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Advanced Stoichiometric Analysis of Metabolic Networks of Mammalian Systems

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ABSTRACT: Metabolic engineering tools have been widely applied to living organisms to gain a comprehensive understanding about cellular networks and to improve cellular properties. Metabolic flux analysis (MFA), flux balance analysis (FBA), and metabolic pathway analysis (MPA) are among the most popular tools in stoichiometric network analysis. Although application of these tools into well-known microbial systems is extensive in the literature, various barriers prevent them from being utilized in mammalian cells. Limited experimental data, complex regulatory mechanisms, and the requirement of more complex nutrient media are some major obstacles in mammalian cell systems. However, mammalian cells have been used to produce therapeutic proteins, to characterize disease states or related abnormal metabolic conditions, and to analyze the toxicological effects of some medicinally important drugs. Therefore, there is a growing need for extending metabolic engineering principles to mammalian cells in order to understand their underlying metabolic functions. In this review article, advanced metabolic engineering tools developed for stoichiometric analysis including MFA, FBA, and MPA are described. Applications of these tools in mammalian cells are discussed in detail, and the challenges and opportunities are highlighted.

KEY WORDS: Metabolic flux analysis, flux balance analysis, metabolic pathway analysis, mammalian cells.

I. INTRODUCTION

Mammalian cells have been extensively used to produce antibodies, hormones, and other pharmaceutical vaccines and proteins because microorganisms do not have adequate mechanisms for post-translational modifications required for medicinally important proteins that should have proper functions.[1] They have been also used in *in vitro* experiments to elucidate toxic effects of drugs and metabolic changes caused by different abnormal metabolic conditions. Lately, isolated whole organs have been increasingly studied to understand the metabolic patterns of different physiological states caused by various diseases or injuries.[2] These experiments performed at the cell and/or organ level are essential for functional characterization to improve the functions of existing systems (such as bioartificial liver).

Recently, metabolic engineering approaches developed for stoichiometric network analysis (Figure 1) have been also applied to mammalian systems to gain a comprehensive understanding of metabolic network properties and to improve the existing processes or systems involving mammalian cells. These approaches are generally used (a) to reconstruct the metabolic networks,[3] (b) to identify metabolic flux patterns using metabolic flux analysis (MFA)[1,4–8] and flux balance analysis (FBA),[9,10] and (c) to characterize the topology of the networks using pathway analysis[11,12] and other optimization-based methods. Table 1 presents the existing bibliography organized according to the system and the method used.

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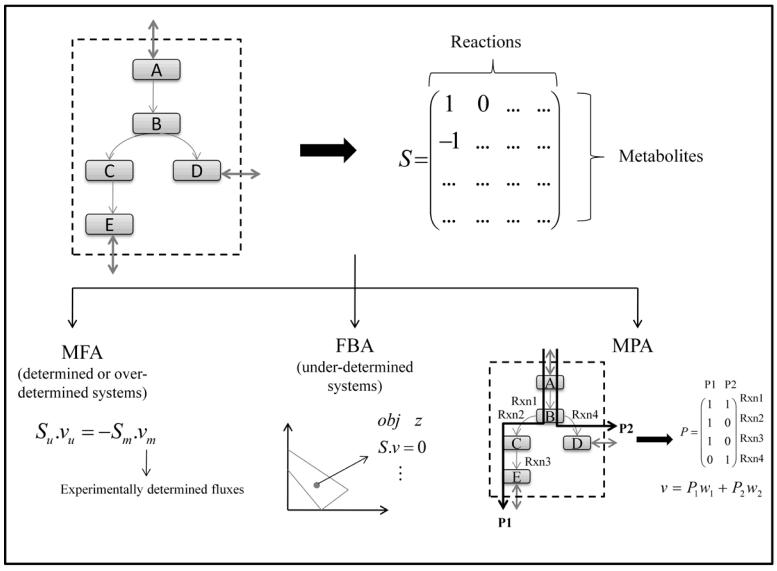

FIGURE 1: Stoichiometric analysis of metabolic networks. For determined or over-determined systems, the unknown fluxes ($\mathbf{v}_u$) are simply calculated using the stoichiometric matrix (**S**) and measured fluxes ($\mathbf{v}_m$). For underdetermined systems, FBA or MPA where an optimization programming is formulated is used to calculate the unknown fluxes. A network can be described by elementary modes or extreme pathways [given in a matrix form (**P**)] with their corresponding weight values (**w**).

In this review article, we mainly focus on the basic techniques and principles of how stoichiometric network analysis is used for mammalian systems, and we comprehensively explain applications and major outcomes of these approaches. We also discuss current challenges faced while applying these techniques.

II. PRINCIPLES OF STOICHIOMETRIC NETWORK ANALYSIS

A. Metabolic Network Reconstruction

Systems biology tools integrating multiple reaction rates to analyze the network and cell behaviors have been applied for mammalian cell types since the early 1990s. Although microbial metabolic networks have been well characterized, applications of metabolic engineering tools for more complex systems have been gaining importance, which is required for cellular physiology and topological characterization. These models provide not only a better understanding of cellular functions but also a detailed description of cellular components and interactions among these components. As discussed in detail in subsequent sections, constructing a metabolic network and application of metabolic network analyses for the complex mammalian systems have provided various advantages, such as characterization of cellular physiology including drug metabolism and understanding the effects of environmental perturbations or inherent disorderedness on the cellular metabolism. To understand the biology of mammalian systems at the system level, first the structure of cellular functions such as biochemical pathways should be well described. This enables us to understand how biochemical networks behave under different conditions using metabolic network principles and experimental data. This is critical for identifying important targets (especially therapeutic

TABLE 1. STUDIES ANALYZING THE METABOLIC NETWORKS OF MAMMALIAN SYSTEMS USING DIFFERENT STOICHIOMETRICALLY BASED APPROACHES.

Cell lines /organs	Method	References
Hybridoma cell line	MFA	[55–60] [54, 147] [88], [53], [49], [52], [51]
	MFA-^{13}C	[154], [50, 92]
	FBA	[92], [109, 110]
CHO cell line	MFA	[44], [43]
	MFA-^{13}C	[47], [46]
	MPA	[45]
Liver (organ) or hepatocytes	MFA	[15, 21, 22] [16, 17] [18, 19], [85] [27], [14], [87]
	MFA-^{13}C	[143], [93, 94] [23, 107], [24]
	FBA	[25, 26], [20] [104], [116] [117, 118], [119]
	MPA	[13]
Red blood cell	FBA	[113]
	MPA	[121, 129] [127], [128]
MDCK cell line	MFA	[41]
	FBA	[42, 111]
Fibroblast cells	MFA	[86]
	MFA-^{13}C	[96]
Skeletal muscle	MFA	[63]
	FBA	[112]
C6 glioma	MFA-^{13}C	[61]
Lung carcinoma cells	MFA-^{13}C	[95]
Adipocytes	MFA	[144–146]
Rat embryonic brain cells	MFA-^{13}C	[97]
Cardiac systems	MFA-^{13}C	[32], [31], [30] [35], [34]
	FBA	[33]

CHO = Chinese hamster ovary; MDCK = Madin-Darby canine kidney; MFA = metabolic flux analysis; MFA-^{13}C = metabolic flux analysis-carbon labeling; FBA = flux balance analysis ; MPA = metabolic pathway analysis.

targets) that can be modified to eliminate the undesired effects of environmental or inherent factors. For example, in our recent study,[13] hepatic metabolic pathways were characterized using elementary mode analysis and data sets obtained from perfused livers of fasted rats receiving burn injury. A set of important metabolites or potential nutrients including glutamine, arginine, and aspartate were identified, which might be utilized to manipulate biochemical environment to reduce physiologic stress caused by burn injury.

