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Preclinical models for interrogating drug action in human cancers using Stable Isotope Resolved Metabolomics (SIRM)

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Abstract

Aims—In this review we compare the advantages and disadvantages of different model biological systems for determining the metabolic functions of cells in complex environments, how they may change in different disease states, and respond to therapeutic interventions.

Background—All preclinical drug-testing models have advantages and drawbacks. We compare and contrast established cell, organoid and animal models with ex vivo organ or tissue culture and in vivo human experiments in the context of metabolic readout of drug efficacy. As metabolism reports directly on the biochemical state of cells and tissues, it can be very sensitive to drugs and/or other environmental changes. This is especially so when metabolic activities are probed by stable isotope tracing methods, which can also provide detailed mechanistic information on drug action. We have developed and been applying Stabile Isotope-Resolve^d Me^ta^bo^lo^mi^cs (SIRM) to examine metabolic reprogramming of human lung cancer cells in monoculture, in mouse xenograft/explant models, and in lung cancer patients in situ (Lane et al. 2011; T. W. Fan et al. 2011; T. W-M. Fan et al. 2012; T. W. Fan et al. 2012; Xie et al. 2014b; Ren et al. 2014a; Sellers et al. 2015b). We are able to determine the influence of the tumor microenvironment using these models. We have now extended the range of models to fresh human tissue slices, similar to those originally described by O. Warburg (Warburg 1923), which retain the native tissue architecture and heterogeneity with a paired benign versus cancer design under defined cell culture conditions. This platform offers an unprecedented human tissue model for preclinical studies on metabolic reprogramming of human cancer cells in their tissue context, and response to drug treatment (Xie et al. 2014a). As the microenvironment of the target human tissue is retained and individual patient's response to drugs is obtained, this platform promises to transcend current limitations of drug selection for clinical trials or treatments.

Conclusions and Future Work—Development of ex vivo human tissue and animal models with humanized organs including bone marrow and liver show considerable promise for analyzing drug responses that are more relevant to humans. Similarly using stable isotope tracer methods with these improved models in advanced stages of the drug development pipeline, in conjunction

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with tissue biopsy is expected significantly to reduce the high failure rate of experimental drugs in Phase II and III clinical trials.

Keywords

SIRM; cell culture; PDX models; tissue slices; metabolism

Introduction

The development of therapeutic agents from concept to clinical use is a lengthy and costly process that often fails at the clinical trial stage (Adams and Brantner 2010; Subbaraman 2011). In oncology, the failure rate is approximately 90% at phase II/III clinical trials (Arrowsmith 2011; Amiri-Kordestani and Fojo 2012), in part attributable to inadequacies of preclinical testing. The concept of a therapeutic target is often based on biological knowledge in model studies that can be incomplete or irrelevant to humans, and without accurate models, it is extremely difficult to predict efficacy, toxicities and differences in an individual patient's response to a particular treatment.

In oncological drug screening, preclinical models typically include panels of cells in vitro, in which the response criteria are typically cell death and/or inhibition of cell growth, and proof-of-concept by manipulation of target gene expression (e.g. shRNAs, gene ablation or overexpression). Similar screening criteria apply to in vivo animal models, most often the mouse model, which are interrogated for tumor regression or growth arrest. The animals may also be used for assessing pharmacokinetic properties of test drugs in vivo, i.e. absorption, distribution, metabolism, excretion, and toxicity (ADMET) (T.W-M. Fan et al. 2012).

Although cell death or growth arrest are key end points to determine the efficacy of a new drug, they provide little information about the underlying molecular mechanism of a drug on target tissues or off-target toxicities. Global gene expression profiles can provide valuable molecular information, but they cannot fully capture alterations in cellular and tissue functions. Metabolism represents the net activity of all live cells, whether they are proliferating or quiescent. As such, the metabolic activity of cells is a natural readout of their functional state. Therefore, analysis of cell metabolism in a tissue context and its response to drug intervention not only provides quantitative measures of response but is also an integral part of deciphering the molecular mechanism of drug action.

Metabolic networks are complex and highly interconnected, and are regulated at the gene, protein, and substrate levels. For example, the activity of an enzyme can be controlled at numerous levels including the rate of transcription, alternative splicing, mRNA stability, translation control, post-translational modification which may be covalent (e.g. phosphorylation (Ruprecht and Lemeer 2014), acetylation (Choudhary et al. 2009), OGlcnAcylation (Zachara and Hart 2004; Hahne et al. 2012) among many others (Huang KY et al. 2016; Hornbeck 2015)) or non covalent “allosteric” interactions, as is commonly observed in central metabolism such as feedback inhibition of phosphofructokinase-1 (PFK-1) by ATP and citrate, up regulation of PFK-1 by fructose-2,6-bisphosphate, feed-forward activation of pyruvate kinase by fructose-1,6-phosphate (Christofk et al. 2008) and

reciprocal activation of pyruvate carboxylase and inhibition of pyruvate dehydrogenase by acetyl CoA (Jitrapakdee et al. 2008). Indeed, the catalytic activities of a large fraction of enzymes in metabolic networks are modulated by interactions with metabolites. From a systems biochemical standpoint, any compound that is transformed via enzymic activity is a metabolite, regardless of size. As such, enzymic modifications of proteins and nucleic acids lie within the realm of metabolic interrogations.

