Biochemical and Biophysical Research Communications 454 (2014) 347–352

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Optimization protein productivity of human interleukin-2 through codon usage, gene copy number and intracellular tRNA concentration in CHO cells

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ARTICLE INFO

Article history:
Received 11 October 2014
Available online 24 October 2014

Keywords:
tRNA abundance
Codon usage bias
Gene copy number
Interleukin-2
DNA vaccine

ABSTRACT

Transfer RNA (tRNA) abundance is one of the critical factors for the enhancement of protein productivity in prokaryotic and eukaryotic hosts. Gene copy number of tRNA and tRNA codon usage bias are generally used to match tRNA abundance of protein-expressing hosts and to optimize the codons of recombinant proteins. Because sufficient concentration of intracellular tRNA and optimized codons of recombinant proteins enhanced translation efficiency, we hypothesized that sufficient supplement of host's tRNA improved protein productivity in mammalian cells. First, the small tRNA sequencing results of CHO-K1 cells showed moderate positive correlation with gene copy number and codon usage bias. Modification of human interleukin-2 (IL-2) through codons with high gene copy number and high codon usage bias (IL-2 HH, modified on Leu, Thr, Glu) significantly increased protein productivity in CHO-K1 cells. In contrast, modification through codons with relatively high gene copy number and low codon usage bias (IL-2 HL, modified on Ala, Thr, Val), or relatively low gene copy number and low codon usage bias (IL-2 LH, modified on Ala, Thr, Val) did not increase IL-2 productivity significantly. Furthermore, supplement of the alanine tRNA or threonine tRNA increased IL-2 productivity of IL-2 HL. In summary, we revealed a potential strategy to enhance productivity of recombinant proteins, which may be applied in production of protein drug or design of DNA vaccine.

1. Introduction

Transfer RNA (tRNA) abundance is a critical factor for controlling protein productivity because it regulates the speed of ribosomal translation. In general, a high level of intracellular tRNA concentration is predicted to result in high translation efficiency because of lower codon missense error rate [1]. For heterologous protein expression, the difference of codon usage bias between recombinant proteins and protein-expressing hosts is an important factor to limit protein expression [2,3]. The favorable codons (but not random codons) of amino acid are generally used for the production of higher expression of genes in a host [4]. It was observed that the "codon usage bias" determines translation efficiency in many species [5–9]. On the other hand, "gene copy number" of tRNA in genomic DNA is usually correlated with tRNA abundance and anticodon–codon interactions [10–12]. In some species, the gene copy number is similar to tRNA abundance and is correlated with codon usage bias [11,13–16]. These observations suggest that the tRNA abundance, codon usage bias and gene copy number are generally conserved in some species [17].

Many therapeutic protein drugs are necessary to be produced in mammalian hosts because of the requirement for correct protein folding and post-translation modification. The baby hamster kidney, human embryonic kidney-293 cells, and Chinese hamster ovary (CHO) cells are frequently used for production of

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http://dx.doi.org/10.1016/j.bbrc.2014.10.097
0006-291X/

recombinant proteins [18]. Modifying DNA sequences of protein products according to gene copy number or codon usage bias are common strategies to increase protein productivity. Besides, there are other factors affecting protein translation [19]. For example, the balanced codon usage and intracellular tRNA pool contributes to eukaryotic translational efficiency [20]. Supplement of tRNAs recognizing rare codons in the protein-expressing host was able to mimic the effect of codon optimization and improve protein productivity in bacterial hosts [21,22]. It might be a strategy to enhance protein expression. However, to the best of our knowledge, there is no report to evaluate whether the strategy is effective in mammalian hosts.

Recombinant human interleukin-2 (IL-2) serves as effective immunotherapy against renal cell carcinoma and melanoma [23]. Because IL-2 supports expansion and maintenance of human lymphocytes *in vitro*, IL-2 can also be used in adoptive transfer of effector T cells for cancer therapy [24,25]. In our previous studies, IL-2 enhances antitumor effect of DNA vaccine and whole cell tumor vaccine in murine tumor models [26,27]. The aim of the present study is to investigate how to enhance protein expression through optimum codon alteration in mammalian hosts. The codon of IL-2 was modified according to high codon usage bias or high gene copy number in CHO cells. In addition, expression level of IL-2 was determined after modulating intracellular tRNA level. This study revealed that modification of IL-2 codons through both high codon usage bias and gene copy number increases IL-2 expression. Furthermore, increasing intracellular tRNA concentration further enhances IL-2 expression.

