IL-21
173 produced by CHO-K1 cells showed similar induction of the secretion of IFN-γ in NK-92 cells
174 (Fig. 4A). However, commercial rhIL-21 produced by bacteria resulted in lower levels of IFN-γ
175 production than those for WT/IL-21 and Az/IL-21 in mammalian cells (Fig. 3A). Furthermore,
176 Western blot analysis showed that Az/IL-21 induced strong STAT3 phosphorylation, even at low
177 concentrations, whereas rhIL-21 resulted in a slight increase in the phosphorylation of STAT3
178 (Fig. 3B). These data suggest that Az/IL-21 has similar biological effects on IFN- γ production
179 and STAT3 phosphorylation to those of WT/IL-21 and better activity than those of commercial
180 rhIL-21.
181

4. Discussion

Most bio-therapeutics are produced in established mammalian cell lines, like CHO cells, which ensure correct glycosylation for the modulation of pharmaceutical efficacy in vivo. However, high production costs by low productivity and time requirements by slow proliferation limit the development and use of these systems [33]. Recently, many studies have aimed to increase the production of recombinant proteins in CHO systems by manipulating various factors [34, 35]. Expression vector engineering technologies, including codon optimization of the gene of interest and selection of appropriate signal peptide, are the most convenient methods to enhance productivity [15, 19].

Previous studies have shown that optimization of various factors, including codon usage bias, codon context, GC content, premature poly A, cryptic spice sites, and CpG dinucleotides content [36]. Our results showed that IL-21 productivity was improved 10-fold by gene optimization (Fig. 2B). Many studies have shown that gene optimization is a powerful method to enhance productivity by improving transcription, translation, and protein folding in non-mammalian or mammalian expression systems [37]. The GC content directly regulates the stability of mRNA and indirectly affects the mRNA level [15]. In this study, gene optimization of *IL-21* resulted in an increase in the COI (0.71 to 0.84), which is a measure of codon usage bias, as well as in the GC content (40% to 51%). Therefore, increased production resulting from gene optimization may be attributed the enhancement of both translation and mRNA level.

Signal peptides regulate efficient transport to the ER, appropriate folding, PTM, and the secretion of translated proteins to the culture supernatant. Several studies suggest that the production of recombinant protein can be improved by the use of alternative signal peptides [19-22]. In this study, we evaluated the efficiency of five signal peptides on the secretion of IL-21.

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Three signal peptides (derived from rat serum albumin, azurocidin, and tissue plasminogen activator signal peptides) resulted in higher productivity than that of the original signal peptide, whereas two signal peptides (derived from human serum albumin and H7 signal peptide) were similar to the control peptide with respect to productivity. Most notably, the azurocidin signal peptide showed the highest IL-21 production activity (Fig. 2B). Consistent with our results, a previous study used the azurocidin signal peptide to potently express various proteins in mammalian cells [21]. However, another study demonstrated that the human albumin signal peptide shows the highest secretion activity for the production of recombinant protein among various signal peptides [22]. This apparent discrepancy may be attributed to differences in experimental design or systems. Results may vary depending on the cell line or organism. Furthermore, the secretion efficiency can be affected by the amino acid sequence downstream of a signal peptide [38, 39].

The biological activities of recombinant IL-21 produced by CHO-K1 cells were assessed by measuring IFN-γ secretion and STAT3 phosphorylation in NK-92 cells (Fig. 3). Codon-optimized Az/IL-21 (IL-21 secreted by the azurocidin-derived signal peptide) had similar activity to that of codon-optimized WT/IL-21 (IL-21 secreted by the original signal peptide). Thus, although the azurocidin-derived signal peptide increases IL-21 production (Fig. 2), it does not affect the biological activity of IL-21 toward NK cells. However, the biological activity of both Az/IL-21 and WT/IL-21 in CHO-K1 increased compared to that of commercial recombinant IL-21 produced in bacteria (Fig. 3). CHO cells regulate PTMs to improve biological activity and reduce the immunological response to the therapeutic recombinant protein. Proper folding of secreted proteins by disulphide bonds, which can be obtained in mammalian cells, is important for their activity [40, 41]. Therefore, these enhanced activities can be explained by PTMs, such as glycosylation, sialyation, and fucosylation, or proper protein folding.

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In conclusion, we demonstrated that codon optimization and Azurocidin signal peptide can improve the production efficiency of human IL-21, which has biological activity, in CHO-K1 cells. Our results provide a potential strategy for the production of recombinant IL-21 as a biopharmaceutical.