Mammalian cells are compartmented both physically and dynamically, such that substrate availability can greatly vary across different compartments. It is often the case that different isoforms of enzymes (e.g. IDH 1,2,3; ME 1,2,3, MDH1,2, GLS1,2, AST or GOT1,2) (Minárik P. et al. 2002; Ren et al. 2014b; I.J. Arinze and R.W. Hanson 1980; I. J. Arinze and R. W. Hanson 1980)), have different coenzyme specificities, and are differentially expressed in various subcellular compartments or have tissue specificity (Uhlen et al. 2015). In addition, some enzymes (e.g. lactate dehydrogenase) have alternative functions unrelated to their catalytic activities, that are contingent on location in other compartments, such as the nucleus (Sriram et al. 2005; Kim and Dang 2005). Furthermore, terminally differentiated cells may express different isoforms compared with the progenitor or stem cells. The de-differentiation of cancer cells in high grade tumors is associated with expression of fetal isoforms, also known as retrodifferentiation (Uriel 1979; Mathupala et al. 2010) (Marin et al. 2009; Lincet and Icard 2015).

Furthermore, the interconnected nature of metabolic networks gives rise to “hub” metabolites, which participate in multiple pathways within a network, such as ATP, NAD⁺, pyruvate or glutamate with numerous inputs and metabolic fates (cf. Fig 1) (Arita 2004; Pfeiffer T. et al. 2005). As hub metabolites are present in multiple compartments, globally measuring steady state levels of metabolites is insufficient for elucidating compartmentalized metabolic networks and their dynamics. For example, as the end product of glycolysis, pyruvate is commonly used to inform on the glycolytic pathway and activity. However, pyruvate can participate in multiple other pathways in different compartments. In the cytoplasm it may be reduced to lactate, or transaminated to alanine. It can be transported into the mitochondrion, and be oxidatively decarboxylated to acetyl CoA (AcCoA), or carboxylated to oxaloacetate (OAA) (Fig. 1A). It is also the product of the decarboxylation of malate in both the mitochondrion and the cytoplasm catalyzed by three isoforms of malic enzyme (Ren et al. 2014b). Moreover, pyruvate is the end product of catabolism of several amino acids, including Ala, Ser, Cys, Thr, Trp, and Gly (Fig. 1B).

With the recent recognition of metabolic reprogramming as a key hallmark of human cancer (Hanahan and Weinberg 2011), there has been increasing interest in developing cancer therapeutics based on specific enzyme target(s), which links understanding of fundamental metabolic dysregulations to drug development. When administered to a cell, a highly specific inhibitor of an enzyme is expected to result in an increase in the substrate concentration and a concomitant decrease in the product concentration. The increase in the substrate level will tend to decrease the activities of enzymes upstream both by thermodynamic effects (as the molecule is the product of the preceding enzyme in the pathway) and by product inhibition. Similarly the decrease in the product will tend to diminish the rate of the downstream reactions as the substrate is less available. In practice

the observed influence of manipulating specific enzyme activities on metabolism may be more complex because very few metabolic pathways are isolated from one another, and the flux through any metabolic segment depends on the properties of all of the enzymes and substrates in that segment. This is sometimes cast in terms of Biochemical Systems Theory (Savageau et al. 1987a, 1987b) and metabolic control theory (Fell 1997; Poulou et al. 2012; Cascante et al. 2012), which can be readily applied to unbranched pathways, though they can also be applied to the more relevant branched pathways in networks (Cascante et al. 1989)). MCA recognizes that the control of metabolic flux is not absolute and may be shared among many enzymes and their regulators, a good example of which is glycolysis. Once glucose is phosphorylated to G6P by ATP via hexokinase (thus linking glucose metabolism directly to ATP metabolism), it may proceed linearly through to the product pyruvate, or its carbon may be diverted at multiple steps including G6P (entry to the pentose phosphate pathway), F6P and GAP (non oxidative branch of the PPP), F6P (entry to the hexosamine phosphate pathway), DHAP (glycerol phosphate and lipid biosynthesis), 3PGA (serine-glycine pathway) (Fig. 1A). The reaction catalyzed by GAPDH uses NAD^+ and generate protons and NADH, linking glycolysis to the cellular redox status. Many of the glycolytic enzymes, especially those at branch points are regulated by covalent modifications and by non-covalent allosteric interaction with metabolites, linking the regulation and flux of glycolysis to many other pathways.

Consider the simple pathway in Scheme 1 (Lane et al. 2008b), the rate of production of different metabolites at steady state is given by the following equations.

$$dl_o(t)/dt = k_1 k_2 k_3 k_4 g / [(k_2 + k_5)(k_3 k_4 + k_7(k_{-3} + k_4))] + k_3 k_4 k_6 b / [k_3 k_4 + k_7(k_{-3} + k_4)] \quad (1A)$$

$$da(t)/dt = k_1 k_5 g / (k_2 + k_5) \quad (1B)$$

$$dc(t)/dt = k_1 k_2 k_7 (k_{-3} + k_4) g / [(k_2 + k_5)(k_3 k_4 + k_7(k_{-3} + k_4))] + k_6 k_7 (k_{-3} + k_4) b / [k_3 k_4 + k_7(k_{-3} + k_4)]$$

(1C)

where l_o , a, c, g, b are the concentrations of Lac_o, G, A and C.