2. Materials and methods

2.1. Cell culture

CHO-K1 (ovary cell line of *Cricetulus griseus*) was obtained from Bioresource Collection and Research Center (Hsinchu, Taiwan). CHO-K1 cells were maintained in Nutrient Mixture F-12 (HAM's F12) medium containing 10% fetal bovine serum, 100 units per milliliter of penicillin and 100 microgram per milliliter of streptomycin. Cell culture materials were from Invitrogen (Carlsbad, CA, USA), except fetal bovine serum, which was from Biological Industries (Kibbutz Beit Haemek, Israel). Cells were cultured at 37 °C with 5% CO_2 incubator.

2.2. Small RNA analysis in CHO-K1 cells (RNA extraction, transfer RNA library preparation, sequencing, and analysis)

Total RNA of the CHO-K1 cell was extracted by Trizol reagent (Invitrogen) according to the instruction manual. The small RNA library construction and transfer RNA (tRNA) sequencing was performed by Welgene biotechnology company (Taipei, Taiwan). Purified RNA was quantified by a ND-1000 spectrophotometer (Nanodrop Technology, USA) and qualitatively analyzed by a Bioanalyzer 2100 (Agilent Technology, USA) with RNA 6000 Labchip kit (Agilent Technology, USA). Purified RNA of the CHO-K1 cell was prepared by Illumina sample preparation kit according to the TruSeq Small RNA Sample Preparation Guide. The 3′ and 5′ adaptors were ligated to total RNA and reverse transcription followed by PCR amplification. The enriched cDNA constructs were fractionated and purified on a 6% polyacrylamide gel electrophoresis and the bands containing the 70–110 nt nucleotide RNA fragments (190–230 nucleotide in length with both adapters). Libraries were sequenced on an Illumina GAIIx instrument (50-cycle single read) and the sequencing data was processed with the Illumina software. The sequences generated went through a filtering process to obtain qualified reads. ConDeTri was implemented to trim or remove the reads according to the quality score [28]. Qualified reads after filtering low-quality data were analyzed by Bowtie2, by which aligning reads to the *C. griseus cell line CHO-K1* from tRNA Genomic database (http://gtrnadb.ucsc.edu). Cuffinks estimates expression levels of known tRNAs (http://cufflinks.cbcb.umd.edu) [29].

2.3. Plasmid construction

All Human IL-2 sequences were synthesized by GENEWIZ, INC (South Plainfield, NJ) and were inserted into the pcDNA3.1(−) vector (Invitrogen) at the EcoRI and KpnI, including human wild type IL-2 (NM_000586, IL-2 WT), modified-IL-2 according to high tRNA gene copy number and high codon usage bias (IL-2 HH, anti-codon of Leu is CAG, anti-codon of Thr is AGT and anti-codon of Glu is CTC), modified-IL-2 according to relatively low tRNA gene copy numbers and high codon usage bias (IL-2 LH, anti-codon of Ala is GGC, anti-codon of Thr is GGT and anti-codon of Val is CAC), and modified-IL-2 according to high tRNA gene copy number and low codon usage bias (IL-2 HL, anti-codon of Ala is TGC, anti-codon of Thr is AGT and anti-codon of Val is AAC). Modified codons are also listed in Table 1 and complete modified sequences are listed in Supplement Table 1 Secreting-GFP vector contained a signal peptide of human IL-2 (1–20 amino acid of human IL-2, forward primer 5′-CCGGGATCCTCTAGAATGTACAGGATGCAACTCCT-3′ and reverse primer: 5′-CTCGCCCTTGCTCACACTGTTTGTGACAAGTG-CAA-3′) and GFP genes which was cloned from EGFP-N1 vector (Clontech) (forward primer: 5′-GTGAGCAAGGGCGAGGAGCT-3′ and reverse primer: 5′-AAGGGAA GCGGCCGCTTACTTGTA-CAGCTCGTCCATGCC-3′). Both fragments were inserted into a pIRES vector (Clontech) with the BD In-Fusion PCR cloning kit (Clontech). For tRNA expression plasmid, the sequence of each tRNA was amplified from genomic DNA of CHO-K1 cells according to the Genomic tRNA database (http://gtrnadb.ucsc.edu/Cgris1/) and the genome of *C. griseus* in National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/genome/?term=Cricetulus+griseus). DNA sequences of $tRNA^{Ala}$ (anti-codon is TGC)

Table 1
Genomic tRNA copy number and tRNA codon usage frequency of CHO-K1 cells.