The metabolism of the specific cell type being studied is constructed based on the knowledge of its genetics and the experimental conditions used for cell-culturing processes. In general, similar reactions or pathways such glycolysis, gluconeogenesis, TCA cycle, and amino acid metabolism were considered in the studies where mammalian cells were investigated. The most commonly used mammalian cell lines in literature are liver,[14–28] hearth,[29–40] Madin-Darby canine kidney (MDCK),[41,42] Chinese hamster ovary (CHO),[43–47] and hybridoma cells (Table 1).[48–60] Portais et al.[61] used a rat tumor cell line, the C6 glioma, and analyzed metabolic fluxes of glycolysis, gluconeogenesis, the citric acid cycle, and a number of reactions corresponding to protein or fatty acid metabolism. Xie and Wang[55–60] studied the central carbon metabolism and energy metabolism of a mouse hybridoma cell line. Lee et al.[16] presented a hepatic metabolic network, including all major pathways of central carbon and nitrogen metabolism, which is expected to be active in the perfused rat livers. The same group experimentally validated the flux estimates by directly measuring pentose phosphate pathway (PPP) fluxes using radioisotope tracing.[16] They also demonstrated that application of the mass balance technique in the metabolic network of livers of fasted rats showed good consistency between the measurements under the assumed biochemistry of perfused liver model.[17] Sepulveda et al.[62] used MFA to characterize the central carbon metabolism of mouse embryonic stem cells, which were differentiated on the surfaces coated with either gelatin or matrigel or by inducing embryoid body formation. Although they identified notable changes in metabolic fluxes between

days 4 and 5 of differentiation, they used a relatively small-scale network, including 15 reactions that described glycolysis, the TCA cycle, oxidative phosphorylation, and glutamine metabolism. Wahl et al.[42] derived a metabolic model for MDCK cells. Two compartments connected with transport reactions, the cytosol and mitochondria, were considered. The developed metabolic model is ''medium'' sized but includes all major reactions of the central carbon metabolism and furthermore considers the transport mechanisms, especially co-transports that are energy-coupled.

The metabolic networks constructed for mammalian cells mentioned above are relatively small-scale networks given the limited experimental measurements and the lack of knowledge regarding the cell physiology. In general, the main reactions or pathways that are considered the most important in terms of the metabolic fluxes have been included, whereas the mechanisms of transport processes have been neglected due to the difficulty of assessing the amounts of material entering via passive and active processes. Because in some instances the energy balance has not been included in the models, the mechanism of the transport reactions might be irrelevant. Moreover, in most studies, it was assumed that there is a single pool of intracellular metabolites; thus intracellular rates represent an average for the whole cell.[14–17,63] This assumption is based on the fact that several metabolites are able to cross the mitochondrial membrane, either directly or via effective shuttle systems, and thus equilibrate between the cytoplasmic and mitochondrial spaces.[15]

Much progress has been made in recent years in the study of large-scale networks. Many metabolic genes and enzymes being individually studied for a long period of time resulted in collective knowledge that facilitates the construction of more comprehensive metabolic networks. The process of metabolic reconstruction requires gathering a variety of genomic, biochemical, and physiological data from the primary literature as well as databases[3] such as Uniprot,[64] BRENDA,[65] BioCyc,[66] KEGG,[67] and the Enzyme Commission database.[68] Based on extensive evaluations of these sources, some generic human metabolic models have been published more recently.[69–72] Duarte et al.[69] manually reconstructed a global human metabolic network (Recon 1) that accounts for functions of 1496 ORFs, 2004 proteins, 2766 metabolites, and 3311 metabolic and transport reactions. The rigorous computational testing of network functionality was performed by means of constraint-based FBA. They further analyzed the potential clinical utility of the model to elucidate a set of reactions related to hemolytic anemia and potential drug targets treating hypercholesterolemia.

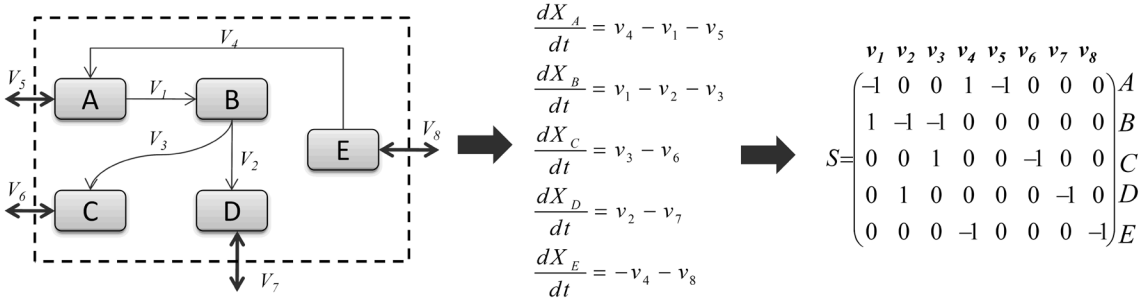

FIGURE 2: A small metabolic network. The network includes four internal reactions (v_1, v_2, v_3, and v_4) and four external reactions (v_5, v_6, v_7 and v_8), which are given as "output" fluxes. The stoichiometric matrix (***S***) is constructed according to material balance equations.

Shlomi et al.[73] studied a computational method by integrating the generic human model with tissue-specific gene and protein expression data to predict 10 different tissue-specific metabolic networks including brain, liver, and kidney. They also used the generic human metabolic model to predict metabolic biomarkers caused by human inborn genomic mutations.[74] Jerby et al.[75] proposed an algorithm generating a tissue-specific model from the generic human model by integrating different tissue-specific data sources such as literature-based knowledge as well as transcriptomic, proteomic, metabolomic, and phenotypic data. They constructed a genome-scale hepatic model and investigated its ability to carry out hepatic metabolic functions. Using a global human metabolic network and manually evaluating the articles, reviews, and biochemical textbooks, Gille et al.[76] presented a comprehensive metabolic network of the human hepatocyte consisting of 777 metabolites in six intracellular and two extracellular compartments and 2539 reactions, including 1466 transport reactions.

For two other mammals, cattle,[77] and mice,[78–80] a central metabolic reconstruction has been also developed. Sheihk et al. characterized a generic metabolic network for *Mus musculus* (mouse)[81] for modeling the hybridoma cell metabolism. The reaction list consists of 872 internal metabolites and 1220 reactions in two different compartments, cytosol and mitochondria. Similarly, Quek and Nielsen[5] based on their published mouse model,[79] developed a medium-size generic mammalian cell network for central carbon metabolism containing 272 reactions and 228 metabolites after certain simplifications and removing the singleton metabolites (dead end). Selvarasu et al.[82] improved and expanded the previous generic model of *Mus musculus* by including additional information on gene–protein-reaction association, and they improved network connectivity through lipid, amino acid, carbohydrate, and nucleotide biosynthetic pathways. After examining the model predictability both quantitatively and qualitatively using constraint-based flux analysis, they investigated the structural and functional characteristics of the mouse metabolism by evaluating network statistics/centrality, gene/metabolite essentiality and their correlation. Sigurdsson et al.[83] mapped the published, detailed reconstruction of human metabolism (Recon 1) to other mammals, including mice. They finalized a functional model via iterative testing until it passed a predefined set of 260 validation FBA tests. The reconstruction is the largest mouse reconstruction to date, involving 2212 gene-associated reactions and 1514 non-gene-associated reactions. Moreover, they showed that the knockout simulation of the lipoprotein lipase gene correlated well with experimental results.

B. Metabolic Flux Analysis (MFA)

Stoichiometric modeling framework is mostly characterized by MFA where a set of measured extracellular metabolite concentration rates of change are fitted to relatively simple mass balance models using the stoichiometric mass balance analysis to derive comprehensive metabolic flux maps. Such quantitative maps have turned out to be extremely useful for comparing the effects of various stressors on metabolism, as they provide a global picture and understanding of the changes in relevant metabolic pathways. MFA has been extensively applied to characterize the physiology of different mammalian cell types (Table 1).[14–19,21,27,30,31,34,35,37,39,43,44,49,51–53,63,84–88] The methodology of MFA used in these studies is explained in the following section in detail.