Under conditions of low enzyme saturation, or high free enzyme concentration, the rate constants can be interpreted as $k_i = k_{cat,i} e_i / K_{m,i}$ (and of equivalent complexity for multisubstrate enzymes) (Poulou et al. 2012; Roberts et al. 1985). e_i is the free concentration of the i^{th} enzyme. If k_2 were greatly decreased, most of the Lac and C now derives from B, and the rate of production of A increases. However if k_2 were smaller than k_5 , inhibiting enzyme 2 has only a small effect on da/dt . In contrast, decreasing k_3 results in a loss of

lactate production, accumulation of intermediate X and an increased flux to C. In the absence of reversibility, the rate of production of A is unchanged.

In order to disentangle and manipulate these multiple levels of control over metabolic activity to achieve desirable outcomes, it is necessary to employ appropriate approaches and tools, including the use of stable isotope tracers for delineating altered metabolic networks and their compartmentation, relevant model systems, bioinformatics (e.g. modeling of network dynamics), and tracer atom-resolved analytical platforms (e.g. NMR and mass spectrometry) as in Stable Isotope Resolved Metabolomics (SIRM) (Sellers et al. 2015b; T.W-M. Fan et al. 2012; Winnike et al. 2012). In the SIRM approach, isotopically enriched precursors such as ^{13}C glucose or ^{13}C , ^{15}N glutamine are provided to the system, and the individual atoms of the source molecule are traced through the metabolic network as a function of time. With NMR and mass spectrometry platforms, the data collection is essentially untargeted, though for testing specific hypotheses, it is usual to choose analytes prior to data collection and analysis, and the remainder can be used for post hoc discovery analyses. This is to be able to control the false discovery rate when multiple under multiple hypothesis testing. The analytical requirements for SIRM are identical to metabolic profiling, which is a subset of the SIRM approach. The main difference lies in the introduction and choice of enriched precursors, and the downstream analysis. Quantification and identification of the metabolites has the same requirements as for profiling, and is described in several publications (T. W.-M. Fan and Lane 2016; T. W.-M. Fan et al. 2008; Lane et al. 2008a; Lane et al. 2009; T.W-M. Fan et al. 2012; Mitchell et al. 2014). A major difference is in the isotopologue and isotopomer distribution analyses, and for pathway reconstruct and flux analysis, the proper choice of modeling (Wolak et al. 2012; Cascante et al. 2012; Buescher et al. 2015). In the following, we illustrate how these approaches and tools can be applied for the purpose of metabolism-based drug development.

Human subject research

From a clinical perspective, the human subject is the gold standard and all new therapeutics must undergo extensive testing through clinical trials. Compared with simple model systems, human subjects research requires an extensive approvals process involving Institutional Review Boards (IRBs) and generally involves extensive coordination of teams of researchers for tissue procurement and biostatistics (Sellers et al. 2015b; Bousamra et al. 2012; T. W. Fan et al. 2009; Lane et al. 2011). The analytical and functional interpretation of human data is also much more demanding due to the system's complexity and very limited ability for experimental manipulations (cf. Table 1). Nevertheless, several groups including ours have successfully embarked on stable isotope tracing in human subjects using either in vivo NMR spectroscopy or analysis of resected tissues that have led to new insights into the metabolic reprogramming in the disease under study (Hensley et al. 2016; Maher et al. 2012; Hyder et al. 2013; Boumezbeur et al. 2010; T. W. Fan et al. 2009; Sellers et al. 2015b) (Nelson et al. 2013; Wilson and Kurhanewicz 2014; Mason et al. 2002). Although such direct human-derived data can be difficult to interpret unambiguously, it does provide an authentic reference with relevant clinical data, against which more detailed and mechanistic information obtained from model systems can be compared, because the latter systems

(including the ex vivo human tissue systems) are generally less complex and have more experimental flexibilities (see below).

Before a clinical trial of a therapeutic agent can be conducted, there are many required steps to show likelihood of efficacy and tolerable toxicities. These involve model systems research as discussed in the following.

Preclinical models

There are numerous preclinical models that are commonly used, as summarized in Table 1. All of these models have significant advantages and limitations.

(i) cell culture

Multiple cell lines, such as the NCI-60 panel, are commonly used to assess overall response to a cancer drug but they often do not recapitulate well the complexities of drug response in real tumors (Niu and Wang 2015; Wilding and Bodmer 2014; Jack et al. 2014), particularly if the appropriate in vivo conditions are not met (Davidson et al. 2016; Xie et al. 2014a), nor can such in vitro models capture the complexity of tumor heterogeneity (Hensley et al. 2016). Nevertheless, such panels are still valuable as they can provide initial guidance on which cancer systems that might be worth investigating in greater details. In addition, established cell lines can be fully manipulated for addressing specific questions, such as environmental (e.g. nutrient and oxygen supplies) relevance of drug action or the functional relevance of metabolic genes by genomic editing of specific genes using CRISPR technology (Sander and Joung 2014) (Ledford 2015), down-regulation of mRNA (Moore et al. 2010), or introduction of an inducible transgene (Pajic A. et al. 2000; Belteki et al. 2005; Saunders 2011; Xie et al. 2014a). They can also be readily used to investigate the influence of metabolic gene mutations, gene amplifications, or splice variants on cancer cell growth, survival, and development. When combined with detailed gene expression profiling and pathway tracing or flux analysis under different conditions (e.g. hypoxia, nutrient deprivation), it is possible to determine the mode(s) of drug action and additional vulnerabilities of the cells that may help refine the drug or suggest synthetic lethal combinations. Furthermore, cell culture systems can be used to define off target effects on normal cell counterparts, or cells derived from different organs. A major problem with many drugs is the off-target toxicity to critical organs, such as liver (Ostapowicz G. et al. 2002), kidney (Herzenberg 2009) or heart (Feenstra et al. 1999).

