with non-stochastic evolution of the antibody/protein inside of an optimized mammalian host cell, increases the likelihood of success and greatly accelerates the process for generating an optimized antibody/protein that will express at high enough levels in mammalian cells desired in manufacturing.

SUMMARY ~~OF THE INVENTION~~

[0005] The present disclosure provides methods of integrating therapeutic protein (including antibodies) generation and/or selection, evolution and expression in a eukaryotic host, such as a mammalian cell host or a yeast cell host, for manufacturing in a single system. Therapeutic proteins, including antibodies, are generated, optimized and manufactured in the same eukaryotic host system. The disclosed system of Comprehensive Integrated Antibody Optimization (CIAO!™) allows for simultaneous evolution of protein performance and expression optimization.

[0005a] In one aspect, there is provided a method of evolution and expression of an antibody in an eukaryotic cell production host, the method comprising: a. selecting a template antibody; b. evolving the template antibody to enable production of a set of mutant antibodies in the host using eukaryotic cell surface display of the mutant antibodies; c. screening the mutant antibodies for at least one predetermined property, characteristic or activity; d. selecting an up-mutant antibody from the set of mutant antibodies based upon optimization of the at least one predetermined property, characteristic or activity as compared to a same property, characteristic or activity of the template antibody; and e. expressing the up-mutant antibody in the same host as in step b.

[0006] In one embodiment the disclosure provides a method of selection, evolution and expression of an antibody in a mammalian cell production host; the method comprising generating an anti-antigen antibody library in a mammalian cell production host with antibody cell surface display; screening the library for at least one predetermined property, characteristic or activity; selecting a template antibody from the library; evolving the template antibody to produce a set of mutant antibodies in the mammalian cell production host with antibody cell surface display; screening the mutant antibodies for the at least one predetermined property, characteristic or activity; selecting an up-mutant antibody from the set of mutant antibodies based upon optimization of the at least one predetermined property, characteristic or activity when compared to the template antibody; and expressing the up-mutant antibody in the same mammalian cell production host as used in the generating step. In one embodiment~~aspect~~, the antigen is pre-selected. In another embodiment~~aspect~~, the anti-antigen antibody library is a humanized anti-antigen antibody library.

[0007] In one embodiment~~aspect~~, the mammalian cell production host is selected from 3T3 mouse fibroblast cells; BHK21 Syrian hamster fibroblast cells; MDCK, dog epithelial cells; Hela human epithelial cells; PtK1 rat kangaroo epithelial cells; SP2/0 mouse plasma cells; and NS0 mouse mouse plasma cells; HEK 293 human embryonic kidney cells; COS monkey kidney cells; CHO, CHO-S Chinese hamster

ovary cells; R1 mouse embryonic cells; E14.1 mouse embryonic cells; H1 human embryonic cells; H9 human embryonic cells; PER C.6, human embryonic cells; S. cerevisiae yeast cells; or picchia yeast cells. In a particular embodimentaspect, the mammalian system is CHO-S or HEK293. In one embodimentaspect, the screening steps utilize fluorescence-activated cell sorting (FACS).

[0008] In another embodimentaspect, the evolving step comprises producing a set of mutant antibodies formed from the template antibody having m complementary determining regions (CDR), wherein m is an integer selected from 1, 2, 3, 4, 5 or 6, each said CDR comprising n amino acid residues, the method comprising generating m x n separate sets of antibodies, each set comprising member antibodies having X number of different predetermined amino acid residues at a single predetermined position of the CDR; wherein each set of antibodies differs in the single predetermined position; and the number of different member antibodies generated is equivalent to m x n x X. In a particular embodimentaspect, m is 6.

[0009] In one embodimentaspect, the evolving step comprises generating n-1 separate sets of mutant polypeptides from the template antibody, each set comprising member polypeptides having X number of different predetermined amino acid residues at a single predetermined position of the polypeptide; wherein each set of polypeptides differs in the single predetermined position; and the number of different member polypeptides generated is equivalent to [n-1] x X. In one embodimentaspect, X represents the 19 naturally occurring amino acid residues not present in a given position of the template polypeptide.