Amino acid	Anti-codon	Codon	Genomic tRNA copy numbers	tRNA codon usage frequency per thousand
Leu	AAG	CUU	5	13.2
	GAG	CUC	0	18.4
	&CAG	CUG	10	38.8
	TAG	CUA	4	7.6
	CAA	UUG	4	14.1
	TAA	UUA	4	6.4
Glu	&CTC	GAG	20	41.4
	TTC	GAA	7	28.4
Thr	&#AGT	ACU	8	14.1
	⁂GGT	ACC	1	20.3
	CGT	ACG	4	4.5
	TGT	ACA	4	15.7
Ala	AGC	GCU	22	22.4
	⁂GGC	GCC	1	25.9
	CGC	GCG	6	5
	#TGC	GCA	15	16.3
Val	#AAC	GUU	13	11.6
	GAC	GUC	0	15.7
	⁂CAC	GUG	9	30.1
	TAC	GUA	5	7.8

Note: symbol "&" indicates codon used for high genomic copy number and High-usage codons plasmid (IL-2 HH); symbol "⁂" indicates low gene copy numbers and relatively high-usage codons plasmid (IL-2 LH); symbol "#" indicates high genomic copy numbers and relatively low-usage codons plasmid (IL-2 HL).

(primer: 5′-TTGTCTGGTGACAGTTAAGCC-3′ and 5′-CAGTTCATTA CAGGCCCTACA-3′), $tRNA^{Val}$ (anti-codon is AAC) (primer: 5′-TGG CCCTGGAATGAAAAGTGT-3′ and 5′-CTGAGCGTTCTTCTGGCACT-3′), and $tRNA^{Thr}$ (anti-codon is AGT) (primer: 5′-CAGCAAACCCTCC TCTTCGG-3′ and 5′-TTTGGTACAAAGTTAAGCA AACTCG-3′) were amplified from genomic DNA of CHO-K1 cells and were cloned into the yT&A vector (YEASTERN BIOTECH CO., LTD., Taipei, Taiwan).

2.4. Determination of IL2 expression with enzyme-linked immunosorbent assay (ELISA)

$2x10^6$ CHO-K1 cells were seeded into 10 cm dish for 18 h and then were transfected with 2.5 μg of each IL-2 plasmid and 2.5 μg of secreting-GFP plasmid using Polyjet™ *In Vitro* DNA Transfection Reagent (SignaGen Laboratories, Rocksville, MD). 24 h after transfection, medium was replaced with 8 ml of serum-free HAM's F12 medium. The medium was collected for another 24 h. IL-2 level was quantitated by ELISA MAX™ Deluxe Sets (BioLegend, San Diego, CA). For GFP detection, 8 ml medium was concentrated to 0.5 ml using Amicon Ultra-15 centrifugal filtration devices with a 3000 NMWL cutoff (Millipore, Billerica, MA). GFP level was quantitated by GFP ELISA kit (Abcam, Cambridge, UK). The relative expression level of IL-2 was normalized by IL-2 and secreting-EGFP ratio.

2.5. RT-PCR

Total RNA was extracted from CHO-K1 cells by using TriPure isolation reagent (Roche) and was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) according to the manufacturer's directions. cDNA was generated with random hexamers and MMLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's directions. For detection of tRNA precursor, $pre\text{-}tRNA^{Ala}$ primers are 5′-TGGGGATGCTGGGAATCA-3′ and 5′-GGGGATGTAGCTC ATTGGT-3′; $pre\text{-}tRNA^{Thr}$ primers are 5′-AGGCCCCGCTGGGATTCG-3′ and 5′-GGCCCTGTGGCTTAGCTGG-3′; $pre\text{-}tRNA^{Val}$ are 5′-AGGCCCCGC TGGGATTCG-3′ and 5′-GGCCCTGT GGCTTAGCTGG-3; and acidic ribosomal phosphoprotein P0 (ARPP P0) primers are 5′-GCACTGGAAGTCCAACTACTTC-3′ and 5′-TGAGG TCCTCCTTGGTGAACAC-3′ were synthesized. For RT-PCR, cycling conditions was 5 min for 95 °C, 22–28 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s in an ABI 2720 thermal cycler (Applied Biosystems, Foster City, CA). The products were analyzed by agarose gel electrophoresis.

