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Genome scale metabolic modeling of cancer

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ABSTRACT

Cancer cells reprogram metabolism to support rapid proliferation and survival. Energy metabolism is particularly important for growth and genes encoding enzymes involved in energy metabolism are frequently altered in cancer cells. A genome scale metabolic model (GEM) is a mathematical formalization of metabolism which allows simulation and hypotheses testing of metabolic strategies. It has successfully been applied to many microorganisms and is now used to study cancer metabolism. Generic models of human metabolism have been reconstructed based on the existence of metabolic genes in the human genome. Cancer specific models of metabolism have also been generated by reducing the number of reactions in the generic model based on high throughput expression data, e.g. transcriptomics and proteomics. Targets for drugs and bio markers for diagnostics have been identified using these models. They have also been used as scaffolds for analysis of high throughput data to allow mechanistic interpretation of changes in expression. Finally, GEMs allow quantitative flux predictions using flux balance analysis (FBA). Here we critically review the requirements for successful FBA simulations of cancer cells and discuss the symmetry between the methods used for modeling of microbial and cancer metabolism. GEMs have great potential for translational research on cancer and will therefore become of increasing importance in the future.

1. Introduction

Cancer is a neoplastic disease, where cells are reprogrammed to avoid the checkpoints in control of nutrient supply, growth, aging, death and dissemination (Hanahan and Weinberg, 2011). This is often caused by genetic events, e.g. mutations and copy number alterations, affecting the cell's signaling system. Cancer cells develop the ability to rapidly evolve, by acquired genomic instability. This allows the cancer cells to adapt to new environments allowing it to metastasize, to evade the immune system and resist cancer treatments. Aberrant energy metabolism is involved in many human diseases (Sangar et al., 2012) and reprogramming energy metabolism is an emerging hallmark of cancer (Hanahan and Weinberg, 2011).

Genome scale metabolic models (GEMs) attempt to collect all metabolites and metabolic reactions catalyzed by the enzymes of the genome in a unified mathematical framework. They have been successfully applied to microorganisms to unravel genotype-phenotype relationships, e.g. the lethality of gene knockouts (Duarte et al., 2004), and a wide range of algorithms have been developed (Lewis et al., 2012). Many methods center around Flux Balance Analysis (FBA), where the metabolic state of the cell is described by the biochemical reaction rates (Fig. 1A–C). GEMs have been extended to human metabolism (Fig. 1D) and employed in the study of cancer, as extensively reviewed before (Edelman et al., 2010; Bordbar and Palsson, 2012; Jerby and Ruppin, 2012; Lewis and Abdel-Haleem, 2013; Sharma and König, 2013; Ghaffari et al., 2015a; Mardinoglu and Nielsen, 2015; Masoudi-Nejad and Asgari, 2015; Resendis-Antonio et al., 2015; Yizhak et al., 2015). These reviews were focused on the reconstruction of generic models as well as cell or disease specific models (Bordbar and Palsson, 2012), applications for patient stratification, personalized treatment and drug target identification (Lewis et al., 2010; Mardinoglu and Nielsen, 2015), the heterogeneity of cancer (Ghaffari et al., 2015a; Resendis-Antonio et al., 2015), the biology of cancer metabolism and known mutations (Sharma and König, 2013; Yizhak et al., 2015) and labeling experiments (Lewis and Abdel-Haleem, 2013). This review aims to cover these topics whilst paying special attention to the requisites for FBA modeling, and how FBA modeling of human cells differs from the modeling of microbial cells.

We begin this review by discussing alterations in metabolic enzymes affiliated with cancer. We then proceed to methods and applications of GEMs, both methods that already have been employed in cancer research and methods that have been developed for microbes

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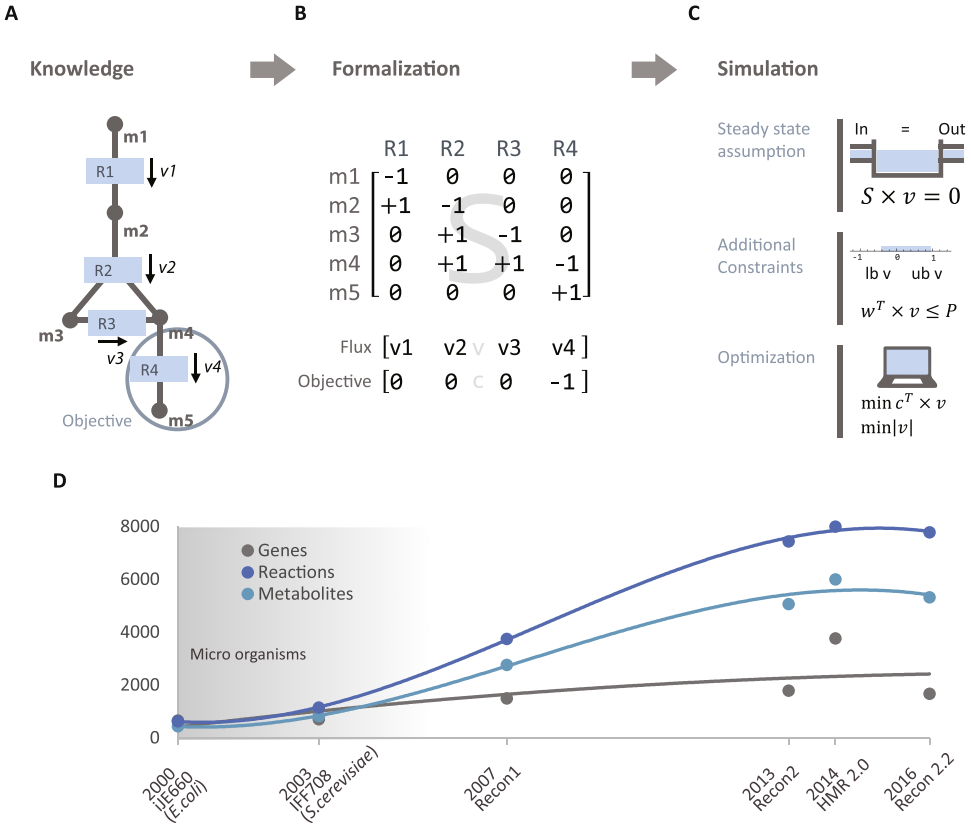

Fig. 1. Biological knowledge is formalized into a mathematical representation on which hypotheses can be tested and simulations run. (A) Assembly of biological knowledge about biochemical reactions, **R**, and rates, **v**, the participating metabolites, **m**, and biological driving forces in form of biomass equations and cellular objectives. (B). Formalization of the metabolic network as a stoichiometric matrix, **S**, where reactions are represented as columns, metabolites as rows and the stoichiometry in the reactions represented as the non-zero elements. (C) Metabolic simulations commonly use the steady state assumption, where the metabolites produced by one reaction are immediately consumed by another reaction, preventing buildup of internal metabolites. Additional constraints may also be added e.g. flux bounds on exchange reactions. Using linear programing, flux distributions can be identified that optimize the flux through the objective function. (D) The amount of genes, reactions and metabolites represented in genome scale models have increased with time and with the shift to more complex organisms. The models we have today are rather comprehensive and new content is added at a slower rate.

with translational potential. We pay special attention to quantitative flux studies using FBA and review the experimental parameters that enable accurate flux predictions. We end the review by evaluating the limitations and possibilities of GEMs in general and FBA in particular. We conclude that GEMs are useful tools to structure knowledge of cancer metabolism and for hypothesis generation, testing and validation.