Primary cells can be cultured under defined conditions for drug testing. However, such cells are generally derived from terminally differentiated tissues with limited cell passage capacity. They are grown in the presence of a cocktail of growth factors to force cell divisions, after which the cells senesce and die. Forcing such cells to proliferate is somewhat artificial, which may confound true drug response. In addition, different batches of the primary cells are distinct, making longer-term comparison more complex.

An alternative to the primary cell is to use immortalized cells lines, or ones that have been freshly immortalized such as the lymphoblastoid cells (Niu and Wang 2015; Jack et al. 2014). The latter are Epstein-Barr Virus (EBV) immortalized peripheral blood lymphocytes

(Neitzel 1986), that are widely used to understand genetic variations in human populations (Cheung. et al. 2003), but may be less suited to studies of drug responses relevant to radically different cell types such as epithelial cells. Furthermore, common methods of immortalization introduce a viral protein (such as SV40 large T antigen, EBV EBNA2, 3C, HPV E6 and E7) that directly or indirectly inactivates proteins essential to the cell cycle control, including pRB, p53 (Sugimoto et al.; Ahuja et al. 2005) or overcoming telomere-dependent senescence via hTERT (K. M. Lee et al. 2004). From a metabolic and functional perspective however, immortalization itself can have a large effect on cell metabolism (S. Telang et al. 2006; S. Telang, Lane, A.N., Nelson, K.K. Arumugam, S. &, Chesney, J.A. 2007), dependent to some extent on the method of immortalization.

(ii) Organoid cultures

2D cultures represent the simplest system that affords the greatest experimental control. The growth of adherent cells on plastic surface and the absence of cell-cell interactions is a major criticism of this system. However, some adherent cells, such as Caco-2, differentiate and organize once they become over-confluent, thereby representing an intermediate level of complexity (Natoli et al. 2012). 3D culture or spheroids can be generated from established cell lines by growing them on an appropriate matrix (such as collagen $\pm$ laminin synthetic extracellular matrix, hydrogel scaffolds, among many others (Lee J et al. 2008; Ravi et al. 2014)), such that they spontaneously form 3D structures that in many cases mimic those found in natural tissue (Bissell et al. 2003) (G. Y. Lee et al. 2007; Wehrle et al. 2000). It is also possible to generate spheroids or whole organoids containing multiple cell types (G. Y. Lee et al. 2007; Willyard 2015; McCracken et al. 2014; Spence et al. 2011), which much more closely mimic the natural tissue, while retaining the flexibility for experimental manipulation in much the same way as 2D cultures (Wehrle et al. 2000; Pawlik et al. 2000). Such systems have been shown to display altered gene expression profiles and drug response compared with the corresponding 2D cell culture systems (Ravi et al. 2014). However, to date little work using metabolic tracing has been performed on these models.

(iii) Mouse models

Mouse models are widely used for proof-of-concept drug testing as well as for analyzing drug metabolism. It should be noted that mice have much higher metabolic rates than humans (roughly 7 times as fast on average) with metabolic rates varying widely among different tissues and physical activity (T. W.-M. Fan et al. 2011; Lane et al. 2011), which is expected to influence drug response and metabolism. In addition, mice may express a variety of enzyme isoforms differently from the human homologues, which are also likely to impact drug response and metabolism. For example the distribution of cytochrome P450 genes in the mouse liver is substantially different from that in human liver (Muruganandan and Sinal 2008), which can lead to distinct drug metabolism. Also different is the distribution of immune cells types between mouse and man (Mestas and Hughes 2004; Yue F 2014; Gould et al. 2015), which would affect responses to immune-modulators.

In many ways the syngeneic mouse models are the simplest animal model, where tumors can be grown either orthotopically or ectopically in the background of an intact host immune system. As such, there are no mismatch between the metabolic properties of the syngeneic

tumor and the host organism. It is also possible to introduce one or a small number of human transgenes into the mouse (GEMMs), to determine how the expression of such gene(s) functions in vivo (Xu and Peltz 2016; Chise Tateno et al. 2015a; Ishida et al. 2015; Hasegawa et al. 2011; C. Tateno et al. 2004; A. Richmond and Y. J. Su 2008). Although such models may be useful for addressing fundamental biological questions, they are often poor models of human diseases, for reasons stated above.

