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Synthetic biology approach revealed enhancement in haeme oxygenase-1 gene expression by codon pair optimization while reduction by codon deoptimization

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Abstract

Haem oxygenase-1 (HO-1) is a ubiquitously expressed gene involved in cellular homoeostasis, and its imbalance in expression results in various disorders. To alleviate such disorders, HO-1 gene expression needs to be modulated. Codon usage bias results from evolutionary forces acting on any nucleotide sequence and determines the gene expression. Like codon usage bias, codon pair bias also exists, playing a role in gene expression. In the present study, HO-1 gene was recoded by manipulating codon and codon pair bias, and four such constructs were made through codon/codon pair deoptimization and codon/codon pair optimization to reduce and enhance the HO-1 gene expression. Codon usage analysis was done for these constructs for four tissues brain, heart, pancreas and liver. Based on codon usage in different tissues, gene expression of these tissues was determined in terms of the codon adaptation index. Based on the codon adaptation index, minimum free energy, and translation efficiency, constructs were evaluated for enhanced or decreased HO-1 expression. The analysis revealed that for enhancing gene expression, codon pair optimization, while for reducing gene expression, codon deoptimization is efficacious. The recoded constructs developed in the study could be used in gene therapy regimens to cure HO-1 over or underexpression-associated disorders.

Keywords: codon deoptimization, codon optimization, codon pair deoptimization, codon pair optimization, Haem oxygenase-1, MFE, tAI, Translation efficiency

Introduction

Haem oxygenase-1 (HO-1) is a ubiquitously expressed 32 kDa protein in many tissues. It degrades the haem group and releases free iron and biliverdin[1]. It is imperative in maintaining cellular homoeostasis, and its imbalance leads to various diseases. The biological role of HO-1 in tumour cells is cell-specific since, in certain tumours, its upregulation leads to cellular death. At the same time, in some, it promotes malignant cell survival and proliferation, for example, in renal cell carcinoma[2], pancreatic cancer[3] and melanoma[4]. High HO-1 expression is correlated with enhanced angiogenesis of human gliomas, suggestive of high HO-1-driven tumour invasiveness and poor clinical outcome[5]. HO-1 inhibition inhibits neuroblastoma growth and liver metastasis[6]. Inhibition of HO-1 through zinc protoporphyrin and tin protoporphyrin IX (SnPP) suppress the proliferation of Pancreatic ductal adenocarcinoma[7]. It is an inducible stress response protein. For heart tissue, intramyocardial insertion of AAV vectors expressing HO-1 results in reduced infarct size in the ischaemia-reperfusion injury rat model[8], and its long-term induction of

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protein expression poses a protective role against chronic heart failure in a rat model[9]. AAV-mediated HO-1 induction in pigs reduced post-ischaemic inflammation[10]. Brain tissue autoimmune neuroinflammation is increased if HO-1 expression is decreased[11]. HO-1 deficiency leads to intracranial haemorrhage in children[12]. Contrary to the neuroprotective role, in Alzheimer patients, 86% of GFAP-positive astroglia showed the presence of HO-1, while only 6–7% fraction of hippocampal astrocytes showed HO-1 in age-matched, cognitively-normal subjects. A constitutive expression of HO-1 in astrocytes results in iron accumulation and mitochondrial dysfunction in the brain, resulting in decreased cognitive ability[13].

HO-1 induction through desoxo-narchinol-A for the pancreas tissue attenuates severe pancreatitis[14]. On one hand, HO-1 induction showed a reduction in pancreatic inflammation; on the other hand, a reduction in HO-1 activity increased the responsiveness of pancreatic cancer cells toward gemcitabine treatment[7].

HO-1 induction by cobalt protoporphyrin is reported to alleviate cholestatic liver disease in a murine model[15] and inhibition of HO-1 activity worsens hepatic steatosis and fibrosis[16]. Overall the impact of over or underexpression is evident in various diseases, and modulation of gene expression may be an effective strategy to fight the disorders. Codon optimization for enhancement and deoptimization for decreasing the gene expression has been utilized to obtain the desired gene expression[17–20].

Glycation is a process that influence various diseases like influence over various diseases' progression such as diabetes, atherosclerosis, cancer, and Alzheimer's disease. HO-1 alleviate advanced glycation end product-induced oxidative stress, inflammation and behavioural disorders.

In the present work, we used codon and codon pair optimization and codon and codon pair deoptimization to enhance and decrease HO-1 expression and systemically designed four constructs. These constructs are evaluated for their efficacy in modulating gene expression in terms of minimum free energy (MFE), codon adaptation index (CAI), and translation efficiency. The constructs developed in the study may be used in modulating the HO-1 gene expression and combat advanced glycation end product-induced oxidative stress, inflammation and behavioural disorders.

HIGHLIGHTS

- Haem oxygenase-1 (HO-1) is a ubiquitously expressed gene involved in cellular homoeostasis, and its imbalance in expression results in various disorders.
- Codon usage and, codon pair bias influence gene expression.
- A synthetic biology approach is adapted to manipulate the gene expression level of HO-1 gene.
- Four constructs have been designed and were evaluated for their possible gene expression modulation using parameters codon adaptation index, minimum free energy, and translation efficiency.
- The analysis revealed that for enhancing gene expression, codon pair optimization, while for reducing gene expression, codon deoptimization is efficacious.
- The recoded constructs developed in the study could be used in gene therapy regimens to cure HO-1 over or underexpression-associated disorders.

Materials and methods

Sequence retrieval

The Homo sapiens hemeoxygenase 1 coding sequence was obtained from National Center for Biotechnology Information (NCBI Reference Sequence: NG_023030.1). The gene had only one transcript; the mature protein is obtained after post-transcriptional modification. The sequence used starts with ATG, ends with a stop codon, and is divisible with three. It had no stop codon in between the sequence.