2. Cancer metabolism

The increased proliferation rates in cancer cells require corresponding adaptations to the metabolic fluxes. It is debated (Ward and Thompson, 2012) whether metabolic changes are driving the cancer progression (the supply model) or if they are merely a response to increased consumption rates (the demand model). It is reasonable to assume that both mechanisms may be involved, and many metabolic genes and regulators of metabolism are implied in cancer (Fig. 2). Metabolism of cancer has been reviewed before (Vander Heiden et al., 2009; Yizhak et al., 2015; Ward and Thompson, 2012; Cairns et al., 2011). We here draw from these reviews to present some key metabolic features of cancer, falling into the two categories; rapid ATP generation, and increased biosynthesis of macromolecules.

2.1. Synthesis of ATP

Free energy in the form of ATP is fundamental to cellular growth and is required for polymerization of macromolecules as well as other growth related activities (Verduyn et al., 1991), and the growth rate of mammalian cells in culture is a function of the ATP formation rate (KILBURN et al., 1969). The synthesis of ATP takes place in glycolysis, the TCA cycle and oxidative phosphorylation. For each glucose molecule metabolized 2 ATP molecules are obtained from glycolysis, 2 from the TCA cycle, and around 21.6–26 by oxidative phosphorylation.

It may therefore seem counter intuitive that most tumors experience the Warburg effect, where oxidative phosphorylation is bypassed and glucose is fermented to lactate (Cairns et al., 2011). The cells appear to compensate for this by increasing the uptake flux, making glucose consumption, measured by positron emission tomography (PET), a reliable marker for tumor detection, present in >70% of all tumors (Cairns et al., 2011). Neither oxygen limitations, nor mitochondrial defects appear to be causing the phenotype (Cairns et al., 2011).

It has been hypothesized that the increased glucose flux mainly serves as a source for reduced carbon required for biosynthesis (Ward and Thompson, 2012; Heiden Vander et al., 2011), but both stoichiometric analysis and labeling experiments have ruled out that option (Shlomi et al., 2011; Hosios et al., 2016; Keibler et al., 2016). A similar phenomenon, dubbed overflow metabolism or aerobic fermentation, is present in many microorganisms, where glucose is converted to different fermentation products, also in the presence of oxygen. Mitochondrial capacity constraints or macromolecular crowding have been proposed drivers (Sonnleitner and Käppeli, 1986; Beg et al., 2007; Molenaar et al., 2009), and metabolic modeling suggest that this also may be the case for tumor cells (Shlomi et al., 2011; Vazquez and Oltvai, 2011). This is corroborated by the fact that also muscle cells and other metabolically active cells produce lactate as a response to increased ATP demand (Vazquez and Oltvai, 2011; Vazquez et al., 2011).

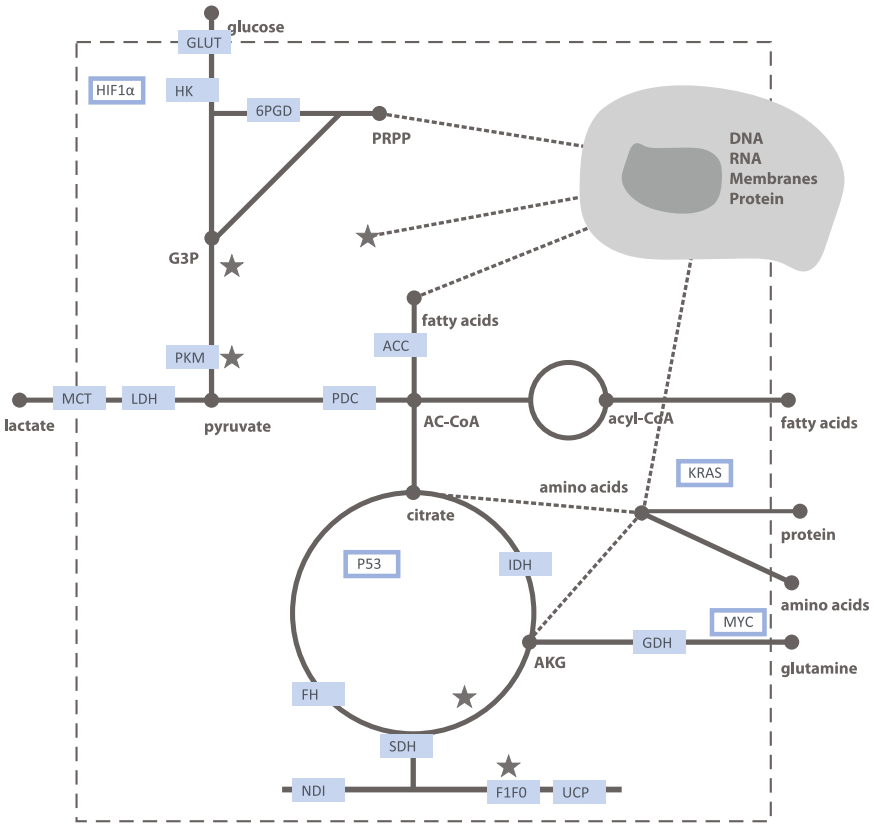

Fig. 2. Schematic representation of intermediary metabolism, which generates energy and precursors for cellular growth. Many genes and signaling systems are perturbed in cancer, metabolic genes are indicated with filled boxes and signaling genes by open boxes, ATP synthesizing steps are indicated with a star. The metabolic pathways from biomass precursors to the building blocks of a new cell are represented by dotted lines, to avoid overlapping lines the drain of ATP for biomass is represented by a lone star. Abbreviations as described in main text.

Cancer affects many genes catalyzing the metabolic steps from glucose to energy. Glucose is first transported into the cell by one or several of the 14 glucose transporters (GLUT) (Carvalho et al., 2011). Upregulation of the GLUT-1 isoform is associated with cancer (Hanahan and Weinberg, 2011), and indicative of increased proliferation, energy expenditure and aggressiveness (Carvalho et al., 2011). It is however absent in several cancer types, and shows variable expression, in most other (Carvalho et al., 2011).