[0010] In another embodimentaspect, the screening step comprises assaying each member polypeptide for at least one predetermined property, characteristic or activity; identifying any change in said property, characteristic or activity of the member polypeptide relative to the template polypeptide; creating a functional map wherein the functional map is used to identify positions and mutations in the mutant polypeptide which result in an up-mutant and/or a silent mutation compared to the template polypeptide.

[0011] In another embodimentaspect, the functional map is used to identify one or more of the group consisting of (a) positions and mutations which do not affect the activity of the mutant polypeptide compared to the template polypeptide; (b) fully mutable

sites compared to the template polypeptide; and (c) positions and mutations which result in an up-mutant compared to the template polypeptide.

[0012] In another embodimentaspeet, the antibody fragment is selected from a heavy chain, light chain, variable domain, constant domain, hypervariable region, complementarity determining region 1 (CDR1), complementarity determining region 2 (CDR2), and complementarity determining region 3 (CDR3).

[0013] In another embodimentaspeet, the generating step comprises subjecting a codon-containing polynucleotide encoding for said template polypeptide to polymerase-based amplification using a 64-fold degenerate oligonucleotide for each codon to be mutagenized, wherein each of said 64-fold degenerate oligonucleotides is comprised of a first homologous sequence and a degenerate N,N,N triplet sequence, so as to generate a set of progeny polynucleotides; and subjecting said set of progeny polynucleotides to clonal amplification such that polypeptides encoded by the progeny polynucleotides are expressed.

[0014] The method of claim 1 wherein the predetermined property, characteristic or activity is selected from reduction of protein-protein aggregation, enhancement of protein stability, increased protein solubility, introduction of glycosylation sites, introduction of conjugation sites, reduction of immunogenicity, enhancement of protein expression, increase in antigen affinity, decrease in antigen affinity, change in binding affinity, change in immunogenicity, or enhancement of specificity.

[0015] In another embodiment, the disclosure provides a method of evolution and expression of an antibody in a mammalian cell production host; the method comprising selecting a template antibody; evolving the template antibody to produce a set of mutant antibodies in a mammalian cell production host with antibody cell surface display; screening the mutant antibodies for the at least one predetermined property, characteristic or activity; selecting an up-mutant antibody from the set of mutant antibodies based upon optimization of the at least one predetermined property, characteristic or activity compared to the template antibody; and expressing the up-mutant antibody in the same mammalian cell production host used in the evolving step.

[0016] In one embodimentaspect, the screening step comprises creating a functional map wherein the functional map is used to identify positions and mutations in the mutant polypeptide which result in an up-mutant and/or a silent mutation compared to the template polypeptide. In another embodimentaspect, the screening steps comprise fluorescence-activated cell sorting (FACS).

[0017] In one embodimentaspect, the mammalian cell production host is selected from 3T3 mouse fibroblast cells; BHK21 Syrian hamster fibroblast cells; MDCK, dog epithelial cells; Hela human epithelial cells; PtK1 rat kangaroo epithelial cells; SP2/0 mouse plasma cells; and NS0 mouse mouse plasma cells; HEK 293 human embryonic kidney cells; COS monkey kidney cells; CHO, CHO-S Chinese hamster ovary cells; R1 mouse embryonic cells; E14.1 mouse embryonic cells; H1 human embryonic cells; H9 human embryonic cells; and PER C.6, human embryonic cells. In a particular embodimentaspect, the mammalian system is CHO-S or HEK293. In another embodimentaspect, the cell production host is selected from CHOK1SV or NSO host cell lines.

[0018] In another embodiment, the disclosure provides a method of evolution and expression of a protein in a eukaryotic cell production host; the method comprising selecting a template antibody; evolving the template antibody to produce a set of mutant antibodies in a eukaryotic cell production host; screening the mutant antibodies for at least one predetermined property, characteristic or activity; selecting an up-mutant antibody from the set of mutant antibodies based upon optimization of the at least one predetermined property, characteristic or activity compared to the template antibody; and expressing the up-mutant antibody in the same eukaryotic cell production host as in the evolving step for any commercial scale.