The flux distribution is calculated using the basic idea of MFA.[89] The mass balances of all internal metabolites can be written as follows:

$$\frac{dX}{dt} = S.v \tag{1}$$

where X is vector of metabolite concentrations, v is the flux distribution vector, and S is the stoichiometric matrix, where rows correspond to the metabolites and columns represent the reaction rates (Figure 2). It is generally assumed that the internal metabolites are at a pseudo-steady state, because metabolic transients are rapid compared to environmental changes.[89] Therefore, the mass balance is rewritten as follows:

$$S.v = 0 \tag{2}$$

The measured fluxes are used to reduce the possible solution space given in Equation (2). Therefore, the vector of reaction v is divided into the vector of measured fluxes v_m, and unknown fluxes v_u. In the same way, the matrix **S** is partitioned in $\mathbf{S}_m$ and $\mathbf{S}_u$. So Equation (2) can be rewritten in the following way:

$$S.v = S_u.v_u + S_m.v_m = 0$$
$$S_u.v_u = -S_m.v_m \quad \textbf{(3)}$$

The rank of the $\mathbf{S}_u$ determines the maximum number of the metabolite balances that are linearly independent. If the number of equations is less than the number of unknown fluxes, the system is undetermined, implying that there are insufficient metabolite balances to determine the intracellular or unknown metabolic fluxes. For a determined system, where enough measurements are available, Equation (3) can be easily used to calculate the unknown fluxes uniquely. In overdetermined or redundant systems, the rank of $\mathbf{S}_u$ is greater than the number of unknown fluxes, which ensures statistical consistency of the measurements. To assess for the presence of measurement errors and consistency of the metabolic network, a test function that is generally assumed to have a χ^2 distribution is calculated. For an overdetermined system, unknown fluxes are usually identified by minimizing the sum of square errors between the measured and the estimated fluxes.

The null space of the stoichiometric matrix, *Nul (S) = {v:Sv=0}*, determines the basis for the null space whose column vectors can give actual flux distribution when combined linearly. This analysis is also used to perform consistency validations of the considered metabolic network.[3] The dimension of the null space of the stoichiometric matrix also identifies the minimum number of fluxes (independent variables) required to determine the unknown fluxes (dependent variables) uniquely. Independent reactions or fluxes should not be necessarily external fluxes, because determining all external fluxes may not necessarily result in the calculation of all the unknown fluxes; metabolic networks are very complex given the cyclic pathways or futile cycles (Figure 3). Therefore, to identify the flux distribution more accurately, fluxes of some internal reactions are measured. In this context, mass isotopomer analysis has been extensively used to quantify internal fluxes with substrates labeled with stable isotopes such as ^{13}C.

In general, metabolic stationary conditions with constant intracellular fluxes are established during the carbon-labeling experiments. Then, NMR and MS techniques are utilized to obtain intracellular isotopomer distribution. Isotope balancing is very similar to metabolite balancing, that is, the sum of labeled carbon atoms entering a given position of a metabolite has to equal the sum of labeled carbon atoms leaving this position.[90] However, isotopomer balancing becomes very complicated when the fraction of molecules having a certain combination of labeled carbon atoms is considered. For a metabolite consisting of n atoms, which might be in labeled or

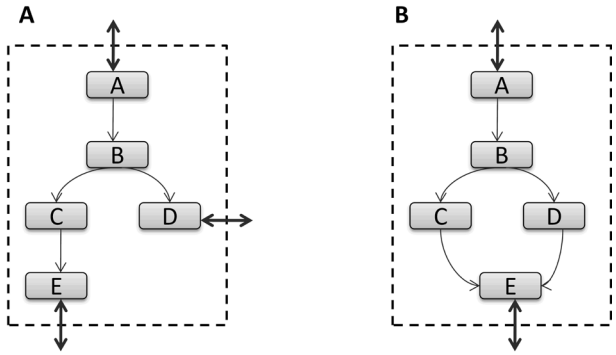

FIGURE 3: Null space of metabolic networks. The null spaces of these two simplified metabolic networks have the same dimension, which is equal to 2. This means that at least two independent variables or fluxes should be known in order to determine the flux distribution vector. Although any two external fluxes (represented by double arrows) in the metabolic network **A** can determine all unknown fluxes, those in metabolite **B** can not identify the flux distribution vector due to the cyclic reactions. This can be determined using either isotope tracers or expanding the model by adding the material balances of energy metabolites such as ATP, NADH if they are associated with cyclic reactions (however, this requires a complete metabolic network).

unlabeled states (e.g., C atom in glucose), 2^n isotopomers are possible. Using a ^{13}C tracer, there are 64 carbon atom isotopomers of glucose and 396 isotopomer models for simulating glucose labeling in the gluconeogenesis pathway.[91] The number of isotopomer equations increases tremendously when different combinations of stable isotopes (such as ^{13}C, ^{2}H, ^{18}O) are used. To determine metabolic fluxes, the residuals between the experimental and the simulated isotopomer distribution is generally minimized. This technique has been described extensively in the literature and used in mammalian systems to characterize the metabolic networks under different environmental conditions and to provide information on specific metabolic pathways or key reactions being investigated.[47,50,61,92–97] Goudar et al.[47] analyzed the physiological state of CHO cells in perfusion culture by determining fluxes through the bioreaction network using ^{13}C glucose and 2D-NMR spectroscopy. They determined the metabolic fluxes using the ^{13}C-Flux software package[98] by minimizing the residuals between the experimental and the simulated NMR data. Portais et al.[61] determined the specific enrichments of glutamate, aspartate, and alanine carbons from ^{13}C-, ^{1}H-NMR spectroscopy, or mass spectrometry data to calculate the metabolic fluxes in a rat tumor cell line. The flux values were similarly estimated by a least-squares fitting procedure. Munger et al.[96] developed a flux measurement approach based on liquid chromatography–tandem mass spectrometry to quantify changes in metabolic activity induced by human cytomegalovirus (HCMV). They constructed an ordinary differential equation model of central carbon metabolism. The model consisted of 69 differential equations, written to maintain flux balance. The model equations describe the rates of loss of unlabeled forms of metabolites and the creation of particular labeled forms after feeding [U-^{13}C]glucose or [U-^{13}C]glutamine media. Hiller et al.,[99] using a lung carcinoma cell line, proposed a novel approach, non-targeted tracer fate detection (NTFD), to detect all measurable metabolites derived from a specific labeled compound. The main part of their algorithm comparatively examines mass spectra of the same compound across all chromatograms to identify labeled fragments. Using the rat hearths perfused *in vitro* with 90% enriched [2-13C]acetate or [3-13C]pyruvate, Chance et al.[30] constructed mathematical flux model, including approximately 200 simultaneous differential equations for analyzing the citric acid cycle and ancillary transamination reactions. However, they observed that nonlinear least-squares fitting of the data to the model gave nonrandomly distributed residuals for the 13C fractional enrichments of glutamate C-4, suggesting an incomplete model. Des Rosiers et al.[35] compared gas chromatography-mass spectrometry (GCMS) and nuclear magnetic resonance (NMR)-determined ^{13}C-isotopomer and flux data obtained from *ex vivo* rat heart perfusion studies with ^{13}C-substrates. They found very good agreement between the ^{13}C-enrichments of GCMS- and NMR-determined citric acid cycle metabolites and glutamate.

Recently, new approaches have been developed to overcome computational difficulties and to reduce the size of the models used to derive steady-state flux distributions from labeling patterns.[91,100] Antoniewicz et al.[91] developed the concept of elementary metabolite units (EMUs). This framework is based on a decomposition algorithm that identifies the minimum amount of information needed to simulate isotopic labeling and describes the relationship between fluxes and isotopomer abundances. Using elementary metabolite unit-based method (EMU), Metallo et al.[95] estimated flux profiles and confidence intervals from simulated measurements in stationary MFA experiments in a tumor cell line. A scoring algorithm was then employed to determine the optimal tracer for the overall model and for subnetworks representing glycolysis, the PPP, and the tricarboxylic acid (TCA) cycle.