Glucose is then phosphorylated by hexokinase (HK) using ATP. The HK2 isoform is up-regulated in hepatoma cells (Rempel et al., 1996) and many other tumors (Solaini et al., 2011). This isoform is bound to the mitochondrial membrane where mitochondrial ATP is directly channeled to the enzyme (Solaini et al., 2011). The affinity for glucose is 250 times higher for the HK2 isoform compared to HK4 from the liver, which operates at glucose concentrations similar to the physiological concentrations in blood, $K_m \approx 5$ mM (Mathupala et al., 2006). The preference for HK2 over HK1 and HK3 with similar glucose affinities may stem from its promoter being activated by the hormone glucagon, meaning that glucose consumption may continue despite signals for low blood sugar, which shuts down the activity of most normal cells (Mathupala et al., 2006).

In the last step of glycolysis, ATP is synthesized by the enzyme pyruvate kinase (PK). The embryonic PKM2 isoform is typically expressed in cancer cells and has a growth advantage over cells expressing PKM1 (Christofk et al., 2008). This has been a matter of controversy, since the PKM2 form is known to have lower activity and is more sensitive to allosteric regulation (Ward and Thompson, 2012). It has been purposed that the enzyme is instead bypassed by a putative pathway over serine and one carbon metabolism (Vazquez et al., 2011). A less drastic explanation may be that the lower substrate affinity of PKM2 increases the concentration of glycolysis intermediates, pushing more flux into nucleotide synthesis through the pentose phosphate pathway (Ward and Thompson, 2012). In the presence of sufficient amounts of allosteric regulators the substrate affinity is greatly increased (Boxer et al., 2010), and the maximum capacity of PKM2 was shown to be sufficiently high to sustain flux in a study using 7 randomly picked cell lines (Xie et al., 2016), potentially resolving the controversy.

At pyruvate there is a branch point where the cell may excrete the carbon as lactate or further metabolize it in the TCA cycle. Since the former is dominating in cancers the proteins lactate dehydrogenase (LDH) and the monocarboxylate transporters (MCT), have been suggested as therapeutic targets (Cairns et al., 2011).

The ATP generating step in the TCA cycle is performed by Succinyl-CoA synthetase, but it is the proceeding enzymes succinate dehydrogenase (SDH) and fumarate reductase (FH) that have received most attention in cancer research. Decreased activity of these enzymes lead to a buildup of succinate which gets transported to the cytosol where it activates HIF-1α signaling (King et al., 2006). HIF-1α induces a glycolytic response by amplifying glucose transporters and glycolytic enzymes (Cairns et al., 2011).

In the first step of oxidative phosphorylation complex I (NDI) uses NADH to generate a proton gradient. The activity and protein content of NDI is downregulated in several cancers and cell lines, which has been associated with changes in the KRAS signaling (further discussed below) (Solaini et al., 2011).

ATP is formed by controlled release of the proton gradient by complex V (F_1F_O), the last step of oxidative phosphorylation. Low content of ATP synthase is observed in clear cell type renal carcinomas and in chromophilic tumors, whilst particularly the F1-ATPase β subunit is affected in a broad range of tumors (Solaini et al., 2011). The content of F1-ATPase β divided by the content of the glycolytic

Table 1
Categorization of cancer related applications of GEMs and the associated algorithms and methods.

Category	Application	Algorithms and Methods
Network reduction	Generation of Cell specific models for personalized medicine or analysis of cell specific functions.	INIT (Agren et al., 2012), tINIT (Agren et al., 2014), mCADRE (Wang et al., 2012), network analysis (Asgari et al., 2013) homogeneity scoring (Ghaffari et al., 2015b)
***In silico* knockouts**	Drug discovery and development. Investigation of synthetic lethality and cocktail effects.	Essential metabolites (Kim et al., 2014), Synthetic lethality (Folger et al., 2011), MOMA (Segrè et al., 2002), MD-FBA (Benyamini et al., 2010)
High Throughput data analysis	Validating or discovering bio markers for screening, diagnostics, prognostics and/or patient stratification.	DIRAC (Eddy et al., 2010), iMAT (Shlomi et al., 2008), GIMME (Becker and Palsson, 2008), MADE (Jensen and Papin, 2011), MCMC (Schellenberger and Palsson, 2009), KIWI (Väremo et al., 2014)
Quantitative flux predictions	Formalizing and testing biological hypothesis. Analyzing sensitivity and importance of system parameters.	dFBA (Mahadevan et al., 2002), capacity constraints (Beg et al., 2007), mass constraints (Adadi et al., 2012), rFBA (Covert and Palsson, 2002), SR-FBA (Shlomi et al., 2007) multi tissue FBA (Bordbar et al., 2011), pharmakokinetic FBA (Krauss et al., 2012), 3DFBA (Cole et al., 2015)

protein GAPDH gives a measure of the tendency to ferment, and was found downregulated in a large number of tumors (Sánchez-Aragó et al., 2012). ATP synthase is involved in the formation of cristae, folding of the inner mitochondrial membrane, which are observed to be less prevalent in carcinomas (Sánchez-Aragó et al., 2012). The proton gradient may also be released without the formation of ATP by the uncoupling proteins, UCP1-5. Thus, UCP2 is shown to be over expressed in several chemo resistant cancers (Solaini et al., 2011).

In healthy cells the TCA cycle may also be fueled by non-glucose derived carbon sources, where fatty acids are converted to acetyl-CoA through beta oxidation. This is consistently downregulated in liver cancer (Björnson et al., 2015). Carbon may also enter the TCA cycle as alpha-ketoglutarate (AKG), through the metabolism of glutamine (DeBerardinis et al., 2007). Glutaminolysis, is a common trait in cancer cells and depends on glutamate dehydrogenase (GDH). Here, glutamate is converted to lactate (60%) or to oxaloacetate to replenish TCA intermediates, i.e. anaplerosis (DeBerardinis et al., 2007). The expression of glutamine transporters is increased by signaling by MYC (Hensley et al., 2013), a transcription factor involved in cell cycle and apoptosis that is commonly deregulated in cancer (Vita and Henriksson, 2006).

2.2. Synthesis of biomass

Cancer cells consist primarily of proteins, lipids and polynucleotides, in similarity with most cells from lean tissues (Keibler et al., 2016). Many amino acids are essential, meaning that they cannot be synthesized by human cells, and therefore amino acid derived carbon constitutes up to 80% of the carbon in cell lines (Hosios et al., 2016). Amongst the non-essential amino acids only 6 could be replaced by *de novo* synthesis without severely affecting proliferation of lung cancer cell lines (Hosios et al., 2016). Amino acids may also be derived from extracellular proteins through macropinocytosis and proteolytic degradation. Inhibiting macropinocytosis reduced the growth in tumor xenografts and extracellular protein may be used in place of glutamine when growing cell lines (Commisso et al., 2013). The ability to utilize external protein appears to be dependent on KRAS signaling. Branched chain Amino Acids (BCAA) are used as nitrogen sources for non-essential amino acid synthesis, and the transamination step in BCCA metabolism is upregulated in glioblastoma (Tönjes et al., 2013).