For cancer research, various immune compromised strains of mice are extensively used, as it is practical to implant human cells or tissues into the mouse host. There are now many such mouse models available, from the early *Foxn1* nude mouse (Fogh 1982) to the more recent SCID/NOD/gamma (NOD.Cg-*Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ*) or NSG and variants (Shultz et al. 2007). These mice lack mature T cells, B cells, and natural killer (NK) cells, are deficient in multiple cytokine signaling pathways, and have many defects in innate immunity. Clearly these are great resources for mouse xenograft-based experiments including the now popular patient derived xenograft (PDX) mouse models, but they suffer from the disadvantage of a largely absent immune system. Furthermore, although genomic stability and expression profiles seem to remain stable over several generations in different PDX models (A. Richmond and Y. Su 2008; Tentler JJ et al. 2012; Wong et al. 2014; Kopetz et al. 2012), significant differences in miRNAs and other genetic properties have been observed by comparing F1 with F0 tumors (Siolas and Hannon 2013).

In some tumors such as pancreatic ductal adenocarcinoma (PDAC), the stroma accounts for the majority of the cell populations in the tumor, which is expected to have major influence on cancer cell metabolism and drug response (Beloribi-Djefalia et al. 2015; Delitto et al. 2015; Guillaumond et al. 2013a). As the fibroblasts and other stromal cells of the original human tumor do not proliferate significantly, whereas the cancerous cells do, an increase in tumor volume is due either to an increase in the cancer/stroma ratio and/or to replacement of human stroma with those of the host. The human stroma-cancer interaction that is characteristic of an individual tumor is lost in later generations, such that drug responses may not be fully recapitulated in such PDX models (Martinez-Garcia et al. 2014).

Indeed, differences in metabolic reprogramming have been observed when comparing cellular xenografts with 2D cultures (Davidson et al. 2016), which point to an influence of the tumor microenvironment (TME). Such an influence is likely to hold true for PDX models once the human stroma has been replaced by mouse stroma, despite the genetic stability demonstrated for PDX tumors. Altered cancer cell metabolism has been implicated in modulating the cancer cell's interaction with TME to enable its escape from immune surveillance (Zamanakou et al. 2007; Ho et al. 2016) and to promote tumor progression, angiogenesis, survival, and metastasis, (Wysoczynski and Ratajczak 2009; Hanahan and Weinberg 2011; Thayanithy et al. 2014; K. Leithner et al. 2014; Caneba et al. 2014; Bonuccelli et al. 2010; Stern et al. 2002; Walenta et al. 2004; Guillaumond et al. 2013b; Sonveaux et al. 2008). Thus, the replacement of human stroma by the mouse counterpart in PDX models may fail to recapitulate the human stroma-cancer cell interactions of individual patients and therefore the drug response.

Drug response of PDX tumors can be further complicated by the observation that the tumor architecture in various immune deficient mice was noticeably different (Maykel J. et al. 2014). It is also unclear whether the orthotopic implantation is more appropriate than ectopic implantation such as subcutaneous or under the kidney capsule. The orthotopic location is designed to mimic the organ environment more closely than ectopic xenografts, though the biochemical and physical mismatch between human and rodents may mitigate this advantage, especially when orthotopic implantation and monitoring is difficult. It seems likely also that the origin of the primary tumor, its heterogeneity (Cassidy et al. 2015), and grade are likely to influence the subsequent physiological and biochemical behavior on implantation, which may need to be carefully characterized in the PDX model on a case by case basis. Even when some case studies have shown that the PDX model can predict the human drug response (Whittle et al. 2015; Maykel J. et al. 2014), the fact remains that the mouse model may not recapitulate the off target effects as there is no corresponding implanted normal human tissues. Finally, the long duration for establishing resected human tumors in PDX model and in some instances the low “take rate” compromises the need for timely feedback on drug testing to inform on efficacious personalized treatment (Hidalgo et al. 2011) (Damhofer et al. 2015; Aparicio et al. 2015).

A possible partial solution to some of these issues with mouse models is to use NSG or NOG mice that have been further engineered to produce humanized tissues (e.g. liver (Azuma et al. 2007; Chise Tateno et al. 2015b; Yoshizato and Tateno 2009) or bone marrow (Azuma et al. 2007; Bility et al. 2012; Michael A. Brehm et al. 2010; Covassin et al. 2013)), many of which are now commercially available. The humanized liver results from the replacement of a substantial fraction of the mouse hepatocytes with human hepatocytes, which express the relevant complement of human proteins, and as such are better models for ADMET studies (Muruganandan and Sinal 2008) (Chise Tateno et al. 2015b; Yoshizato and Tateno 2009) and for monitoring metabolic responses to drugs (C. Tateno et al. 2004). Similarly, engraftment of human hematopoietic stem cells enable mice to produce functional human B and T cells, thereby allowing for the study of a disease in the presence of a (partial) human immune system (Michael A. Brehm et al. 2010; Covassin et al. 2013). By comparing with the fully immune compromised model, the contribution of human immune surveillance can be analyzed. Nevertheless, such humanized models are still inadequate, as there remains the mouse innate immunity, and the levels of both innate and adaptive human immunity are relatively low (M.A. Brehm et al. 2013). This is a very active area of development, and improved humanized models can be expected.