Tissue-wise codon usage determination

Tissue-specific differences in codon usage have been observed for various genes and possibly owing to the difference in tRNA pools[21] in the cells. Since there is a practical implication of gene expression in particular tissues, we determined the codon usage in four tissues heart, brain, pancreas, and liver, using the software TissueCoCoPUTs developed by Kames and colleagues[22]. The software uses transcriptome data from Broad Institute Genotype-Tissue Expression (GTEx) portal. The codon usage table was converted into Kazusa format for further usage.

Determination of CAI

CAI is an index to measure the extent of codon bias. It is widely used as a surrogate for gene expression. For calculating CAI, the relative codon usage of highly expressed genes is used as a reference set[23]. CAI value for the native HO-1 coding sequence was calculated using the CAIcal server developed by Puigbò and colleagues[24]. Similarly, for recoded constructs (meant for HO-1 underexpression and overexpression), the CAI value was calculated[25]. Since codon usage is different in each tissue, CAI values were calculated for native HO-1 sequences and recoded constructs for all four tissues envisaged.

Codon context analysis

The translation of one codon depends on the codon present in its neighbourhood and is called the codon context effect[26]. The codon pair context frequency table was built using Anaconda2 software to statistically test the contingency tables whether the context is significantly biased[27]. The association is tested through the $\chi 2$ test of independence to identify preferred codon pairs[28]. Codon context analysis was done for the HO-1 native sequence to determine the highest contexts.

Construct recoding based on codon and codon pair deoptimization and optimization, respectively

To ameliorate aberrant HO-1 gene expression, we needed constructs that enable both the up and downregulation of gene products. We systemically recoded four constructs from the native sequence. We used codon usage to recode the first and second constructs. The first construct HO-C-DEOPTI is based on codon deoptimization, and the second construct HO-C-OPTI is based on codon optimization. Codon pair manipulation is the strategy for The third and fourth construct recoding. The third construct HO-CP-DEOPTI is based on codon pair deoptimization and the fourth construct HO-CP-DEOPTI, is based on codon pair

optimization. So codon-deoptimized HO-C-DEOPTI, and codon pair deoptimized HO-CP-DEOPTI constructs were recoded and evaluated for underexpression of the gene. On the other hand, codon-optimized HO-C-OPTI and codon pair-optimized HO-CP-OPTI were recoded and evaluated for overexpression of the gene.

In the HO-1 sequence frequency of various codons was determined using Anaconda2 software. Codon frequencies above 3.5% and below 0.5% were considered abundant and rare codons here. Therefore, codons appearing at least ten times in the construct (frequency 5.79 to 3.62%) were chosen for codon deoptimization. Such high frequency eight codons were present in the HO-1 sequence; out of them, one codon encoded trp, which has no synonymous codon. Hence, 09 codons were used for deoptimization and replaced these codons with their respective least abundant synonymous codons, termed HO-C-DEOPTI. The same least abundant synonymous codons were replaced with their respective codon having the highest usage and termed HO-C-OPTI.

Similarly, ten codon pairs with the highest codon context were chosen to disrupt the pair. The 3' codon of the pair was deoptimized with its respective least abundant synonymous codons and termed HO-CP-DEOPTI (3' codon was chosen since in all highest context codon pairs, 5' codon was already least abundant codon and in 05 codon pairs, both the codons of the pair were least abundant synonymous codons). The same ten codon pairs were optimized by optimizing the 5' codon and termed HO-CP-OPTI.

MFE calculation

MFE of transcript is related to the mRNA stability, and the transcripts with highly negative MFE are more stable, and in general, highly expressed genes have highly negative MFE[29,30]. MFE for native sequence and recoded constructs was calculated using the RNAfold server[31].

Codon efficiency

The tRNA Adaptation Index (tAI)[32] values reflect the real translational efficiency changes due to wobble interactions of synonymous codons[33]. Using the algorithm, "ExtRamp" developed by Miller and colleagues[34], overall translation efficacy is the arithmetic mean of codon efficiency of each of the codons concerning their cognate tRNAs may be calculated. Furthermore, the regions of transcripts with slow translation rates also may be identified. While calculating the translation efficacy, a sliding window size was set at nine codons, the average ribosomal size[35]. Here it is possible to identify the local bottlenecks of translation speed that may be optimum to keep ribosomes at a particular space to avoid ribosomal clustering and proper folding of a protein crucial to the biological activity. Less value indicates less adaptive codons[34]. There is a difference in codon adaptiveness in different tissue and the algorithm is used to determine the translation efficacy in four different tissues heart, brain, pancreas, and liver.

Statistical analysis

All graphs are generated in Origin18 software. All the statistical analysis was done using PAST4 software.

Result

The human HO-1 gene encompasses an 867 bp long coding region that encodes for 289 amino acid long protein. The HO-1 system is crucial in cellular defense against numerous diseases, including diabetes, hypertension, heart diseases, inflammation, transplantation, neurodegenerative and aging processes. However, its expression is changed in the diseased conditions and induced modulation in the gene expression may be the key to fighting these diseases. In the present study, we envisaged four vital organs, the brain, heart, pancreas and liver, where we systemically checked the expression level of native HO-1 sequence and the constructs meant for ameliorating the overexpression and underexpression of the gene in diseased conditions.

Codon usage of HO-1 gene in different tissue

Diverse mechanisms regulate tissue-specific protein levels, and tissues specific differences in codon usage have been observed[21]. We calculated the codon usage per thousand for each of the tissues, and the results of two-, three-, four- and six-fold degenerated codons have been demonstrated in Fig. 1. Though there is a clear tissue-wise difference in codon usage of HO-1 gene (Fig. 1), it is not statistically significant.