Acknowledgments

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Conflict of interest

HJ Cho, BY Kim, I Choi, and HG Lee have pending patent applications related to this study. Other authors have no conflict to interest to declare.

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368 Table 1. The origin and amino acid sequence of signal peptides

Name	Signal peptide sequence	Protein	Aceecssion No	Organism
HSA	MKWVTFISLLFLFSSAYS	Human serum albumin	P02768	*Homo sapiens*
RSA	MKWVTFLLLLFISGSAFS	Rat serum albumin	P02770	*Rattus norvegicus*
AZ	MTRLTVLALLAGLLASSRA	Azurocidin preproprotein	NP_001691	*Homo sapiens*
TPA	MDAMKRGLCCVLLLCGAVFVSP	Tissue-type plasminogen activator	P00750	*Homo sapiens*
H7	MEFGLSWVFLVALFRGVQC	Ig heavy chain signal peptide 7		

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Figure 1

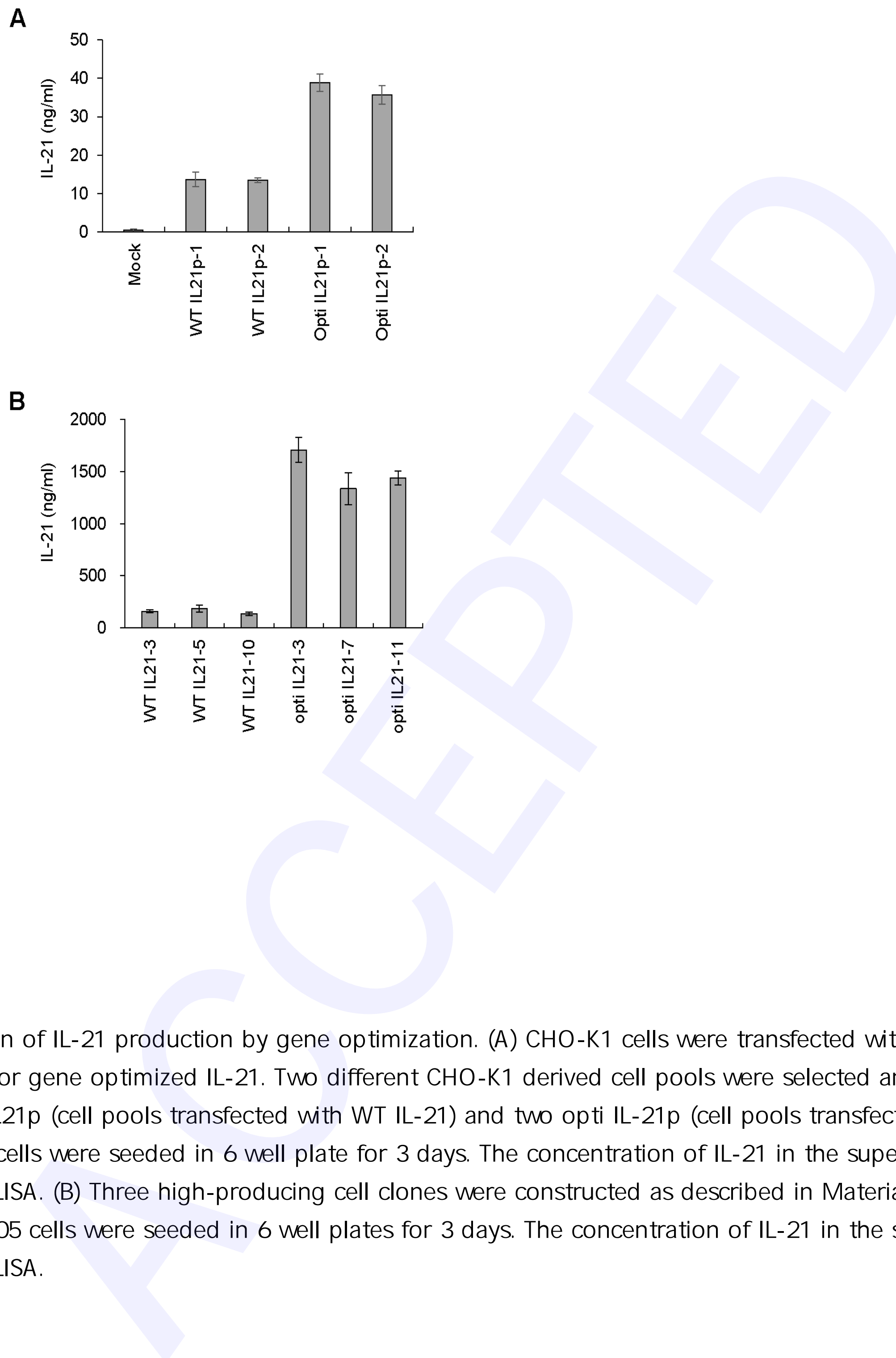

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Fig. 1. Evaluation of IL-21 production by gene optimization. (A) CHO-K1 cells were transfected with the vector expressing WT or gene optimized IL-21. Two different CHO-K1 derived cell pools were selected and defined were two WT IL21p (cell pools transfected with WT IL-21) and two opti IL-21p (cell pools transfected with opti IL-21). 1 x 105 cells were seeded in 6 well plate for 3 days. The concentration of IL-21 in the supernatant was measured by ELISA. (B) Three high-producing cell clones were constructed as described in Materials and Methods. 1 x 105 cells were seeded in 6 well plates for 3 days. The concentration of IL-21 in the supernatant was measured by ELISA.