[0019] In one embodiment, the disclosure provides a method of evolution and manufacturing of a human protein in a cell production host; the method comprising generating a human protein library in a cell production host selected from one of the group consisting of a bacterial or eukaryotic production host; screening the library for at least one predetermined property, characteristic or activity; selecting a template human protein from the library based upon the at least one predetermined property, characteristic or activity; evolving the template human protein to produce a set of mutant human proteins in the cell production host; screening the set of mutant

human proteins for the at least one predetermined property, characteristic or activity and screening for modified expression; selecting an up-mutant human protein from the set of mutant human proteins based upon (1) optimization of the at least one predetermined property, characteristic or activity and (2) modified expression when compared to the template human protein; and manufacturing the human protein comprising expressing the up-mutant human protein in the same production host as in the generating step. In one embodimentaspect, the modified expression in the selecting step is improved expression.

[0020] In another embodimentaspect, the evolving step comprises an evolution technique selected from one of comprehensive positional evolution (CPE); comprehensive positional insertion evolution (CPI); comprehensive positional deletion evolution (CPD); comprehensive positional evolution (CPE) followed by combinatorial protein synthesis (CPS); comprehensive positional deletion evolution (CPD) followed by combinatorial protein synthesis (CPS); or comprehensive positional deletion evolution (CPD) followed by combinatorial protein synthesis (CPS).

[0021] In one embodimentaspect, the human protein is an antibody. In another embodimentaspect, the antibody is a full length antibody.

[0022] In another embodimentaspect, the at least one predetermined property, characteristic or activity in screening step (e) comprises one or more of (1) screening for a silent mutation and (2) screening for a missense mutation; compared to the template antibody.

[0023] In another embodimentaspect, one or more portions of the antibody selected from Fc and Fv; framework; and one or more CDRs are modified in the up-mutant human antibody compared to the template human antibody is evolved.

[0024] In another embodimentaspect, the screening step comprises screening the set of mutant human proteins for the at least one predetermined property, characteristic or activity and screening for modified expression simultaneously.

[0025] In a further embodimentaspect, the human protein for evolution and manufacturing is selected from an enzyme cytokine, receptor, DNA binding protein, chelating agent or hormone.

[0026] In another embodimentaspect, the cell production host is a eukaryotic production host and the evolving step comprises evolving the template human protein to produce a set of mutant human proteins in the eukaryotic cell production host with cell surface display.

[0027] In another embodiment, the disclosure provides a method of evolution for enhanced expression and manufacturing of a human protein in a eukaryotic cell production host; the method comprising selecting a template human protein for evolution; evolving the template human protein comprising generation of mutant codons encoding the template human protein to produce a set of mutant human proteins in the production host; screening the set of mutant human proteins for at least one predetermined property, characteristic or activity and screening for enhanced expression when compared to the template human protein; selecting an up-mutant human protein from the set of mutant human proteins based upon (1) retention or optimization of the at least one predetermined property, characteristic or activity and (2) enhanced expression when compared to the template human protein; and manufacturing the up-mutant human protein comprising expressing the up-mutant human protein in the same production host as in the evolving step.

[0028] In one embodimentaspect, the mutant codons of the up-mutant human protein result in at least one silent mutation and/or missense mutation. In another embodimentaspect, the mutant codons of the up-mutant human protein result in at least one silent mutation.

[0029] In a further embodimentaspect, the template human protein is an approved ethical protein therapeutic drug, and the up-mutant human protein is a biosimilar.

[0030] In another embodimentaspect, the selecting step comprises selecting an up-mutant human protein from the set of mutant human proteins based upon (1) optimization of the at least one predetermined property, characteristic or activity and (2) enhanced expression when compared to the template human protein.