Codon context analysis

Codon context is the tendency of codons to be present in pairs. The codon pairs may be preferred or rejected, and they have been shown to pose a significant effect on the in-vivo translation of protein. Pieces of evidence suggested that instead of limiting tRNAs, the codon context effect accounts for the construct attenuation[26]. In the present study, we found the presence of only positive codon contexts (Fig. 2).

The top 10 positive codon contexts were selected for the study. The highest codon context was present for AAT-GCT, ACA-GTT, ATA-GAA, CAA-GAT, CCG-CAA, CGT-CCG, CGT-GTA, GTT-GCT, TTG-TCA and GAT-TTG codon pairs. ATA-GAA and CGT-CCG codon pairs had the highest codon context and both were rarely used codons among their respective synonymous codons. Constructs HO-CP-DEOPTI, and HO-CP-OPTI were constructed based on the information of codon context (recoded constructs are given in supplementary information 1 and evaluated for gene expression modulation and results are given in below section.

Evaluation of constructs for their expression

The energy stored in mRNA in the form of MFE, codon bias, and tRNA translation efficiency are the parameters determining the gene expression level. Here two constructs HO-C-DEOPTI and HO-CP-DEOPTI have been evaluated for reduced gene expression, while HO-C-OPTI and HO-CP-OPTI were evaluated for increased gene expression based on MFE, CAI and tRNA translation efficiency.

HO-C-DEOPTI has highest while HO-C-OPTI has lowest MFE

MFE is shown to have an inverse relation with mRNA stability, and more negative values have higher stabilities. Furthermore, significantly higher RNA stabilities are linked with highly expressed genes[29]. The MEF of native, HO-C-DEOPTI, HO-C-

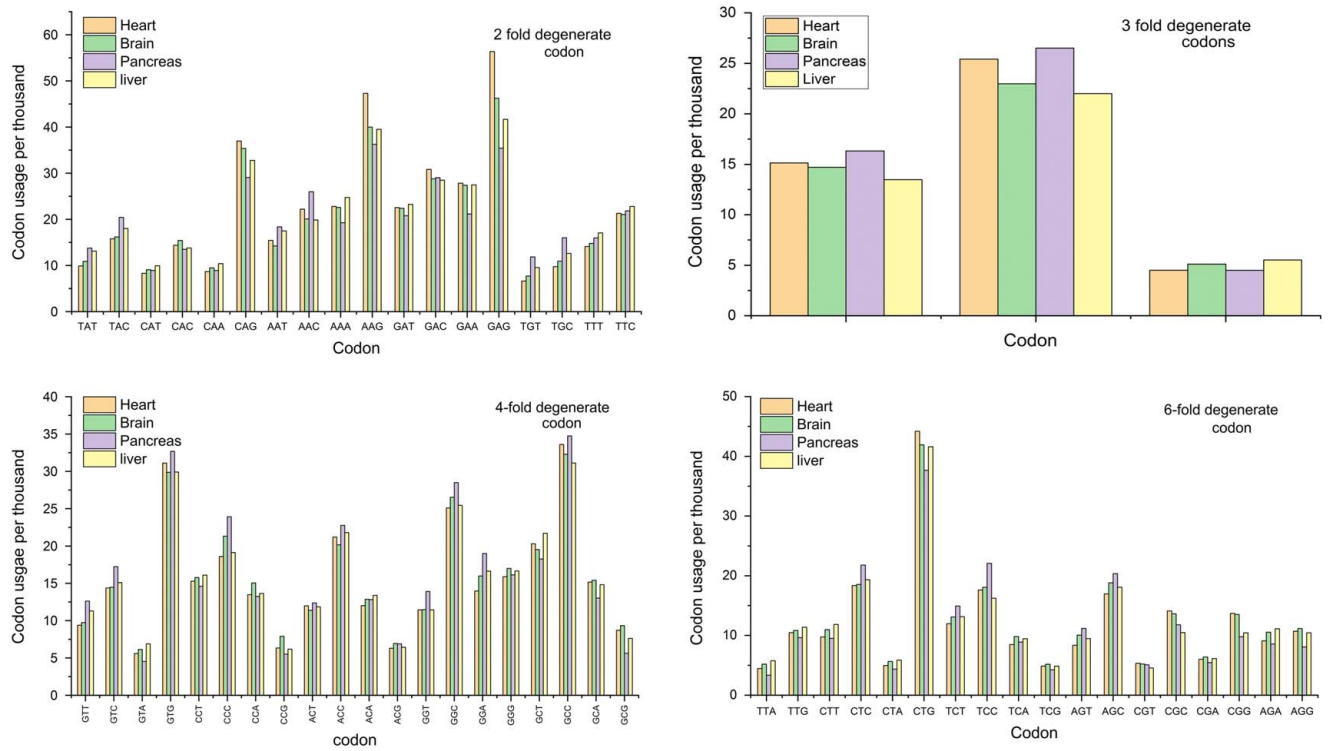

Figure 1. Depiction of codon usage per thousand for two-fold, three-fold, four-fold and six-fold degenerated codons in haem oxygenase-1 gene for heart, brain, pancreas, and liver tissues.

OPTI, HO-CP-DEOPTI and HO-CP-OPTI were – 335.7, – 296.5, – 350.1, – 343.2 and – 346, respectively (Fig. 3).

From the results, it is evident that compared to the native HO-1 sequence, HO-C-DEOPTI construct has the highest MFE and shows low stability and, therefore, low expression. On the other hand, HO-C-OPTI exhibited the highest negative MFE, representing maximum expression.