Fatty acids can be taken up from the medium but many cancers gain the ability to synthesize them *de novo* (Baenke et al., 2013). Although some fatty acids are considered essential for normal human growth, they are not necessarily essential for cancer cells, as it is possible to grow HEP G2 cell lines on deficient medium (Furth et al., 1992). *De novo* synthesis of fatty acids relies on acetyl-CoA or citrate which may be derived from glucose or glutamine (Hosios et al., 2016). Analysis of transcription patterns of fatty acid synthesis in liver cancer show unanimous upregulation of enzymes involved in acetyl-CoA supply (Björnson et al., 2015) and fatty acid synthase (FAS). Some cancers have decreased pyruvate flux from glucose, instead they perform reductive carboxylation, TCA cycle running in reverse, to form lypogenic citrate from glutamine (Ward and Thompson, 2012).

Nucleotides are a critical part of the biomass and are believed to be synthesized *de novo* in human cells (Keibler et al., 2016). Metabolic modeling also suggest an upregulation of the RNA rich ribosomes in response to increased proliferation rates (Vazquez et al., 2011). The ribose ring of the nucleotides can be synthesized *de novo* through the pentose phosphate pathway, with 6-Phosphogluconate dehydrogenase (6PGD) being the third step. Inhibiting this enzyme has demonstrated anti-tumorigenic effects due to impaired nucleotide metabolism (Lin et al., 2015). The inhibition also affects NADPH production, but restoring NADPH to physiological levels does not rescue the phenotype, whilst supplementing the cells with the reactions product (ribulose-5-phosphate) does, suggesting that it is the impaired nucleotide synthesis that limits growth (Lin et al., 2015). The nitrogen atoms in the nucleotides are donated from the amino acids glutamine, aspartate and glycine.

3. GEM methods and applications

The scientific activity currently conducted on human GEMs may been divided into: 1) the reconstruction of the metabolic network, *2) In Silico* Knockouts studies, 3) the use of the network as scaffold for analysis of throughput data and 4) for quantitative predictions of exchange fluxes using FBA (for a summary see Table 1).

3.1. Reconstruction of models of human metabolism

A lot of the early effort went into reconstructing the human metabolic network. The network represents a crystallization of human knowledge (K-base) of metabolism and the GEM Recon 1 was based on 1500 literature sources (Duarte and Becker, 2007; Bordbar and Palsson, 2012). Later reconstructions served to integrate knowledge from other metabolic models (EHMN and HepatoNet1) (Ma et al., 2007; Gille et al., 2010) and focus on the intricate details of fatty acid oxidation, where the same set of enzymes are responsible for a range of different reactions (Thiele et al., 2013). Updated versions of this model have been released which resolve the problems associated with predicted infinite energy production to accommodate more realistic simulation (Swainston et al., 2016). The metabolic model Human Metabolic Reaction (HMR) is an independent reconstruction, with similar scope and detail level, and with functional energy metabolism (Mardinoglu et al., 2014).

The reconstruction serves to identify the metabolic reactions that can take place in the organism of interest. There will inevitably be gaps in this knowledge, since the metabolic function of all genes are not completely characterized. Some qualified assumptions may be required to complete the network, based on chemical properties or analogies to other species. The gap filling processes also highlights limitations in our knowledge. Metabolite transporters is one such category, as membrane bound proteins have been notoriously hard to characterize

and study. An algorithm has been developed to infer exchange reactions from network and expression data (Shlomi et al., 2008). The human serum database (Psychogios et al., 2011) contains information about normal as well as abnormal concentrations of metabolites in blood, and has been used to infer exchange reactions in genome scale models (Mardinoglu et al., 2014). In auxotrofic organisms such as humans, gap filling may require extra caution, since metabolites may be consumed in half processed states and then excreted in detoxified forms.

The aforementioned reconstructions are generic metabolic networks, based on the complete information of the human genome. Different types of network pruning has therefore been used to reduce the network for different applications. Cell specific models is one example, these may be generated for different tissues, but also for individual cancers and disease states (Bordbar and Palsson, 2012; Agren et al., 2012). The resulting reduced network may be useful for topological analysis, and will in general add additional constraints for metabolic modeling purposes. Automatized pruning of 126 normal and 26 cancer tissues, based on mRNA expression, reduced the number of flux bearing reactions from 2500 to 1000 on average (Wang et al., 2012). A network centric analysis of human cancer revealed that all of the reconstructed cancer network satisfied scale-free and small-world properties, meaning that a few metabolites and reactions had a high degree of connectivity. But these parameters were not significantly different from healthy cells (Asgari et al., 2013). This study also noted that well-connected reactions are not over represented as drug targets (Asgari et al., 2013).

Similarities and differences in topology of 11 cell lines was identified through model reconstruction. The cell line that was most dissimilar from the others was the liver cancer cell line HEP G2 (Ghaffari et al., 2015b), potentially due to differences in the tissue of origin. Interestingly the heterogeneity between the cell lines increased even further when connectivity of the network was enforced by simulated metabolic fluxes (Ghaffari et al., 2015b). Care should however be taken to when reducing the metabolic network of cancer cells, due to their high degree of genetic plasticity. It may be advisable to compare results acquired in cancer specific models to the corresponding results in a generic model to evaluate which of the genetic traits the results relies on.

3.2. In silico knockouts for drug development

Many drugs aim to rewire the user's metabolism in a beneficial way and GEMs may therefore be an aid in the engineering of drugs. Cancer specific models may be used to determine if a patient is expected to respond to a specific drug treatment.

In one study immunohistochemical proteomics data from 27 HCC patients was analyzed by pruning a generic GEM to personalized models (Agren et al., 2014). Potential drug targets were predicted using the concept of essential metabolites (Kim et al., 2014), i.e. the predicted lethality of knockouts of reactions connected to a certain metabolite. The effect of the drug on healthy cells was investigating by analyzing the ability to retain 56 metabolic tasks connected to energy production, redox balance, internal conversions, substrate utilization and biosynthesis of products, whilst the cancer cells also were tested for biomass production. The study had both a population wide and individual scope, i.e. identifying both general targets and targets for personalized medicine.