Considering the large distinction between mouse and human metabolic activities, it is crucially important to investigate the metabolic networks and dynamics in mouse xenograft tissues for comparison with the parent human tumor tissues and cells. Such comparison can inform not only the influence of TME but also the metabolic functioning that may underlie differential drug responses. We have performed SIRM experiments on human tumor tissue slices and mouse xenograft models; the latter include ectopic implantation of established cell lines and human tissues (Lane et al. 2015; Xie et al. 2014a; Lane et al. 2011; Sellers et al. 2015a) as well as orthotopic implantation of lung and breast cancer cells (T. W.-M. Fan et al. 2011) (Lane, Fan unpublished data). Figure 2 compares ex vivo patient NSCLC tumor tissue versus its corresponding tumor xenograft in NSG mouse in terms of ^{13}C incorporation

from $^{13}\text{C}_6$ -glucose or $^{13}\text{C}_5$, $^{15}\text{N}_2$ -Gln into glycolytic products ^{13}C -lactate (Lac) and ^{13}C -Ala, the Krebs cycle metabolites ^{13}C -Asp, ^{13}C -Glu, and ^{13}C -Gln, the products of glutathione biosynthesis ^{13}C -GSH+GSSG, and the products of PPP plus nucleotide biosynthesis ^{13}C -1' (riboseyl) -AXP (adenine nucleotides) and -UXP (uracil nucleotides). It is clear that both glucose (glycolysis) and Gln (glutaminolysis) metabolism in the patient lung tumor tissue with mouse stroma (PDX model) is much more active than that in human lung tumor tissue with human stroma (slice model). These data in turn suggest that tumor metabolism in the PDX model is distinct from that in vivo in patient tumor tissues, since the ex vivo metabolism recapitulates that in vivo for human tumor tissues (Lane et al. 2015; Xie et al. 2014a; Lane et al. 2011; Sellers et al. 2015a) (Lane, Fan unpublished data). As such, PDX models may not be predictive for human lung cancer patients in terms of drug responses.

The above SIRM analyses have been performed on tissue extracts. It is also feasible to conduct metabolic measurement (including unidirectional rates of enzymic reactions) on live animals such as the mouse model using localized NMR spectroscopy (D.G. Gadian 1995; D. G. Gadian 1986). Such in vivo metabolic studies can be combined with stable isotope tracing for real-time tracking of metabolic reactions in a number of pathways. These in vivo approaches are particularly useful for tracing brain and liver metabolism as these organs have a high metabolic rate and are comparatively magnetically homogenous. High quality ^{13}C , ^{31}P and ^1H spectra can be acquired with good spatial and time resolution due to the availability of wide bore high field spectrometers (i.e. 9.4 T and above) (P.E. Thelwall et al. 2012; K. R. Keshari et al. 2011; Hu et al. 2012; T. W.-M. Fan and Lane 2016; de Graaf et al. 2011; Patel et al. 2005). In addition to tracers, the mouse can be readily infused with drugs, which enables in vivo analysis of not only metabolic reprogramming in the diseased organ but also its response to therapeutics in terms of metabolic pathways and fluxes (P. E. Thelwall et al. 2005) (Wolak et al. 2012); (T. W.-M. Fan and Lane 2016; T.W-M. Fan et al. 2012). The in vivo tracing approach can also be applied to the humanized mouse models to interrogate human tissue (e.g. liver) metabolism in live animals, with or without a human immune system.

(iv) Tissue/organ cultures

Whole organisms whether human or mouse models are the most complex experimental system to deal with. Apart from the ethicolegal considerations (Bousamra et al. 2012) (and see above), experimental studies with whole organisms must deal with many other complications, such as interactions among multiple organs, lack of precise control of nutrient and tracer supplies or waste removal, and difficulty with time-dependent tissue sampling, to name just a few. An intermediate level of complexity that circumvents many of these problems is to work with isolated perfused organs (such as the Langendorff perfused heart preparation (Chatham and Seymour 2002), everted small intestine (Dixit et al. 2012), perfused kidney (Scaduto and Davis 1985), liver and intestinal slices (deGraaf et al. 2010) or tissue fragments (Hougardy et al. 2008; Kirby et al. 2004; Katharina Leithner et al. 2014). Perfused organ studies are limited to animal studies, but it is possible to use freshly biopsied or resected human tissues, either as small fragments (Katharina Leithner et al. 2014) or as thin slices as originally described by Warburg (Warburg 1923; Berndt 1976; Freeman and Oneil 1984; F. T. Unger et al. 2014; Vaira et al. 2010; F.T. Unger et al. 2015; deGraaf et al.

2010) (Kirby et al. 2004) and further developed for tracer-based metabolic studies (Keshari, Sriram et al. 2013; (T. W.-M. Fan et al. 2016a; Kayvan R. Keshari et al. 2013). It is possible to evaluate tissue metabolism using these models by profiling, or lower metabolic resolution methods that determine changes in NAD(P)H via its autofluorescence. The latter requires time resolved techniques and predicated on the observation that NAD⁺/NADH couples are tightly associated with catabolism (and NADP⁺/NADPH couples with anabolism, and see above), that have single cell resolution, but it is relatively difficult to assign specific metabolic segments from the NAD(P)H levels alone (Stringari et al. 2015; Blacker et al. 2014; Stringari et al. 2012; Rose et al. 2006; Uppal and Gupta 2003). As from the analytical standpoint, SIRM has the same requirements as profiling, there is no speed advantage to profiling alone, though clearly this is advantageous if SIRM cannot be applied to tissue slices.