2.6. Graph and statistical analysis

All of the numerical data and graphs were analyzed with GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego, CA, USA). Pearson correlation coefficients were calculated evaluating correlation between tRNA sequencing results, tRNA gene copy number, and codon usage bias. Student t-test was used for comparing two different IL-2 transfected groups. All tests were two-sided and $p < 0.05$ was considered significant.

3. Results

3.1. Positive correlation between tRNA gene copy number, tRNA codon usage and tRNA abundance in CHO-K1 cells

Because CHO cell line is widely used for protein production in academic research and industry, it was chosen as the model cell in the present study. We first checked the gene copy number of tRNA in genomic DNA or codon usage bias of *C. griseus* in genomic tRNA database (http://gtrnadb.ucsc.edu/Cgris1/) and Codon Usage Database (http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?Species=10029&aa=1&style=N) respectively. The comparison is listed in Supplement Table 2 and the correlation

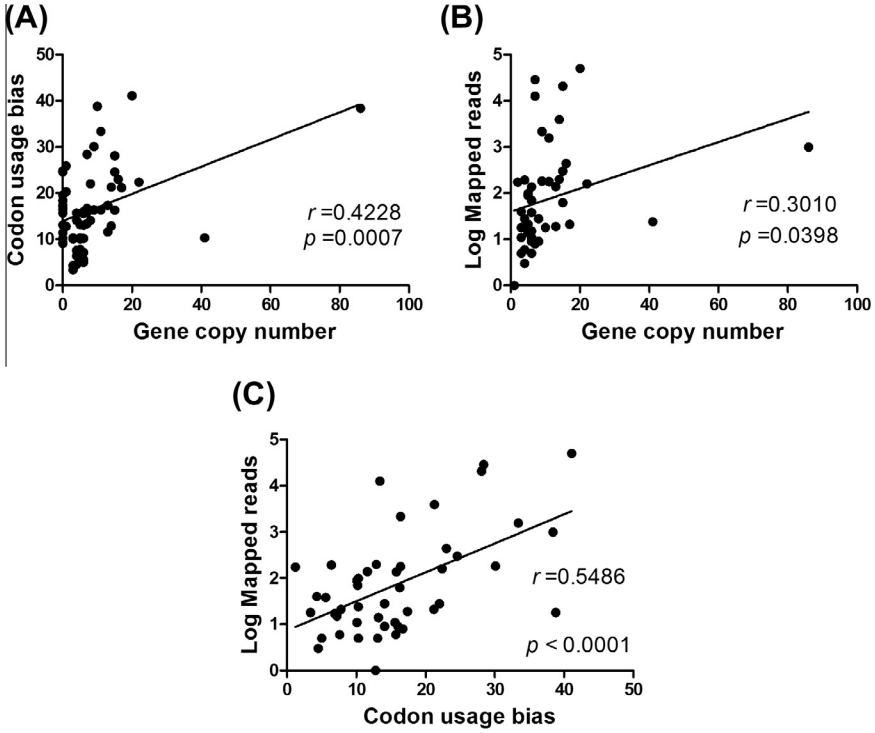

Fig. 1. Correlation between total transfer RNA sequencing, gene copy number and codon usage bias in CHO-K1 cells. (A) Correlation between gene copy number and codon usage bias. (B) Correlation between sequencing reads and gene copy number. (C) Correlation between sequencing reads and codon usage bias. Pearson correlation coefficients and *p*-value are shown in each graphics.

Table 2
Modified codons of IL-2.