The concept of synthetic lethality, i.e. the lethality of two simultaneous knock outs, was used in a study to identify combinations of on the market drugs that may have an effect on cancer (Folger et al., 2011). The metabolic target of the drugs were identified and genome scale modeling was used to calculate a synergy score, measuring the individual effect relative to the combined effect of the *in silico* knockout. A lethality score was also calculated based on how the knockout affected healthy cells, quantified by the effect on ATP production and on cancer, quantified by the effect on biomass formation.

The concept of predicted gene essentiality, i.e. if a knock out is lethal, was critically analyzed in in RCC and prostate adenocarcinoma by comparing essentiality *in silico* and *in vitro* (Gatto et al., 2015). The analysis suggested that the GEM based predictions of gene essentiality were better than chance for RCC and the study also found 5 genes essential for RCC but not healthy cells, identifying them as potential drug targets.

Gene essentiality studies in microbes have been criticized for not taking into account the metabolic adjustments required (Segrè et al., 2002; Raman and Chandra, 2009), and an algorithm minimizing the metabolic adjustment (MOMA) (Segrè et al., 2002) was created to correct for this limitation. The steady state assumption on metabolite pools used when identifying lethality in the metabolic network may sometimes mask the requirement for *de novo* synthesis of currency metabolites (Benyamini et al., 2010; Dikicioglu et al., 2015), e.g. ATP, this has been solved by adding the cofactors to the biomass equation (Dikicioglu et al., 2015) or by adding a small term to all reactions to account for the growth dependent dilution of the free metabolite pools (MD-FBA) (Benyamini et al., 2010). To validate the effect of genetic perturbations, natural gene mutations in form of single nucleotide polymorphisms (SNPs) have been used to identify and constrain reactions in a GEM. The effect on exchange fluxes were studied and compared with literature descriptions of the metabolic phenotype (Shlomi et al., 2009).

3.3. Scaffold for interpretation of high throughput data

GEMs have synergized with the rise of omics, i.e. high throughput data sets of different biological moieties such as genomics, proteomics and transcriptomics. The models have become scaffolds for interpretation of the bio data (Agren et al., 2014), and multiple algorithms have been developed, including the data driven pruning algorithms mentioned above. This may require further modifications for cancer applications, as human metabolism is influenced to a higher degree by post translational modifications (PTM) and signaling, which are not captured by genomics, transcriptomics or proteomics (Shlomi et al., 2008). However, emerging high throughput technologies also capture PTMs e.g. quantification of phosphorylation states of proteins.

Gene-set analysis (GSA) is a common method for condensing the information from transcriptomics studies. GEMs can be transformed to metabolite gene-sets by, for each metabolite, considering all the genes encoding the reactions that are involved with that metabolite. The network visualization tool KIWI (Väremo et al., 2014) allows the user to visualize the interaction between several of these gene sets through their connection in the metabolic network. As a case study the method was used to investigate data from KRAS activation in a mouse xenograft tumor.

A principal component analysis (PCA) of transcriptomics data from a broad range of cancers showed markedly differential expression in RCC patients (Gatto et al., 2014). Analysis of the associated variables did not reveal a clear mechanism and therefore a GEM was used to interpret the data. Using the INIT algorithm (Agren et al., 2012), the topology of RCC was captured in a pruned GEM based on imunohistochemical proteomics. Analysis of the transcriptomics data then revealed unique defects in nucleotide, one-carbon, and glycerophospholipid metabolism, connected to the loss of the Hippel-Lindau tumor suppressor (VHL). The data analysis also pointed to a deregulation of heparin and chondroitin sulfate metabolism, and a diagnostic test based on glycosaminoglycan profiles in blood and urine was later developed based on these findings (Gatto et al., 2016).

Transcriptomics data from liver cancer was analyzed (Björnson et al., 2015) using differential expression (DE) and the differential rank conservation (DIRAC) algorithm (Eddy et al., 2010). DIRAC measures the degree of deregulation in a pathway, which may be an indicator of dysfunction under the studied conditions. A consensus GEM of all

metabolic capacities found in HCC cells was pruned from a generic GEM, and by overlaying the DE and DIRAC data, alterations in fatty acid synthesis and beta oxidation could be identified. Analysis of transcriptomics data of HEP G2, a liver cancer cell line, connected the gene tumor suppressor p53 to hepatic glucose production (Goldstein et al., 2013). The study used the iMAT algorithm (Shlomi et al., 2008), which integrates gene expression levels from several conditions to predict metabolic flux distributions. Four genes in gluconeogenesis were predicted to carry higher flux in genes with functional p53.

In a study stimulated by the discovery of the oncometabolite 2-hydroxyglutarate the authors classified a large data set of gene mutations into gain or loss of function (Nam et al., 2014), and traced the metabolic effect to a small number of suspected oncometabolites. The distribution of feasible fluxes was identified using Markov Chain Monte Carlo (MCMC) sampling (Schellenberger and Palsson, 2009).

3.4. Quantitative flux predictions

Determination of metabolic fluxes provide detailed phenotypic information of a cell, and comparing fluxes in a cancer cell with those of a normal cell can therefore provide crucial information about the mechanisms of cancer, in particular how metabolic reprogramming occurs to sustain increased energy formation and cellular proliferation. GEMs provide an environment for testing hypotheses about mechanisms and biological driving forces underlying cancer development. They also allow sensitivity analysis of individual variables in multi-parameter systems, where experimental investigations would be laborious or unethical. FBA is the most popular method to simulate metabolism.

The flux profile of cancers has been investigated using a small scale metabolic model including only 80 of the most well studied reactions related to cancer and with a simplified biomass equation which included several precursor metabolites and the production of lactate (Resendis-Antonio et al., 2010). By applying dynamically calculated uptake fluxes (dFBA) (Mahadevan et al., 2002) (as illustrated in Fig. 3A), good agreement between predictions and experimentally determined (in the HeLa cell line) temporal profile could be achieved. Based on the model, targets for drug development were suggested. Similar characterization could be envisioned for other temporal changes, e.g. the cell cycle or tumor progression (as illustrated in Fig. 3B–C). For microbes the algorithms rFBA (Covert and Palsson, 2002) and SR-FBA (Shlomi et al., 2007) have been used to characterize the influence of regulation on different steady state behaviors.

For microbial systems there has been growing interest for the hypothesis that proteome limitations is the driving force behind shifts in metabolism associated with increased growth rate or energy production (Beg et al., 2007; Molenaar et al., 2009; Scott et al., 2010; Adadi et al., 2012; Nilsson and Nielsen, 2016). There are several models (Shlomi et al., 2011; Vazquez et al., 2010, 2011; Vazquez and Oltvai, 2011) that have applied this concept to describe cancer phenotypes, e.g. the Warburg effect. These models rely on estimating the protein requirements for a certain flux profile and add global constraints on the share of the total proteome that may be allocated to specific metabolic activities. This additional constraint results in a maximum growth capacity and often in metabolic trade-offs, e.g. production of lactate, due to the higher catalytic efficiency of the glycolytic pathway, i.e. more ATP produced per mass of protein. The models observe qualitative similarities to experimental data in their normalized flux distributions (Shlomi et al., 2011), and may also proceed to predict the mitochondrial and ribosomal content of the cell (Vazquez et al., 2011).