C. Flux Balance Analysis (FBA)

One of the common problems in solving Equation (3) to obtain $\mathbf{v}_u$ is that the rank of $\mathbf{S}_u$ is generally smaller than the number of unknown fluxes, which leads to an underdetermined system. Actually in most cases, the mass balance analysis typically does not yield a unique solution. To overcome this limitation, some metabolic engineering tools based on well-known biological properties have been used

in the literature. The solution space of steady-state flux vectors can be reduced by incorporating additional constraints relying on well-known regulatory mechanisms[101,102] or thermodynamic properties of biochemical reactions.[103,104] Using FBA,[105] it is also possible that a flux distribution can be uniquely determined when an objective function is defined such as maximization of biomass production[105] or minimization of ATP production.[106]

FBA has been widely used to calculate the intracellular fluxes by constructing an optimization problem having a specific objective function (z) restricted by mass balance equations, reaction reversibility constraints and other constraints. A typical optimization formula is given as following:

$$\begin{aligned} &Maximize/Minimize \quad z \\ &Subject\ to \quad S.v = 0 \\ &\qquad v_m^{\min} \le v_m \le v_m^{\max}, \qquad \forall m \in \{\text{measured fluxes}\} \\ &\qquad v_i \ge 0, \qquad\qquad \forall i \in \{\text{irreversible fluxes}\} \end{aligned} \tag{4}$$

The steady-state solution space defined by linear equations and inequalities given in Equation (4) is called a flux cone, and any programming method depending on the formulation of the problem can be used to choose an optimum point described by the objective function z. Various constraints can be incorporated into the stoichiometric modeling. For example, based on the measurement data from different *in vitro* or *in vivo* experiments, the external fluxes can be restricted by their minimum and maximum values. Thermodynamic or reaction directionality constraints can be further added to refine the steady-state flux space. In another example, pathway energy balance obtained from elementary modes weighted by Gibbs energy of reactions was used for hepatic metabolic network to reduce the feasible range of intracellular fluxes.[23,104,107,108]

Flux balance analysis was used in the early 1990s for hybridoma cell lines by Savinell and Palsson to explore the optimum flux distribution corresponding to maximum growth rate.[109,110] Recently, a significant number of studies utilized FBA to describe the metabolic network properties of mammalian cells (Table 1). Wahl et al.[42,111] used the MDCK model to calculate a theoretical flux distribution representing an optimized cell that only consumes a minimum of carbon sources. Heino et al.[112] proposed an effective Markov chain Monte Carlo (MCMC) scheme to explore a two-compartment model for skeletal muscle metabolism. Tekir et al.[113] investigated the human red blood cell (RBC) metabolism by performing FBA via optimization of alternative objective functions and the maximization of production of ATP and NADPH. To observe the relative changes in the flux distribution of the deficient network, they applied two well-known approaches, minimization of metabolic adjustment (MOMA) that minimizes the Euclidean distance from a wild-type flux distribution[114] and regulatory on–off minimization (ROOM) that minimizes the number of significant flux changes with respect to the wild type.[115] Obrzut et al.[33] used FBA to characterize myocardial metabolic phenotypes among non-ischemic dilated cardiomyopathy (NIDCM) patients undergoing cardiac resynchronization therapy (CRT). Arterial and coronary sinus plasma concentrations of oxygen, glucose, lactate, pyruvate, free fatty acids, and 22 amino acids were obtained from 19 male and 2 female patients. Using a metabolic network of the cardiac mitochondria (189 reactions, 230 metabolites), an objective function maximizing ATP production was chosen for the calculations of unknown fluxes. They concluded that analyses of the myocardial metabolic network using FBA may provide unique and clinically useful prognostic information in patients with NIDCM undergoing therapy. Yang et al.[26] demonstrated a proof of principle for the use of constraint-based modeling to achieve enhanced performance of liver-specific functions of cultured hepatocytes during plasma exposure by optimizing amino acid supplementation and hormone levels in the medium. An FBA model was developed for the maximization of urea output. However, because it is possible that alternative flux distribution exists that produces the same maximal output, they applied a recursive mixed-integer linear programming (MILP) to enumerate all solutions. Uygun et al.[116] investigated and identified possible metabolic objectives for hepatocytes cultured *in vitro* using a data-mining procedure that included a multi-level optimization-based algorithm. The multi-level

optimization problem formulated was to identify a minimum set of fluxes that are of major importance to the cells while minimizing difference between the fluxes measured and those predicted by the model and maximizing the summation of weighted fluxes. This problem creates a mixed-integer nonlinear programming (MINLP) problem with multiple inner linear programs, which is computationally challenging and requires a suitable algorithm for the solution. They solved the problem in an iterative scheme where different flux combinations are considered in the objective and tested for sufficient prediction accuracy.

Proposing an objective function for mammalian cells is a major obstacle for FBA. Mammalian cells exhibit various phenotypic states regarding the cell proliferation, differentiation, and organ-specific functions. Metabolic network properties of mammalian cells under different phenotypic states should be further investigated to provide clues regarding the metabolic objectives. Calik and Akbay[20] determined the theoretical flux distributions in the fibrotic and healthy liver cells by maximizing, respectively, the collagen and palmitate synthesis in the objective function for the solution of the model. A mammalian cell type such as hepatocytes might exhibit multiple functions that need to be considered simultaneously when exploring the optimal fluxes. In this case, Pareto-optimal solutions satisfying all the objectives simultaneously can be analyzed.[117–119] Nagrath et al.[117,118] developed a multi-objective optimization approach that couples the normalized normal constraint (NC) with both FBA and energy balance analysis (EBA) to obtain multi-objective Pareto-optimal solutions. They investigated the Pareto frontiers in gluconeogenic and glycolytic hepatocytes for various combinations of liver-specific objectives (e.g., albumin synthesis, glutathione synthesis, NADPH synthesis, ATP generation, and urea secretion). Sharma et al.[119] investigated the importance of amino acids in the supplementation and the criticality of metabolic pathways using a Pareto optimal set of solutions corresponding to liver-specific functions of urea and albumin secretion in the metabolic framework using multiobjective optimization. Furthermore, they used the concept of two-stage stochastic programming to obtain robust solutions by considering extracellular variability. Because the metabolite measurements are subject to variability, uncertainty must be integrated with system analysis to improve the prediction of hepatic function.

A substantial study published by Bonarius et al.[92] illustrates the importance of applications of the metabolic engineering tools. What they have elucidated in their study is the comparison of three different approaches for the analysis of cyclic pathways in the primary metabolism of continuously cultured hybridoma cells. These approaches are (a) MFA including the mass balances of co-metabolites as well (e.g., ATP or NAD(P)H), (b) FBA with linear objective functions, and (c) flux data obtained by isotopic-tracer experiments. They observed that metabolic fluxes estimated using the objective functions "maximize ATP" and "maximize NADH" were relatively similar to the experimentally determined fluxes.

D. Metabolic Pathway Analysis (MPA)

Pathway analysis (based on stoichiometry of the network only) systematically characterizes the structure of a metabolic network and elucidates its important properties by decomposing the highly interconnected reactions into more organized pathways. Metabolic pathway analysis mainly based on extreme pathways and elementary modes has proven to be a useful tool[11,12] and has been extensively applied in the literature. Elementary modes consist of the minimum number of reactions that exist as a functional unit, whereas extreme pathways are the independent subset of elementary modes.[11] Pathway analysis identifies pathways that are important for desired products (yield analysis) and evaluates how much of a flux is carried out by each pathway and to what extent an external metabolite taken by the cell affects the output of the pathway.[13] This analysis can elucidate important information about metabolic regulatory mechanisms and how dominant pathways are controlled.