Figure 2

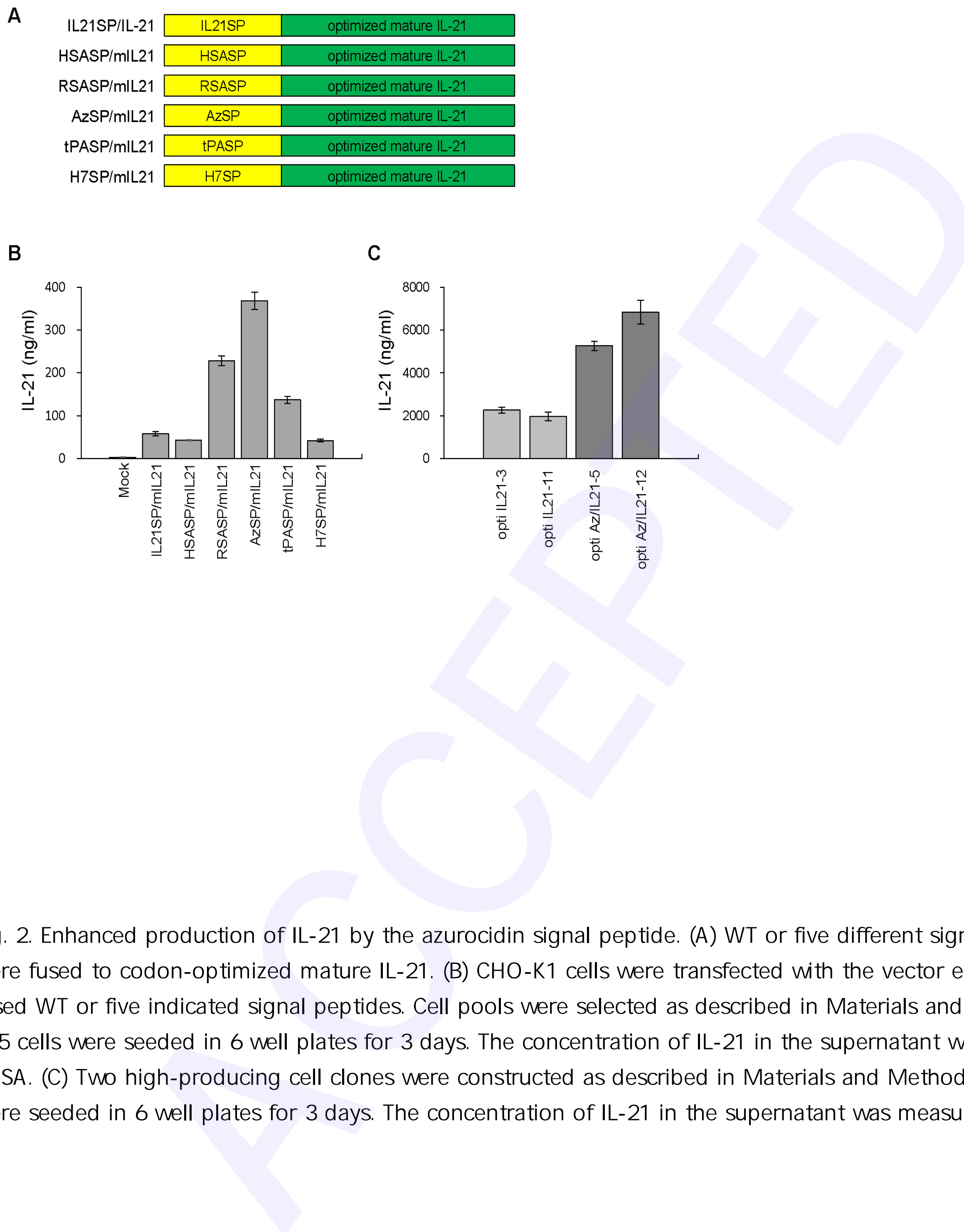

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Fig. 2. Enhanced production of IL-21 by the azurocidin signal peptide. (A) WT or five different signal peptides were fused to codon-optimized mature IL-21. (B) CHO-K1 cells were transfected with the vector expressing IL-21 fused WT or five indicated signal peptides. Cell pools were selected as described in Materials and Methods. 1 x 105 cells were seeded in 6 well plates for 3 days. The concentration of IL-21 in the supernatant was measured by ELISA. (C) Two high-producing cell clones were constructed as described in Materials and Methods. 1 x 105 cells were seeded in 6 well plates for 3 days. The concentration of IL-21 in the supernatant was measured by ELISA.