The protein limited models effectively introduce dynamical upper bounds on the metabolic reactions. Upper bounds on metabolic fluxes (V_{max}) may also be directly applied in the simulations, and may reveal non-canonical metabolic bypasses that could be exploited by cancer cells. An example of such a pathway is the bypass of succinate dehydrogenase and fumarate hydratase, where simulations revealed that the production and degradation of heme could allow continued flux through the TCA cycle despite flux limitations at the succinate step (Frezza et al., 2011).

Multicellular models are gaining popularity in microbial systems (Shoaie et al., 2016), and some attempts have also been made for human cells, with focus on the Cori cycle, i.e. the production and consumption of lactate (Bordbar et al., 2011) and on the production and clearance of ammonia from the blood (Krauss et al., 2012). Both are potentially cancer relevant since the interaction between the exchange of lactate and other metabolites between healthy tissue and cancer cells is becoming more apparent (Semenza, 2008) and since ammonia production is a well-documented trait of cancer (DeBerardinis et al., 2007). Microbial cross feeding, where acetate is produced by rapidly growing cells on the frontier of a colony and consumed by more centrally located cells, has been investigated using spatial FBA modeling (3DFBA) (Cole et al., 2015). In this type of modeling a course grid is generated and each cell in the grid represents a group of cells in the colony. The output from one cell becomes the input of the surrounding cells. The authors suggests that cross feeding of lactate in tissues and tumors may be another application of their model (as illustrated in Fig. 3D).

Flux balance analysis has also been combined with high throughput transcriptional data, to estimate changes in flux from changes in transcription levels. The GIMME (Becker and Palsson, 2008) and MADE (Jensen and Papin, 2011) algorithms where used together with five different glioblastoma transcriptomics data sets and a small set of exchange fluxes from literature (Ozcan and Cakir, 2016). The model captured key metabolic changes and made accurate predictions of growth rate and lactate production.

FBA predicts intracellular fluxes from measurements of exchange fluxes, this can also be done through tracing experiments, where labeled substrates dissipate into different product and biomass pools. Labeling studies are therefore useful to validate flux distributions predicted with FBA. One study (Fan et al., 2014) compared FBA predictions of the fluxes from NADP+ to NADPH with measurements using deuterium labeled substrates. It found that folate metabolism contributed almost as much as the canonical pentose phosphate pathway, and that the total NADPH production rate was 5–20% of the glucose flux. Regular labeling studies rely on labeled substrates reaching a steady state, but for some substrates, e.g. methionine, this does not occur within reasonable time, and methods making use of labeling kinetics have instead been developed (Shlomi et al., 2014). Labeling kinetics have also been determined by culturing cells inside a nuclear magnetic resonance (NMR) spectrometer (Mancuso et al., 2004). This setup has been used to detect the anaplerotic response of the TCA cycle following changes in extracellular substrate.

4. Requirements for implementing FBA of cancer metabolism

Quantitative flux prediction using FBA is one of the most promising applications of GEMs. FBA predicts intracellular fluxes based on constraints on the metabolic network. Maximization of an objective function, commonly growth rate, is often used to find a plausible solution. Growth is defined as flux through a biomass equation, containing the constituents of a cell and the energy required for synthesis. Additional consumption of ATP for maintenance is an additional constrain that is often considered. Solutions can be improved by using experimentally measured exchange fluxes to further constrain the problem (Famili et al., 2003). We here discuss similarities and differences between cancer cell lines and microbes that may affect the FBA simulations.

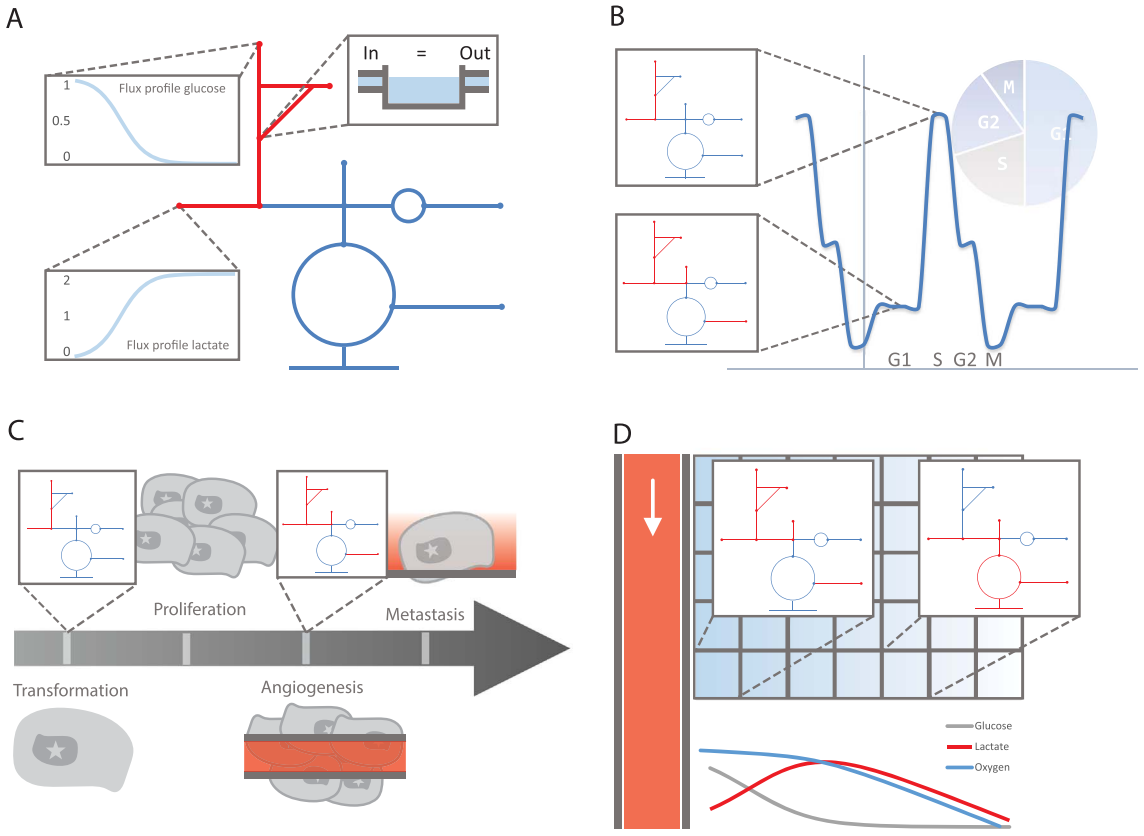

Fig. 3. There are many applications and variants of genome scale modeling. In these schematic representations of the metabolic network red is intended to indicate flux bearing reactions and blue pathways not carrying flux. (A) Dynamic FBA allows the uptake rates to dynamically depend on the extracellular metabolite concentrations, for the internal fluxes steady state is assumed for each uptake rate, this allows prediction on dynamic changes in growth and excretion fluxes. (B) The metabolic tasks may shift over the cell cycle and yield different simulation results. (C) As tumors progress they acquire new metabolic traits. (D) There are spatial differences in external metabolite concentrations potentially resulting in cross feeding between cancer cells.