Notably, every flux distribution can be written as a linear combination of the elementary modes, or extreme pathways, thus a weight can be assigned to each corresponding pathway and can be interpreted

as an indication of the importance of that pathway in the network[120,121]:

$$v = P.w$$
$$w_k \geq 0, \ \forall k \in \{irreversible\ pathways\} \quad (5)$$

where $\boldsymbol{w}$ denotes a vector involving the weight for each elementary mode and $\boldsymbol{P}$ is the matrix of elementary modes. One of the main problems in pathway analysis is that the decomposition of a steady-state flux vector into pathways is not always unique, because for large networks the number of pathways is not usually equal to the dimension of the null space of the stoichiometric matrix (Figure 4).[122] To provide a unique decomposition of the steady-state flux distributions into elementary modes or pathways, different objective functions have been proposed in the literature including maximization of the number of elementary modes, minimization of the elementary mode activity, and the entropy maximization principle.[123–126]

Pathway analysis has been used to characterize the metabolism of red blood cells.[121,127–129] Wiback et al.[129] interpreted the extreme pathways of the red blood cell metabolic network in a biochemical and physiological context and divided them into groups based on different criteria such as cofactor and by-product production, and carbon inputs. They also described how physiological steady-state solutions can be reconstructed from a network's extreme pathways by using a linear optimization problem that maximizes and minimizes the weights of a particular extreme pathway in the reconstruction to find a weight-spectrum (α-spectrum).[121] The α-spectrum showed which extreme pathways can and cannot be included in the reconstruction of a given steady-state flux distribution and to what extent they individually contribute to the reconstruction. Moreover, it was also shown that accounting for transcriptional regulatory constraints can considerably reduce the α-spectrum.[121] Price et al.[128] used the singular value decomposition (SVD) method for the extreme pathway matrix of human red blood cells. They observed that the first five eigenpathways, out of a total of 23, effectively characterize all the relevant physiological states of red blood cell metabolism. Moreover, it was also shown that the dominant features of these first five eigenpathways described key metabolic splits regulated in the human red blood cell. Cakir et al.[127] investigated five enzymopathies (G6PDH, TPI, PGI, DPGM and PGK deficiencies) in human red blood cells using MPA. They analyzed the elementary modes corresponding to each enzyme deficiency case given the functional capabilities.

The basic idea of MPA was also utilized to characterize CHO and hepatic metabolic networks as well. Provost and Bastin[45] translated the elementary flux modes of CHO metabolism into a set of macro-reactions connecting the extracellular substrates and products. Then they built a dynamical model using these macro-reactions. To provide weight values for all possible pathways within hepatic metabolic network, Orman et al.[13] presented different approaches, considering the structural and physiological properties of metabolic network, aiming at a unique decomposition of the flux vector into pathways. These approaches based on optimization functions (including maximization of the number of elementary modes, maximization of the entropy of the system, maximization of activity of short pathways, and minimization of the elementary mode activity) were used to analyze the hepatic metabolism considering the available data sets obtained from perfused livers of fasted rats receiving burn injury. Elementary modes were also used to formulate thermodynamic feasibility constraints for the hepatic metabolic network to shrink the feasible range of intracellular fluxes.[23,104,107,108] The underlying assumption behind pathway-based energy balance is that an exergonic reaction can be a "driving-force" for an endergonic reaction if these two reactions are coupled in the same pathway.

There are other metabolic engineering tools, such as metabolic control analysis (MCA), which has been used to analyze mammalian systems. Different from the methodologies based on the stoichiometry of a metabolic network, MCA might not necessarily need a complete stoichiometric model; therefore the principles of MCA will be briefly reviewed here. MCA can be used to elucidate the parameters responsible for the control of the flux distribution.[130] It is generally used to describe the control coeffi-

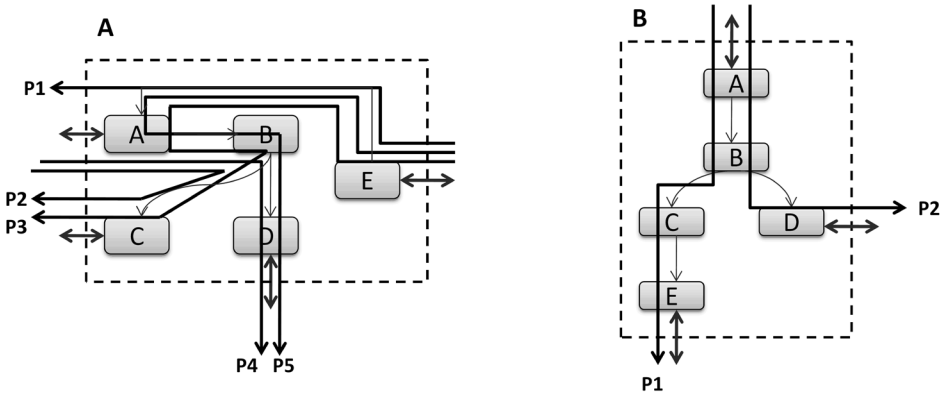

FIGURE 4: Metabolic pathways of two different networks. A. The number of pathways in this network is 5, which is greater than the dimension of the null space of the stoichiometric matrix (equal to 2). The system is redundant and the decomposition of the steady state flux vector into pathways is not unique. **B.** The number of pathways is equal to the dimension of the null space of the stoichiometric matrix, thus the decomposition method is unique.

cients that predict the response of the system to perturbations, that is, fractional changes in the systemic property (e.g., metabolite concentration, cell proliferation or the flux of concerned reaction) over the fractional change in enzyme activity.[131] Because the reactions in a network are dependent on each other, the control coefficients sum to unity, which is called "summation theorem." Elasticity coefficients are other important parameters that indicate the local properties of individual enzymes in the metabolic network.[130] They provide information on how a metabolite can be an activator or an inhibitor of a reaction by analyzing the ratio of the relative change in the reaction rate to the change in the metabolite concentration. MCA is an important tool for the study of drug-target identification, functional genomics, genetic disorders, cancer pathogenesis, and targets for disease therapy.[131] A number of studies regarding the MCA have been published to explain enzyme deficiencies,[132,133] specific pathways such as glycolysis and oxidative phosphorylation under normal[134,135] or disease state,[136,137] and effects of medicinally important drugs on the cell proliferation[138] in mammalian systems. Comin-Anduix et al.[138] investigated the impact of thiamine supplementation on tumor proliferation using the principles of MCA. To induce a considerable change in the activity of enzyme transketolase (TK) involved in ribose synthesis, thiamine (which is metabolized to thiamine pyrophosphate, the cofactor of transketolase) and oxythiamine (an irreversible inhibitor of transketolase) have been used to record the effects of TK alterations on the cell proliferation. With a very high control coefficient, they identified that thiamine could significantly increase tumor growth through transketolase activation. To understand whether there is a tissue variation in the distribution of mitochondrial oxidative phosphorylation control coefficients, Rossignol et al. analyzed the oxygen consumption flux in rat mitochondria isolated from five different tissues under identical experimental conditions.[137] They elucidated that the muscle and the heart are controlled at the level of the respiratory chain, whereas the liver, the kidney, and the brain are controlled mainly at the phosphorylation level by ATP synthase and the phosphate carrier. Soboll et al.[135] analyzed the control coefficients between the pathways of oxidative phosphorylation, gluconeogenesis, ureagenesis, and maintenance ATP consumption in isolated perfused rat liver. They concluded that the processes such as glucose and urea synthesis, which require a significant amount of ATP, are (not exclusively) controlled partly by regulators of ATP production and partly by other ATP-consuming pathways.

III. APPLICATIONS AND OUTCOMES OF STOICHIMETRIC NETWORK ANALYSIS

The methods developed for stoichiometric network analysis have been increasingly applied to mammalian systems to analyze the metabolic network properties of different cell types, to investigate effects of experimental conditions, substrates or other factors such as drugs and toxic compounds, and to improve the systems to increase the yield of the certain product production. Although most studies have used some of the approaches mentioned above simultaneously, we will attempt to categorize the works that appear in the literature regarding this topic in the following sections.