Figure 3

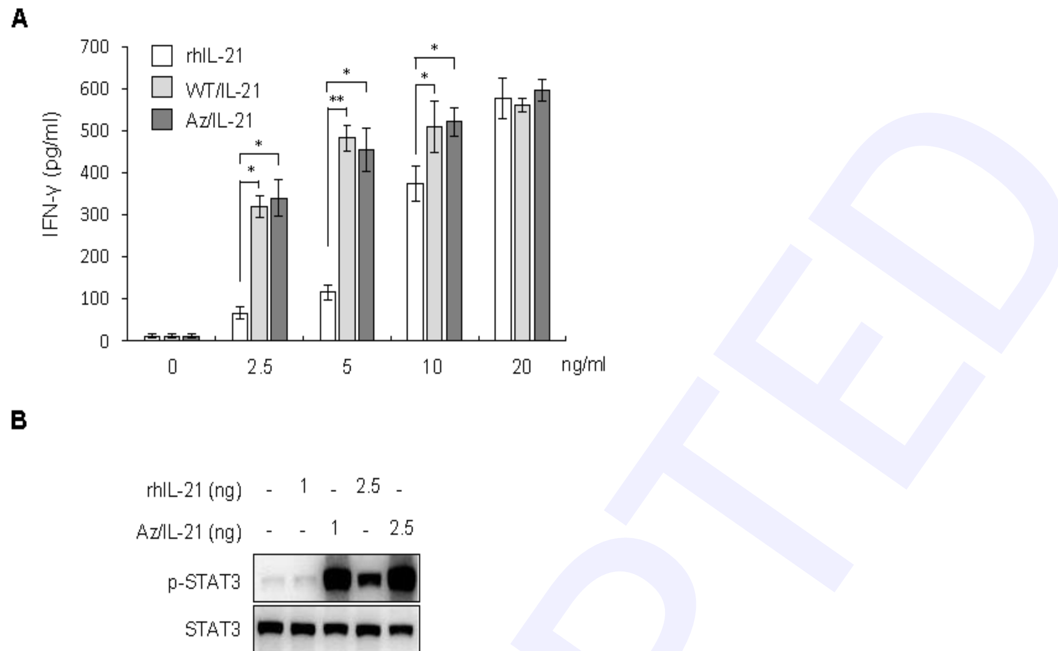

Fig. 3. IFN- production and STAT3 phosphorylation by recombinant IL-21 proteins in human NK-92 cells. (A) NK-92 cells were serum-starved for 18 hours and then treated with indicated concentrations of rhIL-21, WT/IL-21, or Az/IL-21 for 24 hours. The amount of secreted IFN- in the culture supernatant was measured by ELISA. Data represent the mean ± S.D. of three individual experiments. *, $p < 0.05$, **, $p < 0.01$. (B) Serum-starved NK-92 cells were treated with rhIL-21 or Az/IL-21 for 30 min. Cell lysates were immunoblotted with pSTAT3 or STAT3 antibodies.

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