Plasmid	Amino acid (codon)	Amino acid number of IL-2	Unchanged amino acid codons	Changed amino acid codons	Changed percentage of IL-2 codons
IL-2 LH	Ala(GCA)	7	1	6	21 aa./153
	Thr(ACU)	14	3	11	aa. = 13.7%
	Val(GUG)	5	1	4	
IL-2 HL	Ala(GCC)	7	4	3	19 aa./153
	Thr(ACC)	14	2	12	aa. = 12.4%
	Val(GUU)	5	1	4	
IL-2 HH	Leu(CUG)	27	9	18	39 aa./153
	Thr(ACU)	14	2	12	aa. = 25.5%
	Glu(GAG)	12	3	9	

Note: some codons were not necessary to be changed because these codons were the identical with the codons which we wanted to change. "aa." indicated amino acid codons.

between gene copy number and codon usage bias is shown in Fig. 1A. To further determine the intracellular tRNA abundance, the total small RNA sequencing of CHO-K1 cells was performed and the sequencing data was compared with genomic tRNA database. The correlation between tRNA abundance (represented as read numbers in total small RNA sequencing results) and gene copy number or codon usage bias was analyzed (Fig. 1B and C). The results indicated that tRNA abundance was positively correlated with gene copy number and codon usage bias in the CHO-K1 cell line. The correlation is not strong, further suggesting that some high gene copy number is not high codon usage bias (Supplement Table 2).

3.2. Interleukin 2 (IL-2) expression was enhanced by modulation of gene copy number or codon usage bias

In order to determine the influence of gene copy number and codon usage bias in CHO-K1 cells, human IL-2, which contained 153 amino acid residues, served as a model protein. To optimize the influence of altering amino acid codons, we selected to alter the top five of the most abundant amino acids in IL-2: 27 Leu residues, 14 Thr residues, 12 Glu residues, 7 Ala residues, and 5 Val residues. Based on the gene copy number and tRNA codon usage (Table 1), nucleotide sequence of wild type IL-2 was modified to low gene copy number and relatively high codon usage bias (IL-2 LH) and high gene copy number and relatively low codon usage bias (IL2 HL) (Table 1). IL-2 with high gene copy number and high codon bias modification (IL-2 HH) served as a positive control (Table 2). The modified percentage of each IL-2 plasmid is listed in Table 2. Secreting EGFPs, which contain a signal peptide of human IL-2 and EGFP genes, were used as experimental controls.

Each IL-2 plasmid and secreting EGFP plasmid were transfected into CHO-K1 cells and the protein level of IL-2 and EGFP was measured by ELISA assay (Fig. 2A). IL-2 expression of IL-2 HH was significantly enhanced whereas that of IL-2 LH and IL-2 HL were not enhanced (Fig. 2D). The results indicated that modification according to high gene copy number and high codon usage bias (IL-2 HH) contributed to IL-2 production even though only 25.5% codons of IL-2 were modified (Table 2).