This biopsy/slice approach has the advantages of 1) maintaining the native human tissue 3D architecture or TME including resident or infiltrating immune cells; 2) compatible with stable isotope tracer methods; 3) flexibility of tracer use and treatment options; 4) delineation of target tissue responses without systemic influences; 5) complete experimental control of the environment including nutrient supply; 6) circumvention of genetic, physiologic, and environmental complications in elucidating treatment responses by adopting the paired cancerous and surrounding non-cancerous tissue design; and 7) the ability to determine treatment effects on target tissues on an individual patient basis.

For example, we have shown that thin slices of benign and cancerous (CA) lung tissue from the same individual can be kept viable under defined cell culture conditions for at least 48 h, and that the benign and CA slices show metabolic activity commensurate with that observed in vivo (Sellers et al. 2015a). Furthermore, they showed differential metabolic network responses to a variety of agents including the selenium compound selenite (Fig 3), LDH-A inhibitor (Xie et al. 2014a) and a macrophage activating compound β -glucan (T. W.-M. Fan et al. 2016b). These SIRM studies of human lung tissue slices not only validated the known molecular mode of drug action but also helped elucidate other unexpected drug action directly on target human tissues. Both types of knowledge should greatly facilitate preclinical drug testing for efficacy on an individual patient basis.

Clinical considerations

In a clinical setting, the physician must be able not only to diagnose the disease accurately, but also find a treatment regimen that optimally suits the individual. For an aggressive disease such as cancer, time is an important clinical consideration. A drug-testing regimen for individual patients therefore need to be fast as well robust and reliable. 2D cell cultures using lymphoblastoid or established tumor cell lines or equivalent 3D cultures are relatively fast to perform, but cannot recapitulate in vivo conditions (for reasons described above), nor do they take into account individual differences in drug responses. PDX mouse models, especially first generation can circumvent some of these issues but they are intrinsically slow to generate, as it can take weeks for the tumor to become established, which is incompatible with rapid clinical decision-making. The number of mice that can be implanted and tested is comparatively small, which limits the number of drugs that can be tested. In contrast, the ex

vivo target tissue fragments is relatively fast to perform; testing outcome including individualized response and off target effects can be obtained within 2-3 days of the initial biopsy using metabolomics. This time frame is compatible with clinical decision-making. This is assuming 24 h incubation in the tracer solution $\pm$ inhibitors, followed by metabolite extraction and data collection and analysis. For preclinical analyses, more time is available for extensive data work up and analysis in an untargeted approach (e.g. to shed light on what the off-target effects might be). In the clinical setting, the metabolite analyses are more likely to be targeted based on the clinical features of the disease. For example, given the pathological diagnosis of the tumor subtype, the range of drugs to be tested would be expected to have specific metabolic profiles, and those metabolites that directly report on the relevant pathways would be analyzed, according to expected responses based on extensive preclinical databases. Nevertheless, in the pre-clinical and clinical settings, issues of reliability, consistency and reproducibility are paramount, as enshrined in the principle of Good Laboratory Practice (GLP), which covers all aspects of the analytical pipeline from sample collection through to statistical analyses, and the relevant level of training and oversight this implies.

By combining the tissue slice with the SIRM approach, the level of molecular details about the drug action and side effects (by relating to the pathological changes determined on the same tissue fragments using e.g. histochemistry) is unprecedentedly extensive (T. W.-M. Fan et al. 2016b). Such preclinical human models could also be used to evaluate drug resistance and likelihood of recurrence, especially when combined with genomic data.

Conclusions and future directions

Given the stated limitations, no single model of drug screening for developmental or clinical purpose is likely to provide sufficient information regarding the drug efficacy or mode of action. It is clear that any drug development pipeline should employ multiple models (Table 1), and seek to integrate the information from each model. For example, in the early-stage of drug development, cell lines are appropriate model for screening, particularly for proof of principle target validation via molecular genetic manipulation. Once lead compounds are available, more complex models should be adopted such as cell xenograft, GEMM, PDX, or the newer humanized mouse models. When feasible, drug testing on fresh patient tissues provides individualized therapeutic index (e.g. cancer versus benign) in a timely fashion to contribute towards individualized choice of therapeutics by physicians.

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Abbreviations

IRB	Institutional Review Boards
PDX	patient derived xenograft
(m) SIRM	(multiplexed) Stable Isotope-Resolved Metabolomics

Figure 1. Intersecting networks involved in glucose metabolism

A. Glycolysis and associated carbon fates.

G6P: glucose-6-phosphate; F6P fructose-6-phosphate; F1,6BP fructose-1,6-bisphosphate; GAP glyceraldehyde-3-phosphate; DHAP dihydroxyacetone phosphate; 1,3bisPG 1,3-bisphosphoglycerate; 2PGA 2-phosphoglycerate; PEP phosphoenolpyruvate; Pyr pyruvate; OAA oxaloacetate; AcCoA acetyl CoA; Lac lactate; Ru5P ribose-5-phosphate; HK hexokinase; G6Pase glucose-6-phosphatase; PGI phosphoglucose isomerase; PFK1 phosphofructokinase 1; FBPase fructose 1,6 bisphosphatase; ALD aldolase; GAPDH glyceraldehyde-3-phosphate dehydrogenase; PGK phosphoglycerate kinase; PGM phosphoglycerate mutase; ENO enolase; PK pyruvate kinase; LDH lactate dehydrogenase; ALT alanine transaminase; PC pyruvate carboxylase; PDH pyruvate dehydrogenase; PPPox oxidative branch of the pentose phosphate pathway; PPPnx non-oxidative branch of the pentose phosphate pathway

B. Krebs Cycle, anapleurosis and amino acid catabolism Citrate (via ATP dependent citrate lyase), α KG (α -ketoglutarate) and OAA are respectively anabolic precursors for fatty acid, protein, and nucleotide biosynthesis. Replenishment of the Krebs cycle carbon is achieved via glutaminolysis, pyruvate carboxylation or amino acid oxidation. After transamination, the carbon skeletons of the amino acids enter the Krebs cycle at different points. Those in

red are glucogenic, i.e. capable of producing PEP from OAA via PEPCK activity GDH
glutamate dehydrogenase; AST aspartate aminotransferase; ME malic enzyme.