The gene expression of the HO-1 gene was highest in liver based on CAI value

CAI is an index used to measure expression level, and CAI values are predictive of real expression, which has been proved by experimental data[36]. Here we also used CAI values as a predictor of gene expression in different tissues. The CAI value of HO-1 sequence is 0.825, 0.825, 0.809, and 0.834 for the heart, brain, pancreas and liver, respectively. Expression is highest in liver tissue and equal in the heart and brain for native construct. Average CAI for native HO_1 sequences was 0.823, while it was 0.633, 0.842, 0.802 and 0.848 for HO-C-DEOPTI, HO-C-OPTI, HO-CP-DEOPTI and HO-CP-OPTI, respectively. Tukey's test was performed for the CAI values of different constructs, and the log of p value was determined and presented in Fig. 4. The results show that for the HO-C-DEOPTI construct, the gene expression is significantly ($Q = 28.22$, $P < 0.001$) lower than the native construct.

On the other hand, in HO-CP-OPTI, the expression was higher compared to the native construct though not significant ($Q = 3.68$, $P = 0.12$). Overall the results obtained from CAI analysis indicated that for reducing gene expression, codon deoptimization, while for increasing gene expression, codon pair optimization is an effective strategy.

Translation efficiency is lowest for HO-C-DEOPTI

The differences in the expression profile are partially due to differences in GC content, expression levels, or protein lengths[37] and are majorly attributed to the locally available tRNA pool. The translation is a non-uniform rate process and the presence of tRNA copies in the cell where transcripts are being translated affects the translation speed[38]. The translation efficacy also determined the overall protein production because protein expression is altered according to the functional mRNA half-life[39]. With higher translation efficacy, more significant amounts of protein will be produced before completing the functional mRNA half-life. Considering this, we tested our constructs for translation efficiency to modulate the protein expression.

The used algorithm ExtRamp walks over codon by codon, and matches the accompanying tAI to each codon, and generates a list of codon efficiencies. The Codon efficiencies of different constructs are depicted in Fig. 5. Translation efficiency we presented as an arithmetic mean of codon efficiency. From the figure, it is evident that the codon-deoptimized construct HO-C-DEOPT has the least translation efficiency (0.588) and many bottleneck areas where the rate of translation is low are evident. Thus for reducing the protein expression, HO-C-DEOPTI may be used.

On the other hand, for the enhanced expression, HO-CP-OPTI is helpful with the highest translation efficiency (0.879). Here translation efficiency of both HO-C-OPTI and HO-CP-OPTI are very close to each other (0.874 and 0.879, respectively, for HO-C-OPTI and HO-CP-OPTI). However, from the figures, it is evident that the initial 5' ramp with lower efficiency, which is required for proper folding of the protein, is maintained in the