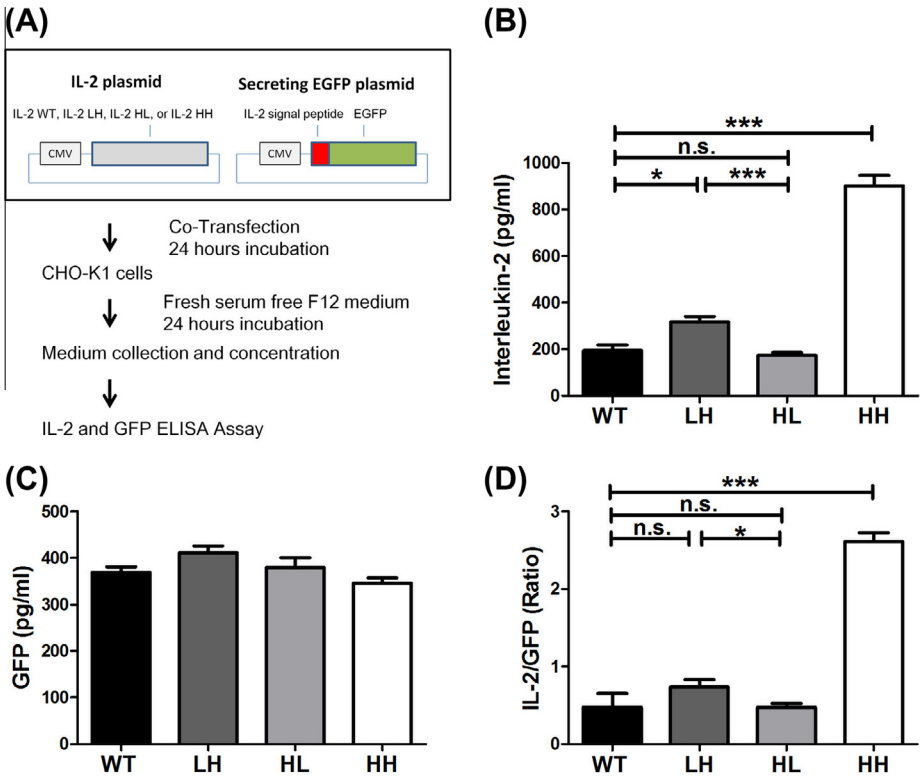

Fig. 2. Evaluation of IL-2 expression with codon modification. Wild type IL-2, modified-IL-2 according to high tRNA gene copy number and high codon usage bias (IL-2 HH), relatively low tRNA gene copy number and high codon usage bias (IL-2 LH), and high tRNA gene copy number and low codon usage bias (IL-2 HL) were constructed into pcDNA3.1 vector and secreting GFP was constructed into pIRES vector. (A) Experimental scheme. (B) 2.5 μg of each IL-2 plasmid and 2.5 μg of secreting GFP plasmid were transfected into 2×10^6 CHO-K1 cells for 24 h. Culture medium was replaced by fresh serum-free HAM's F12 medium for another 24 h. Concentrated medium was performed by GFP and IL-2 EILSA assay. IL-2 expression. (C) GFP expression. (D) Normalized IL-2 expression. Symbol "$*$" indicates a statistically significant difference ($p < 0.05$); Symbol "$***$" indicates a statistically significant difference ($p < 0.001$); and N.S. indicates no significant difference between two groups. At least three independent experiments were performed.

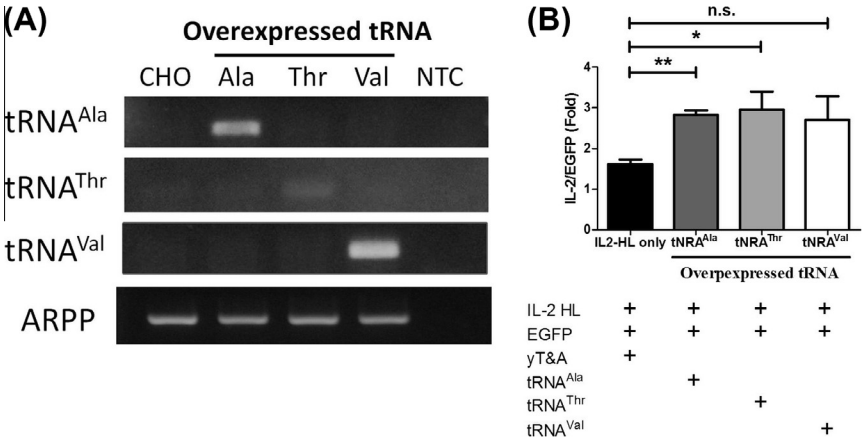

Fig. 3. Modulation of tRNA expression increases IL-2 production. Plasmids of IL-2 HL, secreting GFP, and yT&A, or $tRNA^{Ala}$TGC or $tRNA^{Thr}$AGT or $tRNA^{Val}$AAC were transfected into CHO-K1 cells. Culture medium was replaced by fresh serum-free HAM's F12 medium for another 24 h. RNA and medium was collected. (A) Pre-tRNA expression of Ala, Thr, and Val was analyzed by RT-PCR assay. (B) IL-2 and GFP expression were analyzed by ELISA assay. Symbol "∗" indicates a statistically significant difference ($p < 0.05$); and N.S. indicates no significant difference between two groups. Three independent experiments were performed.