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Figure 2.

Glucose and glutamine metabolism in original lung patient tumor tissues and corresponding PDX tumor tissue differs quantitatively.

A non small-cell lung cancer (NSCLC) patient (UL173) was consented for collection of lung tumor tissues for ex vivo culturing and implantation into NSG mice (PDX). SIRM experiments were performed ex vivo on thinly sliced tissues in DMEM medium containing $^{13}\text{C}_6$ -glucose (**A**) or $^{13}\text{C}_5, ^{15}\text{N}_2$ -Gln (**B**) for 24 has described previously (Lane et al. 2011; T. W. Fan et al. 2011; T. W.-M. Fan et al. 2012; T. W. Fan et al. 2012; Xie et al. 2014b; Ren et al. 2014a; Sellers et al. 2015b). SIRM experiments were also performed in vivo on the third generation PDX mice with 3 consecutive injections of $^{13}\text{C}_6$ -glucose or $^{13}\text{C}_5, ^{15}\text{N}_2$ -Gln over 45 min period as described previously (T. W.-M. Fan et al. 2011; Lane et al. 2011). Tumor tissues from both sets of experiments were processed and extracted for polar metabolites, which were analyzed by 1D HSQC NMR. Despite the much shorter duration of tracer treatment in PDX mice, the level of ^{13}C incorporation from $^{13}\text{C}_6$ -glucose into glycolytic product lactate (^{13}C -3-Lac) or from $^{13}\text{C}_5, ^{15}\text{N}_2$ -Gln into glutaminolytic products Glu (^{13}C -4-Glu) or Asp (^{13}C -3-Asp) was higher in PDX tumor tissues than that in the original patient tumor tissue slices.

Figure 3. Anti-cancer selenite has differential metabolic effects on benign and cancerous lung tissue slices freshly resected from an NSCLC patient

A non small-cell lung cancer (NSCLC) patient (UL143) was consented for collection of paired benign and cancerous (CA) lung tissues for ex vivo culturing. SIRM experiments were performed ex vivo on thinly sliced tissues in $^{13}\text{C}_6$ -glucose-containing DMEM medium under control or 6.25 μM selenite treatments for 24 h. Tissues were processed, extracted, and analyzed by 1D HSQC NMR as described in Fig. 2. Selenite induced a large reduction in ^{13}C incorporation from $^{13}\text{C}_6$ -glucose into glycolytic product lactate (^{13}C -3-Lac), the Krebs cycle metabolites Asp (^{13}C -3-Asp) and Glu (^{13}C -4-Glu), glutathiones (^{13}C -4-Glu-GSH+GSSG), nucleotides (^{13}C -1'-AXP, ^{13}C -1'-UXP), and glycogen (^{13}C -1-Glycogen) in CA tissue slices but not in paired benign tissue slices.

Scheme 1. mini network of central metabolism

Table 1

Property	Cell culture	“organoids”	mouse models	Human tissue culture	Human subject
Experimental control	Maximal-all variables (nutrients tracers, pH, hypoxia).	Near maximal	Low. PDX; syngeneic; transgene.Moderate: (diet, tracer); whole system	Near maximal: all variables (nutrients, tracers, pH, hypoxia); system isolation	Minimal;tracers; whole system
Manipulation	Maximal-gene knockdown or ablation; environment	Maximal-gene knockdown or ablation; environment	Moderate: strain, transgenes, environment	Limited;Environment, not genes	Minimal
Tracers	Any	Any	Any	Any	Limited by cost or toxicity
Complexity	Minimal in 2D cultures. “pure” system; defines possible responses	Cell-cell interactions; possible co-cultures	Full organism with systemic effects; pure strains=> lower variability.NOD mice: minimal immune response; NSG mice humanized immune system or liver.	Retains 3D architecture; cell-cell interactions and microenvironment; resident macrophages; no systemic influences	Full: organism of interest.Systemic influences; greater variability,full immune system
Translation	Human cells; no microenvironment; unrealistic systemically usually poor	Human cells; some microenvironment; unrealistic systemically.?	Not human. PDX may have human tissue/mouse mismatch. Syngeneic is mouse in mouse; transgene is still mouseOften poor	Human tissue; compare with non-lesion from same subject. Expected to be good	Gold standard-Variability & lack of control reduce data quality & quantity
Drug testing	Detailed biochemical phenotype; not suited to ADMET	Detailed biochemical phenotype; not suited to ADMET	ADMET and pharmacodynamics (albeit in mouse)	Detailed biochemistry; acute phase drug response (metabolic readout). Not ADMET	Clinical Trial 90% failure rate in oncology