A. Understanding Cell Physiology

Mammalian cell cultures, including CHO, hybridoma and MDCK cell lines, have been widely used to produce recombinant proteins and other industrial and medical products in batch or fed-batch bioprocesses. Recombinant protein production by mammalian cell lines is more desirable compared to microbial products due to the more efficient post-translational modifications. Therefore, utilizing metabolic engineering tools is required to gain a better understanding of the metabolic networks of different mammalian cell lines. ^{13}C tracing approach has been commonly used to describe metabolic flux distributions in different cell types. Bonarius et al.[50] measured the intracellular fluxes of hybridoma cells to determine the amount of waste products derived from glucose labeled with ^{13}C at different positions. Some of their critical observations were that approximately 20% of the glucose consumed was channeled through the pentose shunt and that the glycolysis pathway contributed the most to lactate production. They also found that the pyruvate–carboxylase flux was negligibly small and that most of the CO_2 was produced by the TCA cycle. Goudar et al.[139] identified that the glucose consumption rate was five-fold higher than that of glutamine, with 41% of glucose channeled through the pentose phosphate pathway in CHO cells. They also observed that the fluxes at the pyruvate branch point were almost equally distributed between lactate and the TCA cycle. Moreover, they demonstrated that most amino acid catabolic and biosynthetic fluxes were significantly lower than the glycolytic and TCA cycle fluxes. Sengupta et al.[46] also used CHO cells for MFA but to analyze the non-growth phase. They showed that almost all of the consumed glucose was diverted toward PPP, with a high NADPH production. Similarly, metabolic fluxes in tumor cell lines using isotope tracers successfully summarized the hallmarks of cancer cell metabolism.[61,95] Portais et al.[61] used ^{13}C isotope to characterize the metabolic network behavior of rat tumor cell line, the C6 glioma. They demonstrated that glucose accounted for approximately 78% of the pyruvate. They also estimated that the pyruvate carboxylase activity and the efflux from the citric acid cycle were very low, suggesting a lack of glutamine production in C6 cells. Santos et al.[97] used [1-^{13}C]glucose and an incubation time of 45 hours to explore specific metabolic pathways in cultured rat embryonic brain cells. They observed an increase in label incorporation in extracellular alanine, lactate, and glutamine, reflecting mainly astrocytic metabolism.

Metabolic engineering tools were also utilized for other mammalian systems. Palsson et al. characterized human red blood cell metabolism and determined the key regulatory points by using extreme pathway analysis, SVD, and Monte Carlo sampling.[121,128,129,140,141] Another of the most widely studied mammalian systems is the hepatic system; the liver has many complex physiological functions including lipid, protein, and carbohydrate metabolism; synthesis of bile; and urea production. Isolated perfused hepatic systems have been extensively used under different disease states; however, animal models in these studies have typically been fasted prior to liver perfusions. Under these conditions, certain simplifying assumptions can be used, such as lack of glycogen storage and inhibition of all strictly glycolytic enzymes to determine the flux distribution easily. A caveat of this approach is that fasting induces metabolic changes that superpose to become disease-related changes. Orman et al.[24] used the flux variability approach, sampling analysi,s and SVD to analyze the data, using the

same metabolic network for perfused liver under fed and fasted conditions. They observed that fasting mostly affects glycolytic and gluconeogenic reactions, aspartate and glutamate metabolism, and PPP. They also elucidated that some glycolytic and gluconeogenic reactions can take place simultaneously (i.e., the reaction rate catalyzed by pyruvate carboxylase was found to be zero in fed state), and protein metabolism only accounts for a small portion of the nitrogen metabolism. Moreover, the same group investigated the effects of oxygen delivery in the perfusion system on liver metabolism by comparing three modes of oxygenation.[142] In the first group, the perfusate was equilibrated in a membrane oxygenator with room air (21% O_2) before entering the liver. In the second group, the perfusate was equilibrated with a 95% O_2/5% CO_2 gas mixture. In the third group, the perfusate included washed bovine red blood cells (RBCs) at 10% hematocrit, equilibrated with the 95% O_2/5% CO_2 gas mixture. They showed that livers perfused with RBCs (10% Hct) consumed oxygen at twice the rate observed using hyperoxic (95% O_2) perfusate without RBCs, and that significant anaerobic glycolysis occurred in the absence of RBCs, even using hyperoxic perfusate. It was demonstrated that the oxygen supply in perfusate without RBCs would be insufficient if it increased above the baseline, as is often the case in response to injury such as burn.[28] Iyer et al.[23] also used FBA to characterize carcinoma hepatic cells under varying levels of glucose (high, low, and glucose-free) and insulin (without and with physiological levels of insulin) for 5 days. They observed that in high- and low-glucose media, glycolysis, glutaminolysis, and oxidative phosphorylation were the main sources of energy (NADH, NADPH, and ATP). In glucose-free medium, due to very low glycolytic flux, the TCA cycle contributed more significantly to energy metabolism.

Using [U-$^{13}C_5$]glutamine as an isotopic tracer, Noguchi et al.[143] analyzed phenotypic changes in rat hepatoma cells treated with either palmitate alone or both palmitate and oleate in combination. They further searched the effects of amino acids on these free fatty acid–induced phenotypic changes. They observed that palmitate inhibited glycolysis and lactate dehydrogenase fluxes while increasing citric acid cycle flux and cytosolic NAD^+/NADH ratio. On the other hand, oleate co-treatment restored most fluxes to their control levels. They also demonstrated that glutamate was the most effective amino acid in promoting reactive oxygen species (ROS). Chatham and Seymour[31] perfused the hearts from Zucker diabetic fatty rats and age-matched controls with ^{13}C-labeled glucose, lactate and pyruvate, and unlabeled fatty acids to test the hypothesis that alterations in cardiac carbohydrate metabolism precede the onset of abnormalities in systolic function shortly after the development of Type 2 diabetes. They identified that the contribution of glucose both to total pyruvate oxidation and to tissue lactate and alanine formation was found to be significantly depressed in Zucker diabetic fatty rats.

More recently, Si et al.[144–146] used MFA to describe the metabolism of adipocytes, which plays a critical role in obesity. They applied this approach to characterize the effects of non-lethal, long-term mitochondrial uncoupling (up to 18 days) on the pathways of the intermediary metabolism of adipocytes.[144] Flux analysis estimated significant up-regulation of glycolysis and down-regulation of fatty acid synthesis, with chemical uncoupling exerting quantitatively larger effects. They also tested the hypothesis that adipocyte TG accumulation could be altered by specifically perturbing the pyruvate metabolism. They treated cultured 3T3-L1 adipocytes with chemical inhibitors of lactate dehydrogenase (LDH) and pyruvate carboxylase (PC). They observed a reduction in TG that was due to decreased *de novo* fatty acid synthesis.

B. Improving Metabolic Network Properties

As previously mentioned, mammalian cells have been extensively used for the production of biopharmaceutics; however, due to slow cell growth and low cell concentrations, these processes generally reach low productivity. Accumulation of waste products such as lactate and ammonia caused by glutamine metabolism and incomplete glucose oxidation can also inhibit the growth of the cells and product formation. On the other hand, in general, excess

amounts of glucose and amino acids for the *in vitro* cultivation of the mammalian cells are required for the metabolic capabilities of the host cell line and for making up the biomass and desired products. Consequently, adjusting the nutrient environment is essential to optimize the system, thus metabolic engineering tools are required to gain a comprehensive understanding regarding the cell metabolism and to analyze the network quantitatively. A feeding strategy derived from these methods can be subsequently employed to control nutrient concentrations at low levels in an effort to reduce ammonia and lactate formation.[55–60] For example, estimating the flux distributions by MFA in MDCK cultures in glutamine-containing and glutamine-free medium revealed that glutamine-free medium favors a more efficient use of nutrients by the cells.[41] MFA analysis was also used to identify a number of steady states resulting in higher concentrations of cells and/or product.[51,53] Follstad et al.[52] observed physiological state multiplicity in continuous cultures of the hybridoma cell line cultivated in glutamine-limited steady-state chemostats. They achieved a high-efficiency steady state or more efficient flux distribution by reducing the dilution rate resulting in conditions of stricter nutrient limitation. Bonarius et al.[49] cultured the hybridoma cells at steady state under both reductive and oxidative stress. They observed that the flux of the NADH-requiring lactate dehydrogenase reaction decreased in response to oxidative and reduction stress. Vriezen et al.[54,147] used different cell lines (mouse hybridoma and mouse myeloma) and bioreactor conditions to analyze the flux distributions. They concluded that the regulation of fluxes in the central metabolism of mammalian cells occurs mainly through modulation of enzyme activity and, to a much lesser extent, by enzyme synthesis.