3.3. Specific tRNA supplement increase IL-2 production

Since IL-2 LH and IL-2 HL modification did not significantly improve IL-2 production, we speculated that insufficient intracellular tRNA concentration in CHO-K1 cells might result in lower translation efficiency. Therefore, the tRNA expression plasmids (tRNA alanine with anticodon TGC, $tRNA^{Ala}$TGC; tRNA threonine with anticodon AGT, $tRNA^{Thr}$AGT; tRNA valine with anticodon AAC, $tRNA^{Val}$AAC) of *C. griseus* were constructed. The expression of each tRNA was confirmed by RT-PCR (Fig. 3A). Approximate 12.5% codons of human IL-2 were modified in IL-2 HL plasmid (3 Ala residues, 10 Thr residues, and 4 Val residues, Table 2). Co-transfection with $tRNA^{Ala}$TGC or $tRNA^{Thr}$AGT and IL-2 HL plasmid significantly enhanced IL-2 expression ($p = 0.0016$ and $p = 0.0420$ respectively) although only one type of tRNA was supplemented. It implied that the effect of tRNA supplement was similar with codon optimization because the rise in the intracellular tRNA resulted in high translation efficiency. The results suggested that increasing a specific tRNA concentration in the intracellular tRNA pool could be a strategy to improve protein productivity.

4. Discussion

Optimizing heterologous protein expression through single index, such as codon usage or gene copy number, may not significantly improve productivity in the host [19]. In the present study, increase of IL-2 productivity was observed by using codons with high gene copy number and high codon usage bias. In addition, supplement of a specific tRNA of CHO-K1 cells further enhanced IL-2 productivity even though the codon was not optimized in CHO-K1 cells. The results suggest that optimization of the codon of recombinant protein and supplement of host's tRNA may be useful in obtaining higher protein expression in CHO-K1 cells.

Previous studies indicate that gene copy number is generally correlated with codon usage bias [11,13–16]. Here, tRNA sequencing data of CHO-K1 cells also showed moderate positive correlation between gene copy number and codon usage bias. Partial optimization of approximate 26% nucleotides improved protein expression of Toll-like receptor 2 [30]. Although IL-2 productivity of IL-2 HL and IL-2 LH was similar with IL-2 WT in CHO-K1 cells, high IL-2 productivity of IL-2 HH was observed even though only 25.5% of the IL-2 codons were changed (Fig. 2D). One possible explanation is that fewer optimized nucleotides (13.7% codons in IL-2 HL and 12.4% codons in IL-2 LH) may be insufficient to increase translation efficiency. The interaction between amino acids, tRNAs, and aminoacyl-tRNA synthetases, which incorporate corresponding tRNA to amino acid for protein synthesis, are known to be involved in regulation of translation efficiency [31]. Manipulating activity of an aminoacyl-tRNA synthetase is one of the strategies to increase synthesis of protein [32]. Additional amino acid supplement improves heterologous protein productivity in yeast [33].

In the present study, it is interesting to note that protein productivity of IL-2 HL (relative high gene copy number and low codon usage bias) was enhanced by an additional tRNA supplement even though only 12.4% of codons of IL-2 were changed. The results may imply that increasing concentration of intracellular tRNAs serves as a strategy to enhance protein productivity in mammalian cells with the non-optimized codon.

IL-2 is a cytokine that activates dendritic cells and downstream immune responses. In addition, IL-2 plays a critical role in cancer immunotherapy including adoptive T cell transfer, whole cell tumor vaccines, and DNA vaccines [24–27]. In general, antitumor DNA vaccine is a plasmid that encodes xenogeneic antigens in a host. High antigen expression of DNA vaccine usually contributes to therapeutic outcome. Therefore, codon optimization has been used to enhance anti-tumor immunity of DNA vaccine [34]. Since supplement of tRNA enhanced protein expression, we propose that co-administration of IL-2 plasmid, host's tRNA expressing plasmids, and DNA vaccines may be a novel strategy of DNA-based immunotherapy.

Because gene length, CG content and secondary structure of mRNA affect translation efficiency [35], it remains to be further determined whether tRNA supplement enhances protein productivity of other cytokines or other genes. In summary, optimization of IL-2 codons and supplement of intracellular tRNAs improved protein productivity in CHO-K1 cells. It may provide a potential strategy to optimize protein productivity in mammalian cells and enhance efficiency of anti-tumor DNA vaccines.

Conflicts of interest and informed consent

The authors note no conflict of interest.

Acknowledgments

This study is supported in part by the grant to Yi-Ling Chen, Meng-Chi Yen and Hui-Ping Hsu MOST 103-2311-B-041-001-from the Ministry of Science and Technology, Taiwan, and in part by

Grant number NSC 97-2320-B-006-003-MY3 to Dr. Ming-Derg Lai from the National Science Council, Taiwan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.097.

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