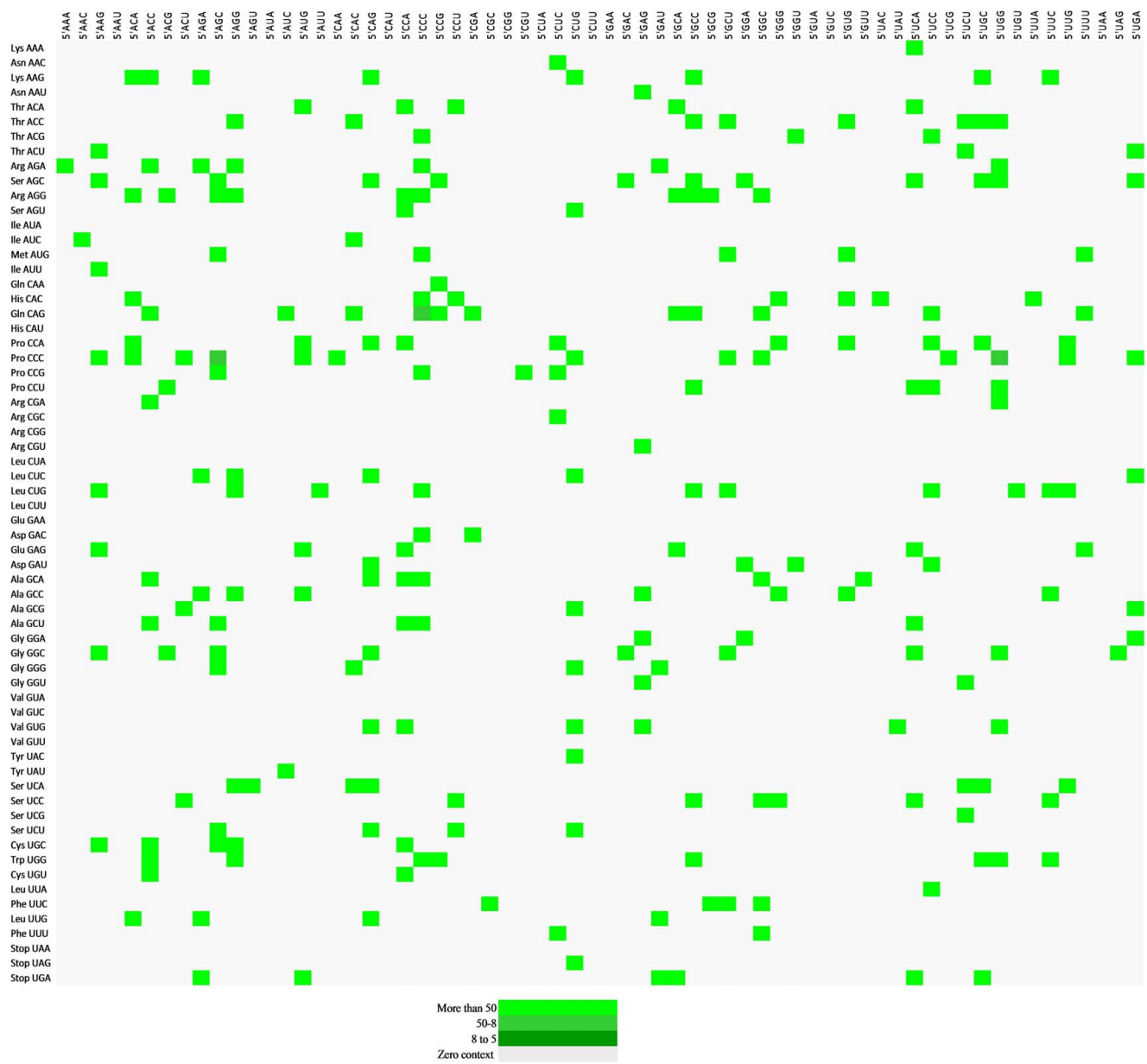

Figure 2. Codon context analysis for haem oxygenase-1 gene showed presence of only positive codon context.

HO-CP-OPTI construct despite high translation efficacy. The slow "ramp" present at 5' end of mRNA forms an optimal and robust means to reduce ribosomal traffic jams, thus minimizing the cost of protein expression[40]. The Maintenance of 5' slow ramp in HO-CP-OPTI construct makes this construct superior to HO-CP-OPTI for high expression of HO-1 protein.

Differences in codon efficiency are high in the case of HO-C-DEOPTI and HO-CP-OPTI for reduced and enhanced expression, respectively.

Codon efficiencies of constructs HO-C-DEOPTI, HO-C-OPTI, HO-CP-DEOPTI and HO-CP-OPTI were calculated and pair-wise compared with the native sequence. The log p values are depicted in Fig. 6. Maximum reduction in translation efficiency compared to native sequence was found with codon-deoptimized construct HO-C-DEOPTI ($Q = 201.7$, $P = 0$) while maximum increment in expression was obtained with HO-CP-OPTI ($Q = 14.92$, $P < 0.0001$). The results suggested that HO-CP-OPTI is suitable for enhancing while HO-C-DEOPTI is for decreasing the expression.

Comparison of translation efficiency between tissues revealed translation efficacy is least for HO-C-DEOPTI

Codon usage bias and resulting expression are varied among different tissues owing to local tRNA pools and tissue-specific RNA binding proteins[37,41]. The results of the investigation for differences in translation efficiency of different constructs at the tissue level are given in Fig. 7. The results suggested a

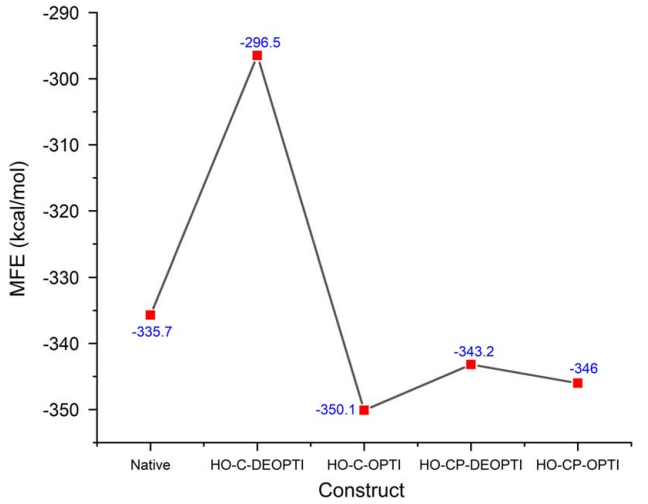

Figure 3. Mimimum free energy for constructs with codon and codon pair deoptimization and optimization, respectively. MFE, mimimum free energy.

very high statistically significant difference in codon efficiency in HO-C-DEOPTI construct and native construct ($P < 0.001$). Furthermore, a statistically significant difference was observed between the native sequence and HO-CP-DEOPTI and HO-CP-OPTI constructs' codon efficiencies for all tissues ($P < 0.05$). The results are suggestive that for codon pairs, (codon pair deoptimized or optimized), the codon efficiency is significantly different in the native sequence and the recoded constructs in all tissues. In contrast, only codon-deoptimized construct demonstrated highly significant changes in codon efficiencies compared to native sequence in all tissues. At the same time, the difference was not significant in the codon-optimized HO-C-OPTI construct for all tissues. In summary, the effects of codon or codon pair optimization/deoptimization are different in different tissues and the results are most pronounced in the codon-deoptimized construct.

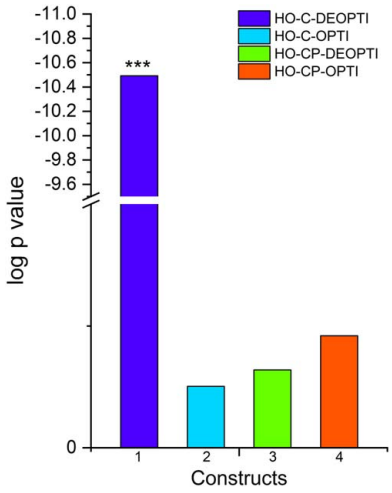

Figure 4. Depiction of log *p* values for comparative analysis of codon adaptation index between constructs.

Comparative analysis of CAI and average codon efficiency revealed high correlation with CAI

We did a correlation analysis between the two and found a very high statistically significant positive correlation between CAI and translation efficiency ($r = 0.985$, $P < 0.0001$). The results show that expression is reduced in the case of codon-deoptimized construct. Table 1 encompasses the CAI and translation efficiency (tAI) of constructs in different tissues with reference to specific codon usage for constructs.