Chan et al.[15,21,22] and Yang et al.[25,26] analyzed the nutrient supplementation for hepatic cell cultures by applying metabolic engineering tools. The rationality behind these studies was to improve hepatic function *in vitro*. The extracorporeal bioartificial liver device (BAL), a promising tool for the treatment of liver failure, has some limitations when exposed to plasma from the patient because hepatocytes are prone to accumulate intracellular lipids and to exhibit poor liver-specific functions. Therefore, based on hepatic intermediary metabolism, Yang et al.[26] used constraint-based modeling to optimize the biochemical environment of hepatocyte cultures toward the desired effect of increased hepatic function. They determined an amino acid supplementation using amino acid flux profiles identified by FBA analysis where performance of liver-specific functions of cultured hepatocytes was maximized. They observed that urea and albumin production under the designed amino acid supplementation was found to be increased compared with previously reported amino acid supplementation.[15,21,22] However, the urea production attained was less than the theoretical value, indicating the existence of pathways or constraints not incorporated in the model. Chan et al.[15,21,22] utilized MFA analysis to quantify the changes in intracellular pathway fluxes of primary rat hepatocytes in response to hormone and amino acid supplementations. They observed that culturing hepatocytes in medium including lower levels of insulin decreased the clearance of glucose and glycerol. Subsequent plasma exposure with amino acid supplementation increased gluconeogenic pathways and fatty acid oxidation.

C. Elucidating Metabolic Changes Caused by Different Disease States

Isolated perfused liver systems as well as MFA have been extensively used to characterize the detailed metabolic changes in various abnormal metabolic conditions (e.g., systemic burns and trauma, infection, and other insults).[14,16–19] A critical component of these investigations was to evaluate the impact of these insults on glucose and nitrogen metabolism because one of the major functions of the liver is to maintain physiological circulating glucose and ammonia levels. Yarmush et al.[16–18] found significant flux increases in the mitochondrial electron transport, the TCA and urea cycles, gluconeogenesis, and PPP in the rat liver metabolism after the burn injury. The same group also analyzed D-galactosamine–induced rat liver failure model to gain a better understanding of the hepatic metabolic pathways affected by fulminant hepatic failure (FHF).[14,27] They

showed that hepatic glucose synthesis was inhibited as a result of a reduction in amino acid entry into the TCA cycle by anaplerosis. FHF also inhibited aspartate synthesis.

In addition to the hypermetabolic state of the liver, it is also well known that systemic inflammation caused by burn injuries and other diseases results in a hypercatabolic state at whole body level due to the muscle wasting (i.e. accelerated muscle protein degradation). Yarmush et al. further investigated the effects of exogenous insulin on skeletal muscle metabolism in rats 4 days after being subjected to a 20% total body surface area (TBSA) burn injury or sham-burn treatment (control of burn treatment).[63] They determined fluxes through intracellular pathways using MFA and extracellular fluxes measured across the perfused hindquarters. They found that burn injury resulted in an increase in the formation and release of glutamine, an increase in the rate of proteolysis, and a negative nitrogen balance. The addition of insulin did not significantly affect the rate of skeletal muscle proteolysis or the nitrogen balance.

There are some other interesting studies where the effects of viruses on the metabolic networks have been analyzed[86,96] because viruses depend on the host metabolic network to provide energy and building blocks for viral replication. Munger et al.[96] quantified changes in metabolic activity of mammalian cells (fibroblasts) induced by human cytomegalovirus (HCMV) using ^{13}C-labeled forms of glucose and glutamine. They observed that infection with HCMV up-regulated flux through the central carbon metabolism (including glycolysis and TCA cycle) and the fatty acid biosynthesis pathway. They also showed that pharmacological inhibition of fatty acid biosynthesis suppressed the replication of both HCMV and influenza A, another enveloped virus, suggesting that fatty acid synthesis is essential for the replication of two divergent enveloped viruses. These results showed that systems-level metabolic flux profiling can identify metabolic targets for antiviral therapy. Nadeau et al.[86] also investigated the metabolism of embryonic kidney cells during an adenoviral vector infection under different feeding strategies. They monitored an increase in amino acid fluxes and more pyruvate entering the Krebs cycle via Acetyl-CoA and the anaplerotic pathway during the infection phase.

D. Effects of Drugs and Medical Treatments on Metabolic Networks

Subtoxic effects of drugs and other medical treatments might result in long-term problems, therefore it is crucial to understand the effects of those substances on the metabolism of liver, the primary organ having a function of detoxification. Niklas et al.[87] analyzed the hepatic metabolism and the effects of three different hepatotoxic compounds: amiodarone, diclofenac, and tacrine. They found that, upon exposure to diclofenac and tacrine, an increase in the TCA-cycle activity was observed which could be a signature of an uncoupling of the oxidative phosphorylation. Recently, Iyer et al.[107] studied the effects of triadimefon, a tumorigenic conazole, on primary rat hepatocytes using FBA. Conazoles are a class of fungicides used to prevent fungal growth in agriculture and for treatment of fungal infections. They are also found to be tumorigenic in rats and/or mice.[107] Iyer et al. demonstrated a switch from fatty acid synthesis to fatty acid oxidation in cells exposed to triadimefon. They interpreted that this might be due to the fact that fatty acid oxidation was active in order to supply energy required for triadimefon detoxification. Maier et al.[93] studied the effects of atorvastatin (a drug lowering the blood cholesterol level) on cholesterol biosynthesis and central carbon fluxes of rat hepatocytes using transient ^{13}C-flux analysis. They showed that the flux through the cholesterol pathway decreased in response to the administration of the drug; however, only minor differences were observed in the central carbon fluxes between cells treated with or without the drug.

Kantor et al.[29] perfused rat hearts using glucose and fatty acids appropriately radiolabeled with either (3)H or (14)C for measurement of glycolysis, glucose oxidation, and fatty acid oxidation to determine the effects trimetazidine, which acts directly and cytoprotectively on the myocardium. It had been found that trimetazidine had no effect on myocardial oxygen consumption or cardiac work under

any aerobic perfusion condition used. It increased the glucose oxidation rate in hearts subjected to low-flow ischemia. They also showed that trimetazidine inhibits long-chain 3-ketoacyl CoA thiolase activity, suggesting a stimulation of glucose oxidation. Lauzier et al.,[32] on the other hand, tested whether the drug ivabradine (IVA) and the β-blockers (METO) reducing the hearth rate alter the substrate metabolism in healthy mice hearts. Using a perfusion system and labeled substrates, IVA was found to selectively reduce the heart rate while preserving the energy substrate metabolism. On the other hand, METO decreases cardiac function and glycolysis.

Banta et al., on the other hand, explored the effects of dehydroepiandrosterone (DHEA), used as a treatment for trauma patients, on hepatic metabolism following burn injury.[19] They showed that DHEA administration appeared to normalize hepatocellular metabolism in burned rats but decreased the PPP flux, which might imply that the liver's ability to recycle endogenous antioxidants was impaired after the burn injury.