4.1. Biomass equation

Human cells, including cancer cells, have a high protein content (up to 80%) compared to microbes (Table 2). The remaining biomass consists mainly of lipids and RNA. The RNA content is markedly lower than for microbes, this is likely due to the lower growth rate (Table 2), since RNA and ribosomes have been shown to correlate with growth (Scott et al., 2010). A similar biomass composition is observed in Chinese hamster ovary (CHO) cells (Martínez et al., 2012), a commonly used model of mammalian cell growth. The amino acid composition is not often characterized in human cells, but shows strong similarity amongst mammals (Wu et al., 1999) and with CHO cells (Martínez et al., 2012). The composition has been estimated computationally, by taking the average amino acid composition of expressed proteins (Dolfi et al., 2013), and the experimental and computational compositions appears to be similar (Fig. 4A). Since few amino acids can be synthesized by human cells most of them are taken directly from the medium (or serum). A study of the metabolic requirements for cancer cells (Keibler et al., 2016) shows that close to half of the biomass components are essential. In addition, several of the non-essential biomass components, including lipids, are preferably absorbed (Hosios et al., 2016). This stands in strong contrast to models of microbial growth on minimal medium, where the synthesis of biomass components contributes significantly to the flux profile.

4.2. Maintenance and synthesis energy

As most of the biomass components are absorbed directly from the environment, energy becomes the primary driver behind the observed flux profiles (Keibler et al., 2016). Energy is expended both for synthesis of biomass, which depends on the growth rate, and maintenance, which is growth rate independent (See Fig. 4B). The maintenance energy is more important in humans than in microbes due to the lower growth rate. Maintenance has been estimated to consume up to 65% of the energy at a rate of approximately 1 mmol ATP/gdw/h, based on measurements in Mouse LS cells (KILBURN et al., 1969). For health humans maintenance energy has been established on organ level (Wang, 2012), and is in the range 0.05–2.25 mmol ATP/gdw/h, assuming a conversion factor of 37.5 mmol ATP/kcal and a water content of 70%.

A large share, approximately 30 mmol ATP/gdw, of the synthesis energy is due to polymerization of proteins from amino acids (Keibler et al., 2016). Several studies (Shlomi et al., 2011; Martínez et al., 2012) use parameter values in this range, 24 and 40 mmol/gdw, which is in agreement with experimental measurements from Mouse LS cells (KILBURN et al., 1969). The synthesis cost has also been estimated in growing infants (Towers et al., 1997), where the values are considerably higher 60 mmol ATP/gdw for fat deposition and 200 mmol ATP/gdw for protein deposition, assuming a conversion factor of 37.5 mmol ATP/kcal. This is consistent with observations in microbes, that the synthesis energy calculated from polymerization is markedly lower than the experimentally observed (Verduyn et al., 1991). A study of glioblastoma (Ozcan and Cakir, 2016), used experimentally determined exchange fluxes (DeBerardinis et al., 2007) and estimated the ATP synthesis rate to 3.1 mmol/gdw/h at a specific growth rate of 0.0069 h^{-1}. This corresponds to a synthesis energy of 270 mmol/gdw, assuming maintenance energy expenditure of brain (Wang, 2012) at 1.23 mmol/gdw/h. (Martínez et al., 2012).

Table 2
Comparison between microbes and cell lines for growth rate, cell size, exchange fluxes and biomass.

Parameter	Cell lines[a]	S. cerevisiae (yeast)[b]	Ecoli[c]
Specific growth rate h^{-1}	0.01 ± 0.003 range 0.004–0.017	0.42	1.3
Cell volume μm^3	2100 ± 900 range 500–4700	50	1.1 range 0.44–1.79
Dry weight *gdw cell*$^{-1}$[d]	630×10^{-12}	15×10^{-12}	0.3×10^{-12}
Examples of uptake rates			
-Glucose *mmol gdw*$^{-1}$ h^{-1}	0.85	11	9
-Oxygen *mmol gdw*$^{-1}$ h^{-1}	0.27	4	30
Biomass composition			
-Protein %	82	60	55
-Lipids %	15	3	10
-RNA %	2	12	20

[a] Growth rates and volume from tabulated values of 60 cell lines (Dolfi et al., 2013), volume estimated from cell diameter data. Metabolic fluxes from glioblastoma cell line (Ozcan and Cakir, 2016). Biomass composition from homogenate of HeLa cells (Bosmann et al., 1968).

[b] Growth rate on glucose (Hoek et al., 1998), volume from article figure (Bryan et al., 2010). Flux data at the highest growth rate (Hoek et al., 1998). Biomass composition under anaerobic growth (Nissen et al., 1997).

[c] Growth on glucose and yeast extract (Han et al., 1992). Volume from bio numbers database (Phillips and Milo, 2009) id 100004. Flux data from article figure (Han et al., 1992). Biomass under aerobic growth from the bionumbers (Phillips and Milo, 2009) database id 111490.

[d] Estimated as *volume* × 0.3.

4.3. Objective function

Evolutionary arguments have been used to justify the use of an optimization algorithm to identify a flux distribution that best describes the condition of interest (Feist and Palsson, 2010). Whilst growth maximization seems intuitive and evidently useful for simulating microbes, its usefulness is unclear for more complex organism, particularly for many of the healthy adult cells that may hardly grow at all. Instead maximization of ATP production, a linear combinations of growth and ATP or a set of biological tasks have been used to model healthy cells (Nam et al., 2014; Folger et al., 2011; Agren et al., 2014). It has also been suggested that we may need unique objective functions for each tissue. (Mardinoglu and Nielsen, 2015). An alternative may be to employ the objective of substrate uptake minimization (Raman and Chandra, 2009), minimizing the use of metabolites to complete a specific task. This has been successfully employed in chemostat studies of microbial cells, where the growth rate is fixed by the experimental setup (Nilsson and Nielsen, 2016). For the case of healthy cells this would correspond to identifying the metabolic rates defining the cells tasks, e.g. maintenance energy expenditure.