To reduce the gene's expression level, codon deoptimization is a suitable technique, while codon pair optimization strategy may be adapted for increasing protein expression. The HO-C-DEOPTI construct based on codon deoptimization had high MFE (– 296.5), low CAI (0.633), and low translation efficiency (0.588) compared to the native sequence. Therefore for a reduction in gene expression, HO-C-DEOPTI may be used.

Based on the results of MFE, CAI, and translation efficiency analysis, it is evident that highly negative MFE (– 343.2 kcal/mol), and highest CAI (0.85 ± 0.01), and the highest translation efficiency (0.87 ± 0.001) was observed for HO-CP-OPTI construct. Although both the CAI and translation efficiency was higher for HO-CP-OPTI, there was not much difference in CAI (0.848 and 0.842 for HO-CP-OPTI and HO-C-OPTI, respectively) and translation efficiency (0.879 and 0.874 for HO-CP-OPTI and HO-C-OPTI, respectively), still we considered HO-CP-OPTI better for enhancing HO-1 expression, where based on the codon efficiencies, a slower ramp at 5' end is observed. The 5' slow ramp is a feature of highly expressed genes[42] preventing detrimental ribosome collision-dependent abortion of protein synthesis and exerting positive effects on translation rates[43,44].

Discussion

The HO-1 gene is an Nrf2-regulated gene playing an imperative role in the functions of oxidative degradation of cellular haem to liberate free iron and biliverdin. Apart role in iron liberation, it exhibits antioxidant and anti-inflammatory functions. Apart from normal physiological roles, its aberrant expression is associated with ailments. For example, HO-1 overexpression in cancer cells promotes proliferation and survival and induces angiogenesis[45]. On the other hand, systemic overexpression of HO-1 gene improves heart function and inhibits aging-induced fibrogenesis, posing a heart-protective role[46]. With that, gene overexpression via pharmacological inducers or viral gene transfer has been reported to inhibit atherogenesis in hypercholesterolemic animal models[47]. Related to brain tissue, on the one hand, HO-1 overexpression protects neurons against oxidative stress-induced damage. On the other hand, its overexpression is linked with the loss of nigral dopaminergic neurons[48]. Overexpression of HO-1 mediated through recombinant adenovirus encoding for HO-1 protects from bacterial infection-mediated cerebral vascular inflammation[49].

HO-1 is found to be significantly overexpressed in pancreatic cancer, which often culminates in fatalities, and the enzyme imparts resistance to chemotherapy[50]. Overexpression of HO-1 is associated with reduced levels of pancreas and liver injury markers[51]. Its induction with hemin is revealed to prevent experimentally induced pancreatitis.

Based on the above facts, both overexpression and reduced expression may be used differently to ameliorate various

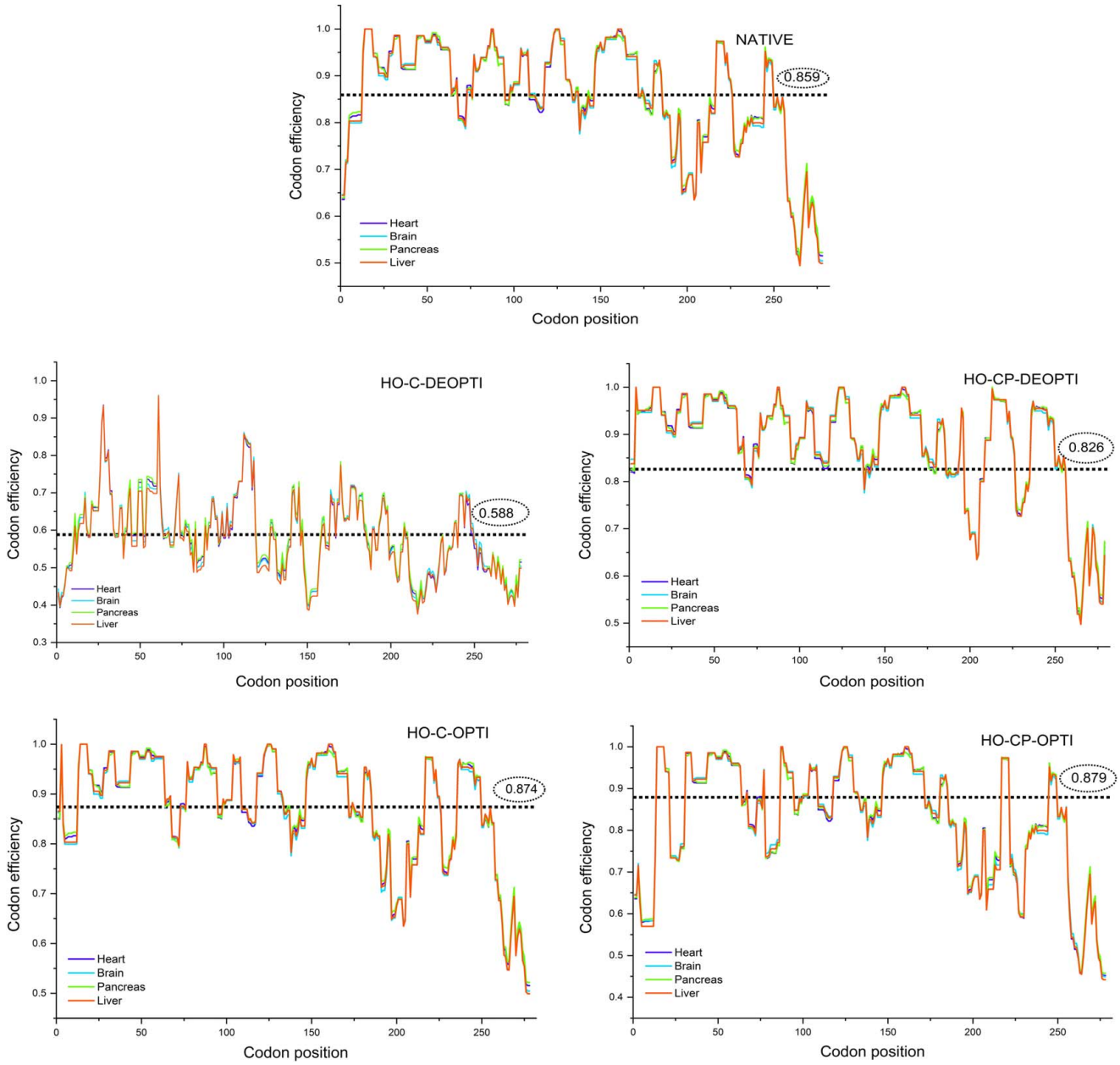

Figure 5. Depiction of codon efficiency of constructs in different tissues. Translation efficiency is shown as a line with its value.

physiological ailments of the heart, brain, pancreas and liver. In the present study, we designed a total of four constructs, two constructs each for enhancement and reduction in expression of HO-1. For high expression, codon and codon pair optimization was implicated, while codon and codon pair deoptimization was performed in recoded constructs for expression reduction. In the present study, we compared which strategy is more effective in modulating gene expression towards over or underexpression.

Codon usage bias[52] and codon context[53] affect gene expression. Codon optimization-based recoded mRNA vaccine candidates against SARS-CoV-2 have been shown enhanced expression[54]. An increase in CAI value is a frequently used strategy to increase the heterologous protein expression and codon optimization is reported to increase protein production by more than 1000 folds[55,56]. On the other hand, codon deoptimization in A24R gene of the vaccinia virus attenuated it and was used as a vaccine candidate[57]. Similar to codon bias, codon pair bias also affects the translation efficacy due to its impact on translation efficiency[58].

In the present study, we used codon and codon pair deoptimization and optimization techniques to generate constructs that may be used to reduce and enhance the protein expression of HO-1 gene, respectively. The protein expression level determination through system biology was based on three criteria; MFE, CAI, and translation efficiency.

MFE is the energy stored in secondary mRNA structures, higher MEF-containing transcripts have poor initiation[59], and lower MFE reflects a more robust RNA structure[60].

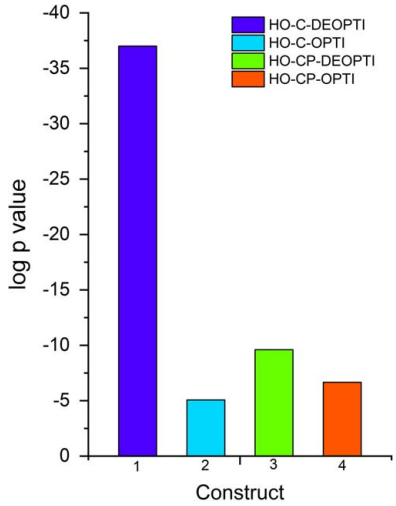

Figure 6. Depiction of log p values for comparative analysis for translation efficiency of different constructs with native haem oxygenase-1 sequence. Analysis revealed a significant difference between the native sequence and HO-C-DEOPTI for a reduction in translation. Similarly larger difference is observed between the native sequence and HO-CP-OPTI for enhancement.

Table 1

CAI and translation efficiency of heart, brain, pancreas, and liver with reference to tissue-specific codon usage for constructs

	Heart	Brain	Pancreas	Liver
Native				
CAI	0.825	0.825	0.809	0.834
TE	0.859 ± 0.12	0.857 ± 0.12	0.86 ± 0.11	0.858 ± 0.12
HO-C-DEOPTI				
CAI	0.629	0.647	0.603	0.655
TE	0.589 ± 0.1	0.589 ± 0.1	0.594 ± 0.1	0.581 ± 0.11
HO-C-OPTI				
CAI	0.842	0.844	0.832	0.851
TE	0.874 ± 0.11	0.871 ± 0.11	0.876 ± 0.11	0.873 ± 0.11
HO-CP-DEOPTI				
CAI	0.803	0.808	0.785	0.813
TE	0.826 ± 0.14	0.826 ± 0.14	0.827 ± 0.14	0.825 ± 0.14
HO-CP-OPTI				
CAI	0.851	0.852	0.837	0.852
TE	0.878 ± 0.11	0.8780.11	0.879 ± 0.11	0.879 ± 0.11

CAI, codon adaptation index; TE, translation efficiency.

Based on the results of MFE, the highest and low MFE was observed for HO-C-DEOPTI and HO-CP-OPTI construct, respectively, suggestive of these constructs as under-expressing and overexpressing constructs.

Further validation of our results was performed with CAI owing to a strong correlation between CAI and real expression level[36]. We predicted the CAI for native HO-1 sequence for the envisaged four tissues and found that expression is higher in the liver and equal in the heart and brain. There are reports where codon bias between tissue-specific gene have been demonstrated. For example, Plotkin and colleagues demonstrated characteristically different codon usage in the brain and liver-specific genes[21], and choice of that codon correlates with the average level of expression of the genes[61]. Therefore, we calculated CAI values for different tissue constructs based on different codon biases in different tissues. In the present study, based on the average CAI for envisaged all four tissues, we found minimum CAI values for the HO-C-DEOPTI construct (0.663) while the maximum for HO-CP-OPTI (0.848). Hence, HO-C-DEOPTI and HO-CP-OPTI constructs may be used for underexpression and overexpression, respectively, for the HO-1 gene.

Translation efficiency is the protein production rate per mRNA transcript in a cellular context[62] and tRNA population in any cell is an essential determinant of translation efficacy[40]. Translation

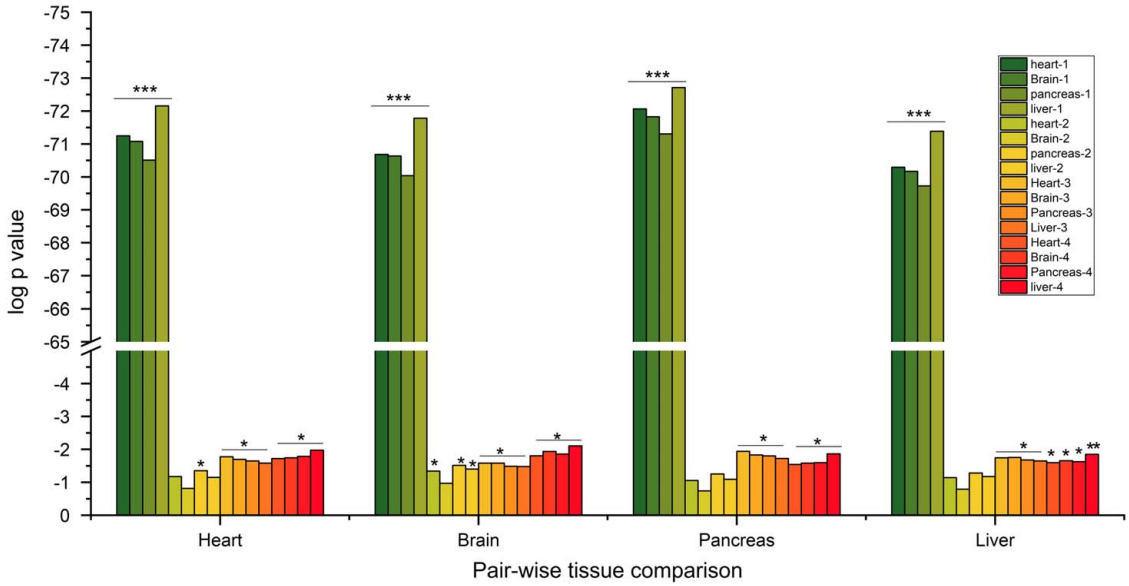

Figure 7. Demonstration of log *p* value from Mann–Whitney pairwise *t*-test between the codon efficiency of native sequence and four constructs in heart, brain, pancreas, and liver. Here (1) HO-C-DEOPTIconstruct; (2) HO-C-OPTI construct; (3) HO-CP-DEOPTI construct; (4) HO-CP-OPTI construct. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

efficiency was lowest and highest for HO-C-DEOPTI and HO-CP-OPTI, respectively, suggesting that for underexpression HO-C-DEOPTI and for overexpression HO-CP-OPTI construct may be used.

The results of all three criteria, which included MFE, CAI and codon efficacy were in concordance with each other and revealed that for reducing the expression level of HO-1 gene, HO-C-DEOPTI construct might be helpful. Similarly, for enhancing expression level, HO-CP-OPTI construct may be useful. Such codon optimization-based certain experiments are in clinical stages also. For example, encouraging clinical results were obtained in gene therapy trials with codon-optimized F8 cDNA in hemophiliaA patients, and circulating factor VIII activity of 52.3% was obtained[63]. In addition, codon-optimized human RPGR cDNA encoded by recombinant adeno-associated virus (rAAV) vector is also in the clinical trial to treat X-linked retinitis pigmentosa[64]. So far, no codon or codon pair deoptimization-based gene therapy clinical trial is going on. Still, these are in use for making vaccine candidates like Codon-Deoptimized RSV Vaccine Candidates are patented by Codagenix[65]. In future pharmaceutical companies are expected to launch such gene therapies also where high expression copy of the gene may be replaced with low expression copy using CRISPR-Cas or gene editing technologies.

In the present study, we did not include a few points that influence the gene expression level, but assessing these factors through a system biology approach is challenging. Such points are local structural constraints like the presence of large introns, which could be a brick on the accelerator[66] or may act for exon-mediated transcription enhancement[67] or other tissue-specific ribosomal binding proteins[68]. With that, we took no extra effort to sustain 5' ramp that is known to impact protein expression positively, but inherently the construct HO-CP-OPTI contained a slow ramp.

The constructs designed in our study will hopefully help curb the ailments like pancreatic cancer with reported HO-1 over-expression, where incorporation of HO-C-DEOPTI construct will reduce the expression level and thus prevent cancer cell proliferation. Likewise, enhancements in the expression levels of HO-1 through HO-CP-OPTI construct during pancreatitis or liver injuries will help improve the diseased condition.

Conclusion

In the present study, out of several methods to reduce or enhance gene expression, we used codon and codon pair deoptimization and optimization methods to under-express or overexpress the HO-1 gene. Our constructs were evaluated systemically based on MFE, CAI and translation efficacy, all suggestive of protein expression in quantitative terms. We found that the codon-deoptimized construct is useful for reducing gene expression. In contrast, the codon pair optimized construct was more valuable for enhancing gene expression.

Ethical approval

Present study does not require ethical approval. The data used in the study are publicly available from the databases like NCBI nucleotide and Genotype-Tissue Expression (GTEx) portal.

Consent

Not applicable.

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Author contribution

R.K.: study concept or design, data collection, data analysis or interpretation, writing and reviewing the paper, editing to give final shape. M.K.P., I.B., A.M.A., P.N., O.P.C.: study concept or design, data collection, data analysis or interpretation, writing and reviewing the paper. A.A.K.: study concept or design, data collection, data analysis or interpretation, writing and reviewing the paper, funding. P.G.: Statistical analysis, Study concept or design, data collection, data analysis or interpretation, writing and reviewing the paper.

Conflicts of interest disclosure

The authors declare no conflict of interest.

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Guarantor

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Available upon request.

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