IV. CURRENT CHALLENGES OF STOICHIOMETRIC NETWORK ANALYSIS

Constructing the metabolic network is time-intensive work requiring collection of a variety of genomic, biochemical, and physiological data from the primary literature as well as available databases. The chemical formula and charge of each metabolite in a reaction should be analyzed carefully to verify that the chemical reaction is balanced correctly. More recently, generic genome-scale metabolic networks for different species, including human and mouse models, have been constructed. Consequently, tissue-specific large-scale networks including liver, kidney, and brain have been also published. These tissue-specific models were constructed either manually using literature and different databases or computationally using the generic metabolic models and genomic data. Recently, "gene to protein to reaction" (GPR) associations are included directly in the stoichiometric models for mammalian systems.[69,82] These associations describe the dependence of reactions on proteins and proteins on genes.[148] Though GPR associations have been used to construct metabolic networks in these studies, more advanced tools are required to analyze mammalian cells using GPR comprehensive networks to identify important targets for drug discovery. Fortunately, platforms such as PhysioLab (Entelos, Inc.) have started to use large-scale, computer-based mathematical models to characterize the disease pathophysiology and the response to therapeutic interventions by integrating genomic, proteomic, physiologic, environmental, and behavior data. Although GPR associations have already been well characterized in well-known microbial systems,[148,149] reconstruction of the networks of more complex mammalian systems from the annotated genome also has some disadvantages because not all genes have been well characterized in mammalian systems. Furthermore, genome-erived metabolic networks might include missing reaction steps, and reaction stoichiometry may vary with physiological conditions.[6] Application of metabolic engineering tools for large-scale networks is a very challenging problem that requires advanced computational and experimental methodologies. For example, to study the central carbon metabolism, Quek et al.[5] reduced the mouse genome scale model (including 2037 reactions), which they previously published,[79] by limiting the inputs and outputs of the network to those metabolites involved in the central carbon metabolism. However, the resultant model still had 1050 reactions and 1154 metabolites with 409 degrees of freedom. Unfortunately, it is very difficult (also expensive) to determine the large number of fluxes experimentally required for MFA. Integration of regulatory and thermodynamic properties and application of FBA or pathway analysis (extreme pathways and elementary modes) methods for large-scale networks are other challenges that should be further explored. FBA methods are generally used to characterize the genome-scale networks, but it is difficult to interpret the objective function describing the optimal flux vector, which should be compared to an experimentally determined flux vector. Moreover, the number of extreme pathways or elementary modes grows exponentially with increasing network size. It is not yet possible to compute them for genome-scale networks.[3]

Most of the metabolic network models used to characterize mammalian cells are small- or medium-scale networks. The models mostly focus on the central carbon metabolism including gluconeogenesis or glycolysis, urea cycle, TCA cycle, fatty acid metabolism, PPP, and some amino acid metabolisms. For small-scale networks, there is a good amount of consistency between the measurements and assumed biochemistry of the model, which requires more experimental measurements to establish a redundant system. Nevertheless, considering macroscopic reactions having large fluxes still gives moderately accurate results. For example, using the isotope tracing method, Goudar et al.[139] showed that most amino acid catabolic and biosynthetic fluxes were significantly lower than the glycolytic and TCA cycle fluxes in CHO cells. Similarly, amino acid and protein metabolisms only accounted for a small portion of flux distribution of rat liver metabolism during a 1-hour perfusion experiment.[16,17] Although, the large-scale metabolic network is more realistic and provides a promising base for analyzing topological properties, it should also be remembered that including more reactions (especially ones not related to experimental measurements) increases uncertainty in the determination of the metabolic flux distribution.

Metabolic flux analysis can be successfully applied to provide valuable insights regarding metabolic networks and metabolic regulatory properties. Only measured fluxes and the stoichiometry of the metabolism are required for this analysis. However, one of the difficulties is obtaining the necessary measurements that result in a determined or overdetermined system. Moreover, MFA does not incorporate the reaction directionality, which may result in a thermodynamically infeasible flux distribution. The metabolic network should also be carefully analyzed to determine the independent variables (i.e., fluxes to be measured experimentally) that are required to determine the dependent variables (or unknown fluxes) to obtain the flux distribution vector. Because the structure of metabolic networks is more complicated (given the parallel metabolic pathways, metabolic cycles, futile cycles and bi-directional reactions), measuring all external fluxes might not identify all internal fluxes. In this case, energy metabolites and other co-factors (e.g., ATP, NADH, NADPH, FAD, FADH, etc.) should be balanced together with other metabolites to estimate the flux distribution, therefore all reaction steps involving those metabolites should be exactly known and included in the model.[7] Moreover, the knowledge of isotope labeling measurements can be used to identify the flux vector correctly. Although incorporation of isotope techniques is very powerful in determining metabolic flux, this technique is only applicable for small-scale networks (mainly central metabolism) or particular metabolic pathways studied. Investigating isotope labeling patterns in the metabolism of microbes grown in minimal media is much easier when compared to that of more complicated mammalian networks that require more complex nutrient media. The number of mathematical equations developed to identify the labeling patterns tremendously increases when the number of isotopes as well as the reactions are increased. This expensive technique also require a rational experimental design for (a) determining the "time span" required for isotopic equilibrium, (b) choosing the appropriate substrate(s) labeled with suitable tracer(s) depending on the pathways or reactions to be analyzed, and (c) mitigating experimental complications in the measurements of labeled atoms when different types of NMR and MS techniques are used.[7]

Flux balance analysis is another technique used to overcome limited experimental data. This approach relies on an objective function together with mass balances and thermodynamic and other regulatory constraints to reduce the number of possible solutions. Although the solution space of steady-state flux vectors can be reduced by incorporating these additional constraints, the complexity of integrating metabolic, regulatory, and thermodynamic constraints into one model is a big challenge. The different time scales of the metabolic and regulatory processes,[150] the incomplete knowledge regarding the intracellular conditions, the lack of thermodynamic data on metabolic reactions (e.g., intracellular metabolite concentrations are still uncertain) are obvious reasons for this problem. Although different objective functions are reasonably applicable for microbial networks, such as maximization of

growth and optimal resource allocation, [151, 152] the applicability of flux balance models is greatly limited in mammalian cells for various reasons (e.g., complex regulatory systems and less proliferation).

Some well-established mathematical tools can be used to analyze the whole solution space instead of choosing an optimal point. One such method is the flux spectrum or variability approach that has been used for mammalian cells.[24,153] In this method, the range of possible values for each flux is determined using linear programming, wherein each flux is maximized or minimized while leaving all other fluxes free. Moreover, Monte Carlo sampling and SVD analysis can be respectively used to determine the size and shape of the steady-state flux space of mammalian cells,[141] and to decompose the steady flux states obtained by random sampling.[24,128,140] The application of SVD resulted in reduction of solution space dimensionality and identification of key branch points that can represent key control points in the network.

Pathway analysis can be also used to characterize the steady-state flux cone. This analysis is mainly based on extreme pathways and elementary modes that are quite similar; systematically independent extreme pathways are a subset of elementary modes. Therefore, analyzing a biological system using a set of extreme pathways can result in the exclusion of possibly important modes.[11,12] Using extreme pathways or elementary modes, a flux distribution can be obtained. However, the "flux decomposition into pathways" is not always unique in larger networks, which requires optimization formulations to solve. Some optimization based methods that have been mentioned in previous sections have already been used to characterize the metabolic networks without biological justifications.[123,124,126] This might be experimentally done using an isotope tracing method, but considering the multiple exchange metabolites, tracing the path of each metabolite in the network will be both experimentally and computationally very challenging. In fact, focusing on consistent results predicted by different methods developed for "unique decomposition of flux vector into pathways" can reveal potentially important properties of the metabolic network.[13]

V. CONCLUSION

Application of metabolic engineering tools developed for stoichiometric network analysis is promising approach for characterizing metabolic networks and for developing novel systems-biology methods which enable us to build more realistic models (such as genome scale metabolic networks) and to understand more complicated systems. The methods reviewed her have been applied recently to mammalian cells, providing greater insight into cell metabolic network properties and including regulatory points and metabolic objectives.

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