Growth may seem a more plausible objective for cancer cells, but some caution may be justified. There are large differences between the doubling times of cell lines and tumors, for tumors in vivo they range from 17 to 343 days (Talkington and Durrett, 2015), which is considerably longer than the average of 1.5 days observed in cultured cell lines (Table 2). This may suggest that only a fraction of the tumor is actively growing, in similarity with microbial colonies (Cole et al., 2015); that the death rates are similar in magnitude as the birth rate; or that growth is restricted by other factors. Nor is it necessarily the case that the best solution identified by metabolic modeling will be biologically viable, since tumor suppressors are likely to target the most obvious metabolic loopholes. High proliferation rates may also be selected against by cancer therapies, or the immune system, that often specifically targets rapidly growing cells. There may also be a tradeoff between metastatic ability and growth, or growth and stress resistance (Jerby et al., 2010).

4.4. Flux normalization

In experiments on cell lines it is common to report metabolic fluxes quantified per cell, whilst fluxes quantified per gram dry weight is more common for microbes. The variability in cell size (Table 2), spanning an order of magnitude, is expected to have a large impact on the measured fluxes. One study (Dolfi et al., 2013) used exchange flux data from a

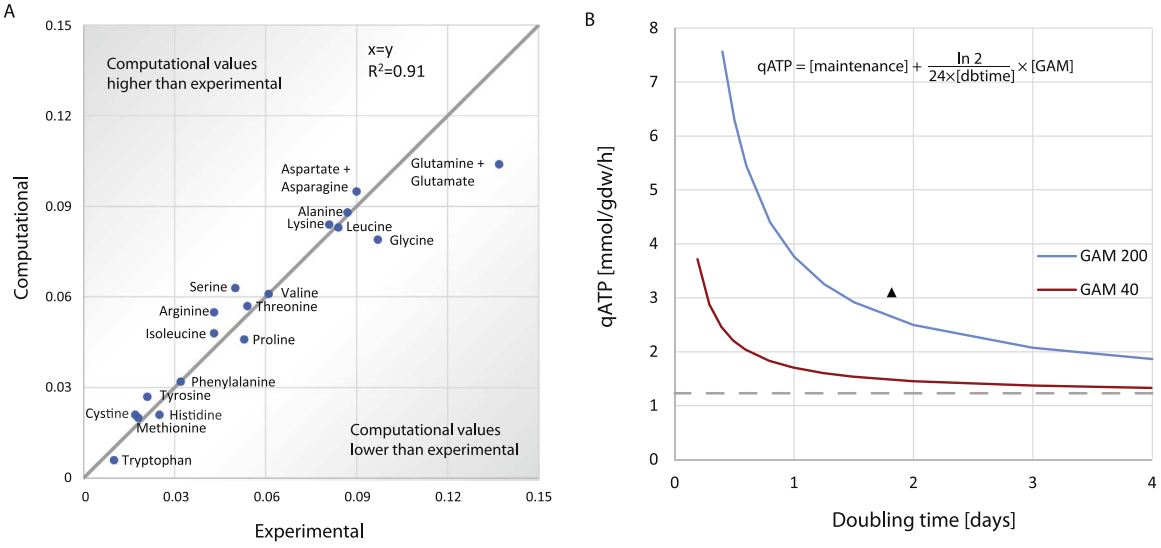

Fig. 4. Parametrization of the biomass equation. A) Comparison of experimentally determined amino acid composition (Wu et al., 1999) with amino acid composition calculated from protein expression patterns (Dolfi et al., 2013). Experimental data from human apart from tryptophan and cysteine which are from pig. The higher experimental values for glycine may be due to measurement issues, as the other mammals have lower values. For glutamate and glutamine the differences may be caused by neglecting the free metabolite pools. B) Comparison of the expected dependency of ATP production on the doubling time and ATP production from literature (triangle) (Ozcan and Cakir, 2016). The maintenance energy (dotted line) from brain cells (Wang, 2012) and the synthesis cost for growth, Growth Associated Maintenance (GAM), taken from protein synthesis in infants (Towers et al., 1997) or as the polymerization cost.

previous study of 60 cell lines (Jain et al., 2012) and normalized them based on the differences in cell size, which partly explained the poor correlation between glucose uptake rate and growth rate observed in the original data. This issue can also be avoided by focusing on the fold changes in concentrations of extracellular metabolites (Aurich et al., 2014). Using this as input to a GEM the authors were able to observe qualitative differences in the glycolytic phenotype between two cell lines. Unfortunately this does not yield accurate estimates of the magnitude of the metabolic fluxes and it is therefore recommended to normalize per gram dry weight as done for microbes.

5. Discussion and outlook

Cancer GEMs have transcended from the early focus on model reconstruction towards applications, through bottom up simulation as well as top down data analysis. GEMs are excellent frameworks to formalize and reconcile biological knowledge from multiple fields and studies, and thanks to their strict mathematical language they force explicit definitions of biological hypothesis with quantitative, verifiable predictions. Cancer is a heterogeneous disease with expression in both the mechanical and information processing dimension. It is therefore desirable to extend GEMs to include as many mechanical processes of the cell as possible, without introducing to much ambiguity and loss of precision, e.g. protein translation, cell division and membrane potential. Similarly it is important to construct an interface between models of signaling and GEMs to enforce a strict mechanistic interpretation of biological circuits and to highlight the limitations of our current knowledge.

Analysis of omics data from cancers reveal a diverse metabolic landscape, where similarity with the tissue of origin many times is bigger than the similarities between cancers (Ghaffari et al., 2015b; Hoadley et al., 2013), and although most tumors origin from the same cancer cell, there is often significant heterogeneity within the tumor (Marusyk and Polyak, 1805). It is a challenge to distinguish incidental differences from important, and metabolic modeling has great potential in mapping out the different metabolic strategies of cancer and cluster divergent genotypic configurations sharing the same mechanism. This will require detailed simulations of both healthy and cancer cells, and may demonstrate how different cell types acquire cancerous traits using a small amount of metabolic adjustment from their healthy origin. Alterations in signaling can in this perspective be seen as a means to reach certain states in the fitness landscape, and by characterizing these states we will start to understand the driving forces behind the metabolic reprogramming in cancer cells.

In conclusion; Genome Scale Metabolic Models of Cancer have been successfully used to interpret high throughput data and to suggest combinatorial therapeutic targets. There still appears to be some challenges regarding quantitative flux predictions relating to uncertainties in the estimated parameters and due to ambiguous normalization of experimentally quantified fluxes. However, the use of additional constraints, in particular protein capacity limitations, appears to be very promising and will likely be used more frequently as data and methods improve. Due to its strict mathematical formulation and the ease by which additional knowledge and constraints can be introduced, we predict that GEMs will become a frequently used tool in the study of cancer metabolism.

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