[0031] In another embodiment, the disclosure provides a method of identifying and producing a target human protein, the method comprising generating an human protein library in a eukaryotic cell production host with protein cell surface display; screening the library for at least one predetermined property, characteristic or activity; identifying a target human protein from the library based upon the at least one predetermined property, characteristic or activity; and expressing the target

human protein in the same eukaryotic cell production host as in the generating step to produce a target human protein. In one embodimentaspect, the target human protein is an antibody. In another embodimentaspect, the antibody is a full length antibody.

[0032] In another embodiment, the disclosure provides a method of evolution of a human protein in a manufacturing host, the method comprising mutating a template human protein to produce a set of mutant human proteins in a manufacturing host; and screening the set of mutant progeny proteins for at least one predetermined property, characteristic or activity. In one embodimentaspect, the method further comprises selecting an up-mutant human protein from the set of mutant human proteins based upon the at least one predetermined property, characteristic or activity. In another embodimentaspect, the method further comprises manufacturing the up-mutant human protein comprising expressing the up-mutant human protein in the same production host as in the mutating step. In another embodimentaspect, the selecting step further comprises selecting an up-mutant human protein from the set of mutant human proteins based upon (1) optimization of the at least one predetermined property, characteristic or activity when compared to the template human protein, and (2) modified expression when compared to the template human protein. In one embodimentaspect, the modified expression is enhanced expression.

[0033] In another embodiment, the disclosure provides a method of evolution and manufacturing of a human protein in a cell manufacturing host; the method comprising mutating a template human protein to produce a set of mutant human proteins in a manufacturing host; screening the set of mutant human proteins for at least one predetermined property, characteristic or activity and screening for modified expression;

selecting an up-mutant human protein from the set of mutant human proteins based upon (1) optimization of the at least one predetermined property, characteristic or activity, and (2) modified expression when compared to the template human protein; and manufacturing the human protein comprising expressing the up-mutant human protein in the same manufacturing host as in the mutating step.

[0034] In a further embodiment, the disclosure provides a method of evolution for enhanced expression and manufacturing of a human protein in a eukaryotic cell production host; the method comprising mutating a template human protein

comprising generation of mutant codons encoding the template human protein to produce a set of mutant human proteins in a manufacturing host; screening the set of mutant human proteins for at least one predetermined property, characteristic or activity and screening for enhanced expression when compared to the template human protein; selecting an up-mutant human protein from the set of mutant human proteins based upon (1) retention or optimization of the at least one predetermined property, characteristic or activity and (2) enhanced expression when compared to the template human protein; and manufacturing the up-mutant human protein in the same manufacturing host as in the mutating step.

[0035] In one embodimentaspeet, the screening step comprises screening the set of mutant human proteins for the at least one predetermined property, characteristic or activity and screening for enhanced expression simultaneously.

BRIEF DESCRIPTION OF THE DRAWINGS

[0036] Figure 1 provides a schematic of the CIAO!™ method of integrating therapeutic protein (e.g., antibody) generation and/or selection, evolution and expression in a eukaryotic host, such as a mammalian cell host or a yeast cell host, for manufacturing in a single system.

[0037] Figure 2 illustrates how comprehensive positional evolution (CPE™) is used to generate a molecule specific database (EvoMap™).

[0038] Figure 3 shows a schematic of Comprehensive Positional Synthesis (CPS™) which can be used to combine up-mutants from CPE™.

[0039] Figure 4 shows a schematic of Comprehensive Positional Insertion (CPI™) evolution.

[0040] Figure 5 illustrates one combination of evolution methods: a lengthened nucleic acid from CPI™ evolution is subjected to Comprehensive Positional Evolution (CPE™) and used to generate a molecule specific database (EvoMap™).

[0041] Figure 6 shows a schematic of Comprehensive Positional Deletion (CPD™) evolution.

[0042] Figure 7 illustrates another combination of evolution methods: a shortened nucleic acid from CPD™ evolution is subjected to Comprehensive