Frontiers in Metabolism
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Frontiers in Metabolism

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A computational study of the Warburg effect identifies metabolic targets inhibiting cancer migration

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Abstract

Over the last decade, the field of cancer metabolism has mainly focused on studying the role of tumorigenic metabolic rewiring in supporting cancer proliferation. Here, we perform the first genome-scale computational study of the metabolic underpinnings of cancer migration. We build genome-scale metabolic models of the NCI-60 cell lines that capture the Warburg effect (aerobic glycolysis) typically occurring in cancer cells. The extent of the Warburg effect in each of these cell line models is quantified by the ratio of glycolytic to oxidative ATP flux (AFR), which is found to be highly positively associated with cancer cell migration. We hence predicted that targeting genes that mitigate the Warburg effect by reducing the AFR may specifically inhibit cancer migration. By testing the anti-migratory effects of silencing such top predicted genes in four breast and lung cancer cell lines, we find that up to 13 of these novel predictions significantly attenuate cell migration either in all or one cell line only, while having almost no effect on cell proliferation. Furthermore, in accordance with the predictions, a significant reduction is observed in the ratio between experimentally measured ECAR and OCR levels following these perturbations.

Introduction

Altered tumor metabolism has become a generally regarded hallmark of cancer (Hanahan & Weinberg, 2011). The initial recognition that metabolism is altered in cancer can be traced back to Otto Warburg’s early studies, showing that transformed cells consume glucose at an abnormally high rate and largely reduce it to lactate, even in the presence of oxygen (Warburg, 1956). Over the last decade, much of the field of cancer metabolism has focused on the role of the Warburg effect in supporting cancer proliferation (Vander Heiden et al, 2009). However, the role of this process in supporting other fundamental cancer phenotypes such as cellular migration has received far less attention.

Contemporary cytotoxic cancer treatment has been mainly based on drugs that kill proliferating cells generally unselectively and are therefore accompanied by many undesirable side effects. Drug targets that can inhibit migration but leave cellular proliferation relatively spared may be able to avoid such side effects. Such targets may have the additional benefit of reducing the selection for more resistant clones that occurs due to the elimination of treatment-sensitive cells. The growing availability of high-throughput measurements for a range of cancer cells presents an opportunity to study a wider scope of dysregulated metabolism across many different cancers. Here, we aim to

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integrate pertaining data with a genome-scale mechanistic model of human metabolism to study the role of the Warburg effect in tumor progression and its potential association with cellular migration.

Genome-scale metabolic modeling is an increasingly widely used computational framework for studying metabolism. Given the genome-scale metabolic model (GSMM) of a species alongside contextual information such as growth media and ‘omics’ data, one can obtain a fairly accurate prediction of numerous metabolic phenotypes, including growth rates, nutrient uptake rates, gene essentiality, and more (Covert et al., 2004). GSMMs have been used for various applications (Oberhardt et al., 2009; Chandrasekaran & Price, 2010; Jensen & Papin, 2010; Szappanos et al., 2011; Wessely et al., 2011; Lerman et al., 2012; Nogales et al., 2012; Schuetz et al., 2012) including drug discovery (Trawick & Schilling, 2006; Oberhardt et al., 2013; Yizhak et al., 2013) and metabolic engineering (Burgard et al., 2003; Pharkya et al., 2004). Over the last few years, GSMMs have been successfully used for modeling human metabolism as well (Duarte et al., 2007; Ma et al., 2007; Shiomi et al., 2008; Gille et al., 2010; Lewis et al., 2010; Mardinoglu et al., 2013). Specifically, GSMM models of cancer cells have been reconstructed and applied for predicting selective drug targets, as well as for studying the role of tumor suppressors and oxidative stress (Folger et al., 2011; Frezza et al., 2011; Agren et al., 2012, 2014; Jerby et al., 2012; Goldstein et al., 2013; Gatto et al., 2014). In the context of studying the Warburg effect, the original human metabolic model does not predict forced lactate secretion under maximal biomass production rate, even when oxygen consumption rate equals zero. This renders it unsuitable for studying the Warburg effect as is, as already noted by (Shiomi et al., 2011). While the addition of solvent capacity constraints has been shown to overcome this hurdle in principle (Shiomi et al., 2011), this addition requires enzymatic kinetic data which are still largely absent on a genome-scale.

In this study, we utilize individual genome-scale metabolic models tailored separately to each of the NCI-60 cancer cell lines to study the role of the Warburg effect in supporting cancer cellular migratory capacity. We first test and validate the individual models against both existing and novel bioenergetic experimental data. Then, we examine the extent of the Warburg effect occurring in a given cancer cell line, by quantifying the glycolytic to oxidative ATP flux ratio (AFR). We find that the AFR is highly positively correlated with cancer cell migration, emphasizing the role of glycolytic flux in supporting the more aggressive metastatic stages of tumor development. To determine whether a causal relation exists between AFR levels and cell migration, we predict gene silencing that reduce this ratio. These potential targets are then filtered further to exclude those predicted to result in cell lethality. Reassuringly, the predicted targets are found to be significantly more highly expressed in metastatic and high-grade breast cancer tumors. Experimental investigation of the top predicted targets via siRNA-mediated knockdown shows that a significant portion of them truly attenuate cancer cell migration without inducing a lethal effect. Furthermore, in accordance with the predictions, a significant reduction is observed in the ratio between ECAR and OCR levels following these gene silencing perturbations.

**Results**

**Stoichiometric and flux capacity constraints successfully capture the coupling of high cell proliferation rate to lactate secretion across individual NCI-60 cancer models**

As a starting point for this study, we developed a set of metabolic models specific for each of the NCI-60 cell lines. We built these models using a new algorithm we have recently developed termed PRIME, for building individual models of cells from pertaining omics data (Yizhak et al, submitted, Supplementary Information and Supplementary Fig S1). PRIME uses the generic human model as a scaffold and sets maximal flux capacity constraints over a subset of its growth-associated reactions according to the expression levels of their corresponding catalyzing enzymes in each of the target cell lines.

An important hallmark of cancerous cells is the production of lactate through the Warburg effect (Warburg, 1956). As a first step in validating the basic function of our NCI-60 models, we assessed whether maximizing biomass forces production of lactate, which would signify proper coupling of biomass production with lactate output as seen in cancer cells. We found that the models indeed must secrete lactate under biomass maximization (Supplementary Information and Supplementary Fig S2). Hence, in contrast to the original generic model of human metabolism, they enable us to systematically assess the extent of lactate secretion and study the Warburg effect across a wide range of cancer cell lines without needing to add (mostly unknown) solvent capacity constraints, thus identifying its functional correlates on a genome scale.

**Comparing predicted versus experimentally measured bioenergetics capacity**

We compared the predicted lactate secretion rates across all cell lines to those measured experimentally by Jain et al (Jain et al, 2012), obtaining a moderate but significant correlation (Spearman correlation $R = 0.36$, $P$-value $= 5.7e^{-3}$, Fig 1A, Materials and Methods). To further test the models’ performance under different environmental conditions, we measured lactate secretion rates in four breast cancer cell lines, T47D, MCF7, BT549, and Hs578T (Supplementary Dataset S1), under both normoxic and hypoxic conditions (see Materials and Methods). Utilizing the corresponding cell line models from the NCI-60 set, we found a high correlation between measured and predicted lactate secretion levels across both conditions (Spearman correlation $R = 0.95$, $P$-value $= 1.1e^{-3}$, Fig 1B).

The ratio of glycolytic versus oxidative capacity in a cell can be quantified using its extracellular acidification rate (ECAR, a proxy of lactate secretion) and its oxygen consumption rate (OCR). To further examine how well our cell line models capture measured Warburg-related activity in response to genetic perturbations, we utilized measured ECAR and OCR levels in response to perturbations in two NCI-60 lung cancer cell lines (A549 and H460), and compared the results to predictions from our models (Materials and Methods) (Wu et al., 2007). Qualitatively similar ECAR and OCR changes are found in response to various enzymatic perturbations along the glycolytic pathway. Specifically, increased glycolytic inhibition resulted in reduced ECAR and elevated OCR levels in both cells, while the maximum cellular respiration increase in H460 cells
observed after all glycolysis inhibitors was lower than the corresponding increase in A549 cells (Fig 1C).

Quantifying the Warburg effect and its relation to proliferation and migration across the NCI-60 cell lines

While ECAR and OCR are the commonly used measures for experimentally quantifying the bioenergetic capacity of the cell and thus the Warburg effect, the genome-wide scope of GSMMs enables us to examine other putative measures as well. One promising such measure we examined is the ratio between the ATP flux rate in the glycolysis versus its flux rate in OXPHOS (AFR). Clearly, higher AFR values denote more ‘Warburgian’ cell lines and vice versa. A comparison of our new AFR metric versus the aforementioned state-of-the-art ECAR/OCR ratio (EOR) (Materials and Methods and Supplementary Dataset S2) showed a significant correlation across the NCI-60 models (Spearman correlation $R = 0.66$, $P$-value $= 2e^{-8}$). Testing both measures using a genome-wide NCI-60 drug response dataset (Scherf et al, 2000), we find that the model-predicted wild-type AFR levels across all cell line models are significantly correlated (Spearman $P$-value $< 0.05$; FDR corrected with $a = 0.05$) with G50 values of 30% of the compounds across these cell lines (empirc $P$-value $< 9.9e^{-4}$), whereas the model-predicted EOR measure accomplish this task for only 19% of the compounds (Materials and Methods). Interestingly, we find that out of the 30% AFR-G50-correlated compounds, 97% are positively correlated, suggesting that the more ‘Warburgian’ cell lines are less responsive and therefore require higher dosage of compound to suppress their

![Image](image_url)
growth. The effect of most of these compounds is also negatively correlated with the cells’ growth rates, suggesting that slowly proliferating cells are more resistant to treatment (similar results were previously shown for compounds targeting cell growth (Penault-Llorca et al, 2009; Vincent-Salomon et al, 2004)). Interestingly, the response to many compounds in this dataset shows a significant association with the AFR measure while having no association with the cells’ growth rate. 133 such compounds were identified (Supplementary Dataset S3), possibly suggesting that their mechanism might be related to the Warburg level of the cells rather than to their proliferation. Finally, predicted AFR values correctly separate between epithelial and mesenchymal breast cancer cell lines (with the more aggressive mesenchymal cell lines exhibiting larger Warburg effect (Sarrio et al, 2008), Fig 2A). Once again, the AFR was more predictive of this experimental observation than the EOR (Supplementary Dataset S2).

We next turned to our primary objective of examining the relation between the Warburg effect and tumor proliferation and migration. To this end, we experimentally measured the migration speed of six NCI-60 breast cancer cell lines (Fig 2B and C, Materials and Methods, Supplementary Fig S3, and Supplementary Dataset S2) and utilized publically available measured growth rates for these cell lines. While the AFR correlates markedly negatively with cell growth rate (Spearman correlation of $R = -0.55$, $P$-value $= 4.53e^{-6}$, Figure 2A), it correlates even more significantly with growth and migration rates measured experimentally. Both measures represent a negative association with the AFR measure while having no association with the cells’ growth rate.

![Figure 2](image_url)

**Figure 2.** Association between AFR levels and cell proliferation and migration.

A The 20 cell lines that are predicted to exhibit the Warburg effect to the greatest/least extent according to the AFR measure. The x-axis and y-axis represent the mean and SD of the normalized ATP flux rate in glycolysis and OXPHOS, respectively (Materials and Methods). The AFR measure correctly separates between mesenchymal (orange) and epithelial cell lines (green), showing that the former (which are known to be more aggressive) have higher AFR levels.

B We analyzed a panel of six breast cancer cell lines for their migration capacity using live cell imaging. Differential Interference Contrast (DIC) images of the six cell lines in the order of their respective migration speed (from low to high), scale bar is 100 μm (Materials and Methods).

C The average migration speed of cells followed for 12 h in complete medium. Error bars represent SEM; the number of samples is between $n = 100$ and $n = 200$.

D The correlation of predicted model-based EOR and AFR measures to growth and migration rates measured experimentally. Both measures represent a negative correlation with growth and a positive correlation with migration rates. Significant results ($P$-value $< 0.05$) are marked with an asterisk.
between epithelial and mesenchymal breast cancer cell lines (with association with the AFR measure while having no association with response to many compounds in this dataset shows a significant proliferating cells are more resistant to treatment (similar results were correlated with the cells’ growth rates, suggesting that slowly dividing cells are more resistant to treatment). A similar association between lactate secretion and growth rate has been recently found in an experimental study by Jain et al (Jain et al, 2012) across the entire NCI-60 collection (Spearman correlation of $R = -0.22$, $P = 0.09$). Furthermore, previous studies have shown that high concentrations of lactate correlate with a high incidence of distant metastasis (Hirschhaeuser et al, 2011). The overall picture portrayed by these correlations is that while glycolytic carbon diverted to biosynthetic pathways may support cell proliferation, non-diverted glycolytic carbon supports cell migration and metastasis (Supplementary Fig S4).

### Predicting drug targets that revert the AFR and hence may inhibit cancer migration

The congruence between AFR levels and disease severity led us to ask if we could build upon this association to identify potential new drug targets. We searched for drug targets predicted to reduce the AFR ratio by simulating the knockout of each metabolic reaction across the NCI-60 models, and examining the effects of the knockouts on biomass production, lactate secretion, and the AFR. As lactate secretion is a basic indicator of the Warburg effect, we first identified a set of 113 reactions whose knockout is predicted to abolish lactate secretion rate in all cancer cell lines under biomass maximization. Interestingly, the set of enzymes catalyzing these reactions is significantly more highly expressed in the NCI-60 cell lines than the background metabolic genes (one-sided Wilcoxon $P-value < 1.6e–8$), indicating the potential oncogenic nature of these genes.

To avoid selecting for drug-resistant clones it would be advantageous to develop drugs that reduce the virulence of cancer cells but avoid killing them. The knockout of 12 of 113 lactate-reducing reactions reduces the AFR but relatively spares biomass production (Materials and Methods and Supplementary Table S2). Importantly, the knockout of these 12 reactions according to models of healthy lymphoblast cells built by PRIME (Choy et al, 2008) also spares their biomass production (Materials and Methods). Moreover, we found that none of the lymphoblast cell lines show the forced lactate secretion that is observed in cancer cells. While the Warburg effect is sometimes referred to the literature as occurring in highly proliferating cells in general, our analysis finds that this phenomenon is apparently more prominent in cancer cells, at least with regard to the lymphoblastoid cell population studied here.

The final list of predicted gene targets includes 17 metabolic enzymes that are associated with the final 12 reactions, spanning glycolysis, serine, and methionine metabolism (Fig 3A). 10 of the predicted targets have significantly higher expression levels in metastatic versus non-metastatic breast cancer patients (Chang et al, 2005) (one-sided Wilcoxon $P-value < 0.05$, Fig 3B). Moreover, 9 of the predicted targets exhibit higher expression levels in grade 3 tumors than in grade 1 tumors (Miller et al, 2005) (one-sided Wilcoxon $P-value < 0.05$, Fig 3C). Finally, lower expression of nine of the predicted targets is significantly associated with improved long-term survival (Curtis et al, 2012) (log-rank $P-value < 0.05$, Fig 3D), testifying for their potential role as therapeutic targets. All $P$-values are corrected for multiple hypothesis using FDR with $\alpha = 0.05$.

### siRNA-mediated gene knockdown experiments testing the predicted targets

To experimentally test our predictions we silenced the 17 predicted AFR-reducing genes and examined their phenotypic effects in the MDA-MB-231, MDA-MB-435, BT549, and A549 cell lines. Knockdown experiments were performed with SmartPools from Dharmacon using a live cell migration and fixed proliferation assays (Materials and Methods). 8–13 out of the 17 enzymes (8–10 out of 12 metabolic reactions) were found to significantly attenuate migration speed in each cell line (two-sided $t$-test $P-value < 0.05$, FDR corrected with $\alpha = 0.05$, Fig 4, Materials and Methods and Supplementary Dataset S4). This result is highly significant as only 17% of the metabolic genes were found to impair cell migration in a siRNA screen of 190 metabolic genes (Fokkelman M, Rogkoti VM et al, unpublished data, Bernoulli $P-value$ in the range of $3.9e–3$ and $1.18e–7$). Of note, the association between the gene expression of the predicted targets and the measured migration speed is insignificant for all targets but one, testifying for the inherent value of our model-based prediction analysis (Supplementary Table S3). It should also be noted that the knockdown of the three splices of the enolase gene have almost no significant effect on these cells’ migration speed, possibly because of isoenzymes backup mechanisms. Importantly, most of the gene knockdown experiments do not manifest any significant effects on cell proliferation (Fig 4). In accordance with the findings of Simpson et al (Simpson et al, 2008), we found that the correlation between the reduction in migration speed and reduction in proliferation rate is mostly insignificant (Supplementary Dataset S4), suggesting that the reduced migration observed is not simply a consequence of common mechanisms hindering proliferation, but rather it occurs due to the disruption of distinct migratory-associated metabolic pathways.

### ECAR and OCR levels following selected gene silencing

To further study the association between reduced AFR levels and impaired cell migration we used the Seahorse XF96 extracellular flux analyzer to measure both ECAR and OCR fluxes in the MDA-MB-231 cell line, following knockdown of a selected group of targets (Materials and Methods and Supplementary Fig S6). As the AFR measure is very difficult to measure experimentally, we tested the conventionally measured EOR (ECAR/OCR) as its proxy. We focused on a subset of seven genes (Fig 5) whose knockdown is predicted to have the highest effect on cell migration and span all three predicted metabolic pathways. As shown in Fig 5, a significant EOR reduction versus the control is found for all seven examined genes (two-sided $t$-test $P-value < 0.05$, FDR corrected with $\alpha = 0.05$, Materials and Methods and Supplementary Table S4). The silencing of the four glycolytic genes (HK2, PGAM1, PKG2, and GAPDH) results in both decreased ECAR and increased OCR levels, while the silencing of the serine- and methionine-associated genes
(PSPH, AHCY, and PHGDH) results with decreased ECAR solely (Fig 5A). Furthermore, a matching significant difference in experimentally measured EOR levels is found between the lowest and highest AFR-reducing genes (one-sided Wilcoxon P-value = 0.05). Overall, taken together our results testify that, as predicted, the knockdown of the top-ranked genes results in attenuated cell migration that is accompanied by reduced EOR and AFR levels.

**Discussion**

In this study we explored the role of the Warburg effect in supporting tumor migration, going beyond recent investigations focusing on its role in assisting cancer proliferation. A model-based investigation across cancer cell lines shows that the ratio between glycolytic and oxidative ATP flux rate is significantly associated with cancer migratory behavior. Gene silencing perturbations predicted to reduce this ratio were indeed found to attenuate cell migration, and result with a significant reduction in ECAR to OCR levels. Of note, our modeling approach relies on gene expression differences between the cells and does not take into account specific uptake rates. It is therefore more suited for capturing qualitative rather than exact quantitative differences between the cells, as demonstrated throughout the paper. Moreover, the lion share of our analysis is focused on the simulations of perturbations where specific uptake rates are not available. Nonetheless, utilizing such uptake measurements can significantly increase the correlation to the measured lactate rates (Spearman correlation $R = 0.67$, $P$-value $= 1.5e^{-8}$), suggesting that uptake rates measurements under perturbation states can significantly increase the models’ prediction power.

Our AFR measure is conceptually analogous to a bioenergetic (BEC) index previously introduced by Cuevza et al (Cuevza et al, 2002). In that study, the ratio between the expression of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
and the β-catalytic subunit of ATP synthase forming the BEC index was found to have a prognostic value in assessing the clinical outcome of patients with early-stage colorectal carcinomas. The AFR measure and the BEC index (as computed by its corresponding RNA levels) are significantly correlated (Spearman $R = 0.58$, $P$-value $= 1.6e-6$) across the NCI-60 cell lines, and the BEC index is perfectly correlated with migration speed across the six breast cancer cell lines (Spearman $R = 1$, $P$-value $= 2.8e-3$). However, the BEC index has inferior performance in predicting drug response (Supplementary Table S1).

The finding that enhanced glycolytic activity plays a key role in cancer cell migration is also in line with a very recent study by De Bock et al., showing that glycolysis is the major source of ATP production in endothelial cells and that the silencing of the glycolytic regulator PFKFB3 impairs the cell migration capacity and interferes with vessel sprouting (De Bock et al., 2013). In addition, silencing of PFKFB3 was shown to suppress cell proliferation in about 50% (De Bock et al., 2013). Overall, the results presented in this study, as well as findings reported by others (Simpson et al., 2008), suggest that proliferation and migration are not mutually exclusive, and the effect of potential targets on both processes should be carefully examined.

Some of our predicted targets have been previously studied in the context of cell proliferation as well (Cheong et al., 2012). Possemato et al (Possemato et al., 2011) have showed that suppression of PHGDH in cell lines with elevated PHGDH expression, but not

**Figure 4.** Normalized to control mean speed per SmartPool gene silencing of the predicted targets. A–D The four different cell lines that were analyzed: MDA-MB-231, MDA-MB-435s, BT549, and A549. Significant results (two-sided t-test, $P$-value < 0.05 after correcting for multiple hypothesis using FDR with $α = 0.05$) are marked with an asterisk. Two different controls are used: (1) non-targeting siRNA (= negative control); and (2) a positive control DNMT which is known to block both migration and proliferation (Ezratty et al, 2005). Left panel shows migration speed and right panel shows nuclear count. Error bars represent SD, the number of samples is $n = 3$. 

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Identifying anti-migratory metabolic drug targets

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in those without, inhibits cell proliferation. Accordingly, as PHGDH is not amplified in the cell line MDA-MB-231 which was examined in both studies, its suppression is indeed non-lethal. However, we show that its suppression significantly attenuates cell migration, suggesting that metabolic enzymes can promote different cancerous phenotypes in different cancer cells.

Remarkably, analyzing the model-predicted flux rates has successfully uncovered a fundamental association between the AFR and cancer migration, even given the relatively small set of cell lines for which migration was measured. Our analysis has also revealed other potential associations between individual fluxes and cell migration (Supplementary Fig S4). However, future studies measuring cellular migration data across a much wider array of cell lines (of the order for which we already have proliferation data) are needed to determine the actual significance of these potential leads. As this study has shown, cellular proliferation and migration have distinct underlying metabolite correlates; understanding the metabolic correlates that are strongly associated with cell migration may lead to new anti-metastatic treatment opportunities. It is important to note, however, that while the inhibition of migration alone might be a good strategy for avoiding the adverse side effects of cytotoxic treatment, cell migration is a crucial process also in normal physiology, for instance, in immune response and tissue repair (Förster et al., 1999; Ridley et al., 2003). Therefore, future anti-migratory drugs may pose different drug selectivity challenges that should be carefully addressed in the future studies. Irrespectively, they may result in lesser clonal selection, and as a result, their usage may be accompanied with lesser rate of emergence of drug-resistant clones.

Materials and Methods

Computational methods

Genome-scale metabolic modeling (GSSM)

A metabolic network consisting of m metabolites and n reactions can be represented by a stoichiometric matrix $S$, where the entry $S_{ij}$ represents the stoichiometric coefficient of metabolite $i$ in reaction $j$ (Price et al., 2004). A CBM model imposes mass balance, directionality, and flux capacity constraints on the space of possible fluxes in the metabolic network’s reactions through a set of linear equations:

$$S v = 0$$

$$v_{\text{min}} \leq v \leq v_{\text{max}}$$

where $v$ stands for the flux vector for all of the reactions in the model (i.e. the flux distribution). The exchange of metabolites with the environment is represented as a set of exchange (transport) reactions, enabling a pre-defined set of metabolites to be either taken up or secreted from the growth media. The steady-state assumption represented in equation (1) constrains the production rate of each metabolite to be equal to its consumption rate. Enzymatic directionality and flux capacity constraints define lower and upper bounds on the fluxes and are embedded in equation (2). In the following, flux vectors satisfying these conditions will be referred to as feasible steady-state flux distributions. Gene knockouts are simulated by constraining the flux through the

Figure 5. ECAR and OCR levels of top predicted gene targets.

A Mean and SEM (normalized to nuclear count) ECAR and OCR levels after silencing of seven different genes (HK2, PGAM1, PGK2, GAPDH, PSPH, AHcy, and PHGDH) compared to the control. Silencing of the four glycolytic genes results in both a decrease in ECAR levels (x-axis) and an increase in OCR levels (y-axis), while the serine- and methionine-associated genes show only a decrease in ECAR levels. Error bars represent SEM. The number of samples is $n = 18$.

B Mean and SD of computed ECAR/OCR (EOR) levels for control and selected gene silencing (Materials and Methods). For all genes a significant reduction in EOR levels is observed. Error bars represent SD. The number of samples is $n = 18$. 
corresponding metabolic reaction to zero. The biomass function utilized here is taken from (Folger et al., 2011). The media simulated in all the analyses throughout the paper is the RPMI-1640 media that was used to grow the cell lines experimentally (Lee et al., 2007; Choy et al., 2008).

**Building cell-specific metabolic models and computing lactate secretion**

Our method to reconstruct the NCI-60 cancer cell lines (see Supplementary Material, based on the yet unpublished methods in Yizhak et al., submitted) required several key inputs: (a) the generic human model (Duarte et al., 2007), (b) gene expression data for each cancer cell line from (Lee et al., 2007), and (c) growth rate measurements. The algorithm then reconstructs a specific metabolic model for each sample by modifying the upper bounds of growth-associated reactions in accordance with their gene expression (Note: the growth rates were used only to determine which reactions should be used in constraining the models, in order to obtain models that were as physiologically relevant as possible; they were not used to determine reaction bounds). A similar procedure was used to reconstruct the lymphoblastoid metabolic models (Choy et al., 2008) for comparison against normal proliferating cells. A more detailed description is found in the Supplementary Material.

Simulations of the Warburg effect include the examination of minimal lactate production rate under different demands for biomass production, glucose, glutamine, and oxygen uptake rates (Supplementary Material). We examined the minimal value of lactate secretion as it testifies whether or not the cell is enforced to secrete lactate under a given condition (Supplementary Fig S1). All the correlations reported in the paper are Spearman rank correlations and their associated P-values are computed using the exact permutation distribution.

**Calculating wild-type and perturbed lactate secretion rates and OCR levels**

For simulating lactate secretion under normoxic conditions (when comparing to Jain et al (Jain et al., 2012), Wu et al (Wu et al, 2007) and the breast cancer data collected in this paper), oxygen maximal uptake rate was set to the highest value under which minimal lactate secretion is positive. Since metabolic models are designed to maximize growth yield rather than growth rate, using an unlimited amount of oxygen in GSMM simulations will result in a state where the minimal lactate secretion rate equals zero. However, it’s important to note that even under the limited oxygen levels simulated here, the generic human model doesn’t show lactate secretion (as opposed to the NCI-60 cancer cell line models described above). For simulating the hypoxic conditions measured here for the breast cancer cell lines, we lowered the oxygen maximal uptake rate by 50% of its normoxic state as described above. Under each of these conditions, we sampled the solution space under maximal biomass yield and obtained 1,000 feasible flux distributions (Bordel et al., 2010). The predicted lactate secretion rate is the average lactate secretion flux over these samples. For emulating the perturbation experiments in Wu et al we gradually lowered the bound of the corresponding compound target (from the maximal bound to 0) and repeated the procedure described above for computing the ECAR (lactate secretion) and the OCR, which in a similar manner is defined as the average oxygen consumption flux across all samples.

**Calculating the EOR and AFR measures for assessing the Warburg level of the cell lines and using them to predict drug response**

The EOR and AFR measures were calculated in a similar manner to that described above. Specifically, the EOR is calculated as the mean over lactate secretion across all samples divided by the mean over oxygen consumption across all samples. Similarly, the AFR is calculated as the mean flux carried by the reactions producing ATP in glycolysis versus the mean flux carried by the reaction producing ATP in OXPHOS. To determine an empiric P-value in the drug response analysis we randomly shuffled the drug response data 1,000 times, each time examining the resulting Wilcoxon P-value over the original set of cell lines.

**Predicting the effect of reaction knockouts**

Each metabolic reaction in each cell line model is perturbed by constraining its flux to zero. Under each perturbation the minimal lactate secretion (under maximal growth rate) and the maximal growth rate is calculated. The set of reactions that eliminate forced lactate secretion while maintaining a level of cell growth that is > 10% of the wild-type growth prediction is further tested for the AFR level. The mean AFR level for each cell line under each of these perturbations is calculated over 1,000 flux distribution samples as described above. The final set of predicted reactions includes those whose knockout reduces the AFR to below 60% of its wild-type level.

**Datasets**

Growth rate measurements and drug response data were downloaded from the NCI website.

- Growth rate: http://dtp.nci.nih.gov/docs/misc/common_files/cell_list.html

**Experimentally measuring lactate secretion rates of breast cancer cell lines**

**Cell Culture**

The MCF7, T47D, Hs578T and BT549 breast cancer cell lines were obtained from the American Type Culture Collection and London Research Institute Cell Services. Cells were cultured in DMEM/F12 (1:1), with 2 mM L-glutamine and penicillin/streptomycin. Medium was supplemented with 10% FCS (GIBCO) for the cancer cell lines and 5% horse serum, 20 ng/ml EGF, 5 µg/ml hydrocortisone, 10 µg/ml insulin, and 100 ng/ml cholera toxin for the non-malignant cell lines.

**Lactate secretion measurements**

Cells were cultured under normoxic (20% O₂) and hypoxic (0.5% O₂) conditions for 72 h. Cells were starved of glucose and glutamine for 1 h and full medium was added for 1 h. Lactate secretion was determined from normoxic and hypoxic cells and normalized to cell growth (increase in total protein during the 72 h incubation in normoxia). Lactate concentrations in media incubated with or without cells were determined using lactate assay kits (BioVision). Total protein content determined by Sulforhodamine B assay was used for normalization. Two experiments were performed with three or four biologically independent replicates (total of seven replicates).
Cell culture for live cell imaging and cell migration assays

T47D, MCF-7, MDA-MB-435, BT549, MDA-MB-231 and Hs578t were cultured in RPMI ( Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 10% FBS (PAA, Pasing Austria) and 100 International Units/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA).

Gene silencing

Human siRNA SmartPools (a combination of four individual singles) for the 17 predicted genes were purchased in siGENOME format from Dharmacon (Lafayette, CO, USA). Plates were diluted to 1 μM working concentration in complementary 1× siRNA buffer in a 96-well plate format. A non-targeting siRNA was used as negative control. A 50 nM reverse transfection was performed according to manufacturer’s guidelines. Complex time was 20 min and 5,000 cells were added. The plate was placed in the incubator overnight and the medium was refreshed the following morning. After 48–72 h cells were used for various assays. Cell migration and metabolic flux assay experiments were performed in duplicate while the cell proliferation assay was performed in triplicate.

Live cell imaging random cell migration assay

Glass bottom 96-well plates (Greiner Bio-one, Monroe, NC, USA) were coated with 20 μg/μl collagen type I (isolated from rat tails) for 1 h at 37°C. 48 h after silencing, the MDA-MB-231 cells were re-plated onto the collagen-coated glass bottom plate. 24 h after seeding, cells were pre-exposed for 45 min to 0.1 μg/μl Hoechst 33342 (Fisher Scientific, Hampton, NH, USA) to visualize nuclei. After refreshing the medium, cells were placed on a Nikon Eclipse TE2000-E microscope fitted with a 37°C incubation chamber, 20× objective (0.75 NA, 1.00 WD) automated stage and perfect focus system. Three positions per well were automatically defined, and the Differential Interference Contrast (DIC) and Hoechst signals were acquired with a CCD camera (Pixel size: 0.64 μm) every 20 min for a total imaging period of 12 h using NIS software (Nikon). All data were converted and analyzed using custom-made ImagePro Plus macros (Roosmalen et al., 2011). Cell migration was quantified by tracking nuclei in time. Changes in migration speed per knockdown were evaluated via a two-sided t-test comparing the speed for every individual cell followed overtime for 16 h and the corresponding control values. Data shown are normalized to control and represent only one replicate. Of note, for all four cell lines both replicates showed a $R^2 > 0.75$. Genes achieving $P$-value < 0.05 after correcting for multiple hypothesis using FDR with $α = 0.05$ are considered as hits.

Proliferation assay

Cells were directly transfected and plated onto micro-clear 96-well plates (Greiner Bio-one). After 5 days of incubation, the cells were stained with Hoechst 33342 and fixed with TCA (Trichloroacetic acid) allowing both a nuclear counting and/or Sulforodamine B (SRB) readout. Whole wells were imaged using epi-fluorescence and the number of nuclei was determined using a custom-made ImagePro macro. Plates were further processed for SRB staining as described earlier (Zhang et al., 2011). SRB data showed a complete overlap with the nuclear count so this measure is used in all figures. Changes in proliferation rates upon knockdown when compared to control were evaluated in triplicate via a two-sided t-test. The mean proliferation rate after knockdown between all three replicates was calculated and normalized to the non-targeting siRNA (= control). Genes achieving $P$-value < 0.05 after correcting for multiple hypothesis using FDR for multiple hypothesis using FDR with $α = 0.05$ are considered as hits.

Metabolic flux assay

The bioenergetics flux of cells in response to gene silencing was assessed using the Seahorse XF96 extracellular flux analyzer (Seahorse Bioscience). About 8,000 MDA-MB-231 cells per well (Seahorse plate) were treated with siRNAs or control for 72 h. Each gene (in total 7) was knockdown in six different wells and the experiment was performed twice (so a total of six replicates per plate and two plates). Prior to measurement, the medium was replaced with unbuffered DMEM XF assay medium. The basal oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were then determined using the XF96 plate reader with the standard program as recommended by the manufacturer: three measurements per well were done (so for each gene 18 measurements were obtained for both OCR and ECAR). After the measurements were completed, the plates were live stained with Hoechst 33342 for 1 h and fixed with TCA allowing both a nuclear counting and/or SRB readout. Whole wells were imaged using epi-fluorescence and the number of nuclei was determined using a custom-made ImagePro macro. Plates were further processed for SRB staining as described earlier (Zhang et al., 2011). SRB data showed a complete overlap with the nuclear count so this measure was used for normalization. All values are normalized to nuclear count. EOR for control and each gene knockdown is computed by dividing the corresponding ECAR and OCR values. A two-sided t-test is applied to examine significant changes between control and knockdown-induced EOR.

Supplementary information for this article is available online: http://msb.embopress.org

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Author contributions

KY and ER conceived and designed the research. SLD, VCB, CF, and BvdV designed the experimental procedures. FB and AS contributed the lactate secretion data. KY performed the computational analysis and the statistical
computations. SLD, VMR and VCB performed the experimental procedures. KY, SLD, BvW, and ER wrote the paper.

Conflict of interest
The authors declare that they have no conflict of interest.

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Identifying anti-migratory metabolic drug targets


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Principles of targeting endothelial cell metabolism to treat angiogenesis and endothelial cell dysfunction in disease

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Abstract

The endothelium is the orchestral conductor of blood vessel function. Pathological blood vessel formation (a process termed pathological angiogenesis) or the inability of endothelial cells (ECs) to perform their physiological function (a condition known as EC dysfunction) are defining features of various diseases. Therapeutic intervention to inhibit aberrant angiogenesis or ameliorate EC dysfunction could be beneficial in diseases such as cancer and cardiovascular disease, respectively, but current strategies have limited efficacy. Based on recent findings that pathological angiogenesis and EC dysfunction are accompanied by EC-specific metabolic alterations, targeting EC metabolism is emerging as a novel therapeutic strategy. Here, we review recent progress in our understanding of how EC metabolism is altered in disease and discuss potential metabolic targets and strategies to reverse EC dysfunction and inhibit pathological angiogenesis.

Keywords angiogenesis; endothelial cell dysfunction; metabolism

Introduction

Blood vessels perform many functions that are critical for tissue homeostasis (Carmeliet, 2003). The endothelium, a single layer of endothelial cells (ECs) that lines the blood vessel lumen, controls vessel function. EC functions include the regulation of vascular tone and barrier, leukocyte trafficking, blood coagulation, nutrient and electrolyte uptake and neovascularization of hypoxic tissue, to name only a few (Cines et al, 1998; Pober et al, 2009; Potente et al, 2011). Many diseases are characterized by pathological blood vessel responses or formation. The inability of ECs to perform their physiological function (a condition termed EC dysfunction) contributes to cardiovascular disease and diabetes (Davignon & Ganz, 2004), whereas diseases such as cancer and age-related macula degeneration are characterized by new blood vessel formation (a process termed angiogenesis) (Carmeliet & Jain, 2011). Targeting ECs to prevent dysfunction or inhibit pathological angiogenesis is potentially beneficial for a wide variety of diseases, but current treatment modalities, focusing primarily on growth factors, receptors, signaling molecules and others have limited efficacy or specificity (Bergers & Hanahan, 2008; Versari et al, 2009; Lee et al, 2012).

An emerging but understudied therapeutic target is EC metabolism. It has been long known that risk factors for cardiovascular disease (hypercholesterolemia, hypertension, dyslipidemia, diabetes, obesity and aging) cause EC-specific metabolic perturbations leading to EC dysfunction (Davignon & Ganz, 2004; Pober et al, 2009). Similarly, the links between EC metabolism and angiogenesis are apparent as angiogenic ECs migrate and proliferate in metabolically challenging environments such as hypoxic and nutrient-deprived tissue (Harjes et al, 2012). Moreover, the growth factor-induced switch from a quiescent to an angiogenic phenotype is mediated by important adaptations in EC energy metabolism (De Bock et al, 2013a,b; Schoors et al, 2014a,b). EC metabolic alterations are therefore not just innocent bystanders but mediate pathogenesis. In this review, we summarize existing data on the role of EC metabolism in mediating vascular disease and discuss how metabolism may be targeted for therapeutic benefit.

General endothelial metabolism

Despite their close proximity to oxygenated blood, ECs rely on glycolysis instead of oxidative metabolism for adenosine triphosphate (ATP) production (Parra-Bonilla et al, 2010; De Bock et al, 2013b). In fact, under physiological conditions, over 80% of ATP is produced by converting glucose into lactate (Fig 1). Less than 1% of glucose-derived pyruvate enters the mitochondria for oxidative metabolism through the tricarboxylic acid cycle (TCA) and subsequent ATP production via the electron transport chain (ETC) (Fig 1) (Culic et al, 1997; De Bock et al, 2013b). However, ECs retain the ability to switch to oxidative metabolism of glucose, amino acids

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Glossary

1C metabolism
A complex metabolic network characterized by the transfer of carbon from serine/glycine for folate compound chemical reactions and involved in nucleotide, lipid and protein biosynthesis, redox homeostasis and production of methylation substrates.

Advanced glycation end products (AGEs)
Proteins or lipids that have been non-enzymatically glycated, often as a result of hyperglycemia and/or oxidative stress, that cause damaging intracellular and extracellular dysfunction.

Angiogenesis
Growth of new blood vessels from existing microvasculature.

Endothelium
Continuous inner lining of all vasculature composed of endothelial cells (ECs), which regulates physiological vascular function and angiogenesis.

EC dysfunction
Inability of endothelial cells to fulfill their physiological role as mediators of the blood barrier and vasotone.

Fatty acid oxidation
Metabolism of fatty acids in mitochondria into acetyl-CoA to fuel the TCA cycle.

Glycolysis
Anaerobic metabolism of glucose producing ATP and pyruvate

Glycosylation
A post-translational modification that enzymatically adds glycans, or oligosaccharides, to proteins and lipids.

Hexosamine biosynthesis pathway
Side pathway from glycolytic intermediate fructose 6-phosphate (F6P) that produces substrates for glycosylation.

Isoprenoid
Mevalonate pathway intermediates used for the production of cholesterol and as substrates for prenylation.

Metabolic flux
Flow of metabolites through a given metabolic pathway.

Metabolic flux analysis
Quantification of metabolic flux by tracing the fate of isotope-labeled substrates.

Metabolism
The spectrum of organic and chemical cellular reactions dedicated to the production of energy and building blocks for general maintenance and functionality.

Methylglyoxal pathway
Glycolytic side pathway from dihydroxyacetone phosphate (DHAP) that results in production of methylglyoxal and/or AGEs.

Oxidative metabolism
Aerobic metabolic pathways that break down substrates through oxidation for energy production and biosynthesis.

Pentose phosphate pathway
Metabolic pathway important for redox homeostasis and biosynthesis which utilizes glucose-derived glucose-6-phosphate (G6P) for production of NADPH through its oxidative branch, and fructose 6-phosphate (F6P) and 3-phosphoglycerate (3PG) for nucleotide production in its non-oxidative branch.

Polyol pathway
Pathway implicated in diabetic endothelial dysfunction by reduction of glucose into sorbitol and then fructose to fuel production of AGEs.

Prenylation
Post-translational addition of isoprenoids such as farnesy1 or geranyl-geranyl to a protein.

Quiescence
Cell state defined by a lack of activity.

Reactive nitrogen species
Highly reactive nitrogen-containing molecules that often interact with ROS, promote oxidative stress and reduce bioavailability of nitric oxide.

Reactive oxygen species (ROS)
Highly reactive molecules that contain oxygen (produced by aerobic metabolic processes) and are involved in normal cell homeostasis and signaling, but whose accumulation, termed oxidative stress, leads to cell damage.

Stalk cell
Endothelial cells that trail migratory tip cells and proliferate to extend growth of a new blood vessel during sprouting angiogenesis.

Tip cell
Migratory endothelial cells that lead spouting microvessels up a chemokine gradient during angiogenesis.

and fatty acids in case of reduced glycolytic rates (Krutzzfeldt et al, 1990; Dranka et al, 2010).

ECs lining peripheral tissue vessels or the blood brain barrier (BBB) express multiple members of the two major families of sugar transporters, that is, glucose transporters (GLUT) and sodium/glucose co-transporters (SGLTs), but the high-affinity GLUT1 is considered to be the main route of glucose uptake in ECs (Fig 1) (Mann et al, 2003; Gaudreau et al, 2004, 2008; Sahoo et al, 2014). Phosphorylation of intracellular glucose by hexokinase (HK) destines it for metabolic utilization, predominately by conversion to lactate via glycolysis (Fig 1) (Park et al, 2005; De Bock et al, 2013b). Glycolytic intermediates also serve as precursors for biosynthetic pathways including the pentose phosphate pathway (PPP), hexosamine biosynthesis and glycosogenesis (Fig 1, for an extensive review see (De Bock et al, 2013a,b)).

The PPP consists of oxidative and non-oxidative branches, and its overall flux is determined by the rate-limiting enzyme glucose-6-phosphate dehydrogenase (G6PD) (Fig 1). Partially regulated by VEGF signaling, G6PD destines glucose-6-phosphate (G6P) for utilization in the PPP (Pan et al, 2009). The oxidative branch of the PPP converts G6P into ribulose-5-phosphate (Ru5P) and produces NADPH from NADP+ , thereby generating reducing power to maintain EC redox balance and biosynthetic reactions (Dobrina & Rossii, 1983; Jongkind et al, 1989; Spolarics & Spitzer, 1993; Spolarics & Wu, 1997; Vizan et al, 2009). The non-oxidative branch converts Ru5P into xylulose-5-phosphate (Xu5P) and ribose-5-phosphate (RSP), the latter is necessary for nucleotide biosynthesis (Pandolfi et al, 1995). However, PPP intermediates may also be converted back into glycolytic intermediates via the action of transketolase (TKT) and transaldolase. These reactions are reversible, allowing biosynthesis of macromolecules from glycolytic metabolites via the non-oxidative arm.

The hexosamine biosynthesis pathway starts with the conversion of the glycolytic intermediate fructose-6-phosphate (F6P) into glucosamine-6-phosphate (GlcN6P) (Fig 1). GlcN6P is then metabolized to uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), a key substrate for glycosylation reactions that control many aspects of EC function (Benedito et al, 2009; Laczky et al,
VEGF signaling, G6PD destines glucose-6-phosphate (G6P) for synthetic pathways including the pentose phosphate pathway (PPP), 2013b). Glycolytic intermediates also serve as precursors for biosynthesis of macromolecules from glycolytic metabolites via the hexosamine biosynthesis pathway. Glucose co-transporters (SGLTs), but the high-affinity GLUT1 is considered to be the main route of glucose uptake in ECs (Fig 1). Phosphorylation of intracellular glucose by hexokinase (HK) and functionality.

For clarity, not all metabolites and enzymes of the depicted pathways are shown. Abbreviations: 3DG: 3-deoxyglucose; 3PG: 3-phosphoglycerate; 6PGD: 6-phosphogluconate dehydrogenase; AGE: advanced glycation end-product; AR: aldose reductase; ARG: arginase; ATP: adenosine triphosphate; CPT: carnitine palmitoyltransferase; DHAP: dihydroxyacetone phosphate; eNOS: endothelial nitric oxide synthase; ETC: electron transport chain; F6P: fructose-6-phosphate; F1,6P2: fructose 1,6-bisphosphate; F2,6P2: fructose 2,6 bisphosphate; FA: fatty acid; G6P: glucose-6-phosphate; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GFAT: glutamine-6-phosphate amidotransferase; GS: glutamine synthetase; glutathione; GluNAc: N-acetylglucosamine; GluN6P: glucose-6-phosphate; GLS: glutaminase; GLUT: glucose transporter; GS: glutathione; GSH: glutathione; hCYS: homocysteine; HMG-CoA: hydroxymethylglutaryl coenzyme A; IDH1: isocitrate dehydrogenase; IDH2: lactate dehydrogenase; MCT: monocarboxylate transporter; ME: malic enzyme; MET: methionine; mTHF: 5,10-methylene-tetrahydrofolate; FDA: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3; PFK: phosphofructokinase; PGK: phosphoglycerate kinase; PGK: reactive oxygen species; RPI: ribose-5-phosphate isomerase; SAM: S-adenosylhomocysteine; SLC1A5: uridine diphosphate N-acetylglucosamine.

Figure 1. Overview of general EC metabolism.

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2009; Croci et al., 2014). The polyol pathway and methylglyoxal pathways are glycolysis side-paths that are mostly known for their role in cardiovascular disease (Fig 1; see below) (Goldin et al., 2006).

Other metabolic pathways are less well characterized in ECs. Fatty acid (FA) oxidation (FAO) and glutamine oxidation have been implicated in replenishing the TCA cycle to produce ATP via oxidative phosphorylation (Fig 1) (Leighton et al., 1987; Hinshaw & Burger, 1990; Dagher et al., 1999, 2001; De Bock et al., 2013b). However, since ECs predominately rely on glucose metabolism to provide ATP, the energetic function of FAO and glutamine oxidation is not clear (De Bock et al., 2013b). FAs and amino acids can serve as precursors for biomass production, but such a role in ECs has not been demonstrated using isotope tracer labeling studies. FAO produces significant amounts of nicotinamide adenine dinucleotide phosphate (NADPH), which is an important co-factor in many biosynthetic reactions and essential to maintain redox balance. In addition, FAO generates acetyl-CoA which is another important precursor for biomolecule production.

For example, acetyl-CoA is used, among other things, for the synthesis of cholesterol via the mevalonate pathway (Fig 1). Although endothelial cholesterol metabolism has been poorly studied, perturbations in cholesterol homeostasis are known to affect key EC functions such as intracellular signaling, inflammatory activation, nitric oxide synthesis and angiogenesis (Boger et al., 2000; Ivashchenko et al., 2010; Whetzel et al., 2010; Xu et al., 2010; Fang et al., 2013). ECs express all the cholesterol biosynthesis enzymes and the LDL receptor for extracellular uptake (Fig 1). These proteins are under transcriptional control of the sterol regulatory element binding protein (SREBP1 and -2) and liver X receptors (LXRs) (Noghero et al., 2012). SREBP1 and LXRs inhibit cholesterol synthesis and absorption, whereas SREBP2 induces synthesis and inhibits cholesterol efflux via transcriptional repression of the ATP-binding cassette (ABC) transporter 1 ABCA1, which together with ABCG1 mediates cholesterol efflux from ECs (Hassan et al., 2006). Notably, endothelial SREBP2 also controls expression of arginine metabolism enzymes, although the physiological significance of this interaction between cholesterol and arginine metabolism remains to be determined (Zeng et al., 2004).

Arginine and glutamine are the best studied amino acids (AAs) in ECs. Arginine is a metabolite in the ornithine cycle and converted into citrulline and nitric oxide (NO) by endothelial nitric oxide synthase (eNOS) (Fig 1) (Sessa et al., 1990). Alterations in arginine and eNOS metabolism are among the best-characterized causes of EC dysfunction and a prime therapeutic target (Leiper & Nandi, 2011). Glutamine is the most abundant AA in the peripheral blood and preferentially taken up by ECs via the solute carrier family 1 member 5 (SLC1A5) transporter (Fig 1) (Herskowitz et al., 1991; Pan et al., 1995). Glutamine-utilizing pathways are mainly biosynthetic and can be divided into those that utilize the γ-nitrogen (nucleotide biosynthesis, hexosamine biosynthesis, asparagine synthesis) and those that use the α-nitrogen or carbon backbone (DeBerardinis & Cheng, 2010). The latter reactions use glutamine-derived glutamate rather than glutamine itself and include glutathione (GSH) synthesis, anaplerotic refueling of the TCA cycle and biosynthesis of polyamines, proline and other non-essential AAs (NEAAs) (Fig 1) (DeBerardinis & Cheng, 2010).

Serine and glycine are especially interesting examples of glutamate / glutamate-derived NEAAs, not only because of their direct effects on ECs (Weinberg et al., 1992; Rose et al., 1999; Yamashina et al., 2001; Mishra et al., 2008; den Eynden et al., 2009; McCarty et al., 2009; Stobart et al., 2013), but also since their synthesis requires both the glutamate α-nitrogen and the glycolytic intermediate 3-phosphoglycerate (3PG) (Fig 1) (Locasale, 2013). Hence, serine and glycine metabolism integrates metabolic input from central carbon (glycolysis) and nitrogen (glutamine) metabolism. Moreover, the reversible interconversion of serine and glycine is directly coupled to one-carbon metabolism, intermediates of which are considered important targets to treat cardiovascular disease (Fig 1; see below) (Locasale, 2013). In fact, while EC metabolism is largely understudied, several of the above-mentioned metabolic pathways have been implicated as mediators of pathological angiogenesis or EC dysfunction.

**EC metabolism in diseases characterized by angiogenesis and EC hyperproliferation**

**Cancer**

Tumors need blood vessels to supply oxygen and detoxify waste products (Jain, 1987; Papetti & Herman, 2002; Welti et al., 2013). When tumors become too large to allow adequate diffusion of oxygen and nutrients from local vasculature they secrete pro-angiogenic growth factors to induce angiogenesis (Bergers & Benjamin, 2003). Pharmacological inhibition of growth factor signaling (primarily vascular endothelial growth factor (VEGF) signaling) is the only clinically approved anti-angiogenic strategy, but the benefits are limited as tumors acquire resistance within months after treatment initiation (Bergers & Hanahan, 2008; Carmeliet & Jain, 2011; Ebos & Kerbel, 2011; Welti et al., 2013). Escape from anti-angiogenic therapy is mediated by increased secretion of pro-angiogenic factors, activation of alternative angiogenic signaling pathways, recruitment of pro-angiogenic accessory cells and other mechanisms (Loges et al., 2010; Sennino & McDonald, 2012). A recent report indicated that glycosylation-dependent interactions of galecin-1 with VEGF receptor 2 (VEGFR2) could activate pro-angiogenic signaling even when the VEGF ligand is blocked (Fig 2A) (Croci et al., 2014). Hence, angiogenic signaling is robust and redundant, and inhibition of individual signaling molecules and growth factors can be overcome by escape mechanisms.

The switch from a quiescent to an angiogenic phenotype (as occurs in cancer) is metabolically demanding and mediated by adaptations in EC metabolism (Fig 2A). While the changes in metabolic fluxes of ECs, freshly isolated from tumors, have not been characterized yet, ECs in tumors and inflamed tissues likely resemble highly activated ECs. Lactate dehydrogenase B (LDH-B) is upregulated in tumor endothelium, and VEGF signaling increases glycolytic flux by inducing GLUT1 and the glycolytic enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKF3B) (Fig 2A) (van Beijnum et al., 2006; Yeh et al., 2008; De Bock et al., 2013b). PFKFB3 catalyzes the synthesis of fructose-2,6-bisphosphate (F2,6P2), which is an allosteric activator of 6-phosphofructo-1-kinase (PFK-1) (Van Schaftingen et al., 1982). PFK-1 converts fructose-6-phosphate (F6P) to fructose-1,6-bisphosphate (F1,6P2) in the rate-limiting step of glycolysis. EC-specific PFKFB3 deletion diminishes...
retinal and hindbrain vasculization in mice, showing that increased glycolytic flux is required for growth factor-induced angiogenesis (De Bock et al, 2013b). Moreover, PFKFB3 overexpression in zebrafish drives EC specification into sprout forming tip cells, even in the presence of tip cell-inhibitory Notch signals that promote proliferating stalk elongating cells (De Bock et al, 2013b). Increased glycolysis not only provides energy for proliferation and biosynthesis, but also for locomotion. Specifically, PFKFB3 and other glycolytic enzymes co-localize with F-actin bundles in filopodia and lamellipodia to produce ATP needed for rapid actin remodeling, underlying locomotion and tip cell formation (De Bock et al, 2013b).

The important role of glycolysis in angiogenesis provides opportunities for therapeutic targeting. Indeed, pharmacological blockade with 3-[(3-pyridinyl)-1-[(4-pyridinyl)-2-propen-1-one (3PO) or EC-specific genetic silencing of PFKFB3 inhibits tumor growth in vivo (Xu et al, 2014). In addition, 3PO inhibits glycolytic flux partially and transiently and has recently shown efficacy in reducing pathological angiogenesis in a variety of disease models (Schoors et al, 2014b; Xu et al, 2014). The systemic harm caused by inhibiting glycolysis is minimal, however, showing that even moderate, short-term impairment of glycolysis renders ECs more quiescent without overt detrimental side effects (Schoors et al, 2014b). The finding that partial and transient reduction of glycolysis may be sufficient to inhibit pathological angiogenesis provides a paradigm shift in our thinking about anti-glycolytic therapies, away from complete and permanent blockade of glycolysis, which can induce undesired adverse systemic effects.

Aside from serving as an energy source or building blocks for biosynthesis, glycolytic metabolites can also modulate angiogenesis by acting as bona fide signaling molecules. This is evidenced by the observation that glycolytic tumor cells secrete lactate, which is taken up by ECs through the monocarboxylate transporter 1 (MCT1) (Fig 2A) (Sonnevaux et al, 2012). Instead of being metabolized,
lactate induces HIF-1α activation leading to increased expression of VEGFR2 and bFGF (Sonveaux et al., 2012). Moreover, lactate competes with α-ketoglutarate for binding to the oxygen sensing prolyl hydroxylase-2 (PHD-2), resulting in diminished PHD-2 activity and subsequent hypoxia-inducible factor-1α (HIF-1α) stabilization (Fig 2A). Stabilized HIF-1α induces pro-angiogenic signaling pathways such as nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB)/interleukin 8 (IL-8) leading to increased angiogenesis (Fig 2A) (Hunt et al., 2007; Vegran et al., 2011; Sonveaux et al., 2012). Exploratory studies found that lactate induces angiogenesis in vitro and that pharmacological blockade of MCT1 inhibits angiogenesis and reduces tumor growth in mice (Sonveaux et al., 2012). Together, these data suggest an intricate relationship between classical pro-angiogenic signals such as VEGF, HIF-1α and hypoxia, and EC glucose metabolism. Targeting EC glucose metabolism to inhibit tumor angiogenesis is in its infancy as a therapeutic strategy, but recent evidence suggests its viability.

Pulmonary arterial hypertension
Idiopathic pulmonary arterial hypertension (PAH) is characterized by heightened pressure in pulmonary arteries caused by excessive EC proliferation and vascular dysfunction (Xu & Erzurum, 2011). Emerging evidence indicates that metabolic abnormalities underlie PAH (Fig 2B) (Sutendra & Michelakis, 2014; Zhao et al., 2014). In line with recent findings that glycolysis regulates angiogenesis, hyperproliferative PAH ECs rely on increased glycolytic flux and reduced oxygen consumption, which may be related to HIF-1α overexpression (Fig 2B) (Xu et al., 2007; Fijalkowska et al., 2010; Majmundar et al., 2010; Tuder et al., 2012). Human pulmonary ECs expressing mutated bone morphogenetic protein receptor 2 (BMPR2), which confers PAH, show altered expression of several glycolytic enzymes including GLUT1 and phosphoglycerate kinase 1 (PGK1). PAH ECs also show increased expression of enzymes of the PPP (R5P isomerase, Ru5P-3-epimerase) and polyamine biosynthesis pathway (ornithine decarboxylase (ODC), spermine synthase (SMS)). These metabolic changes may underlie the rapid proliferation of PAH ECs, since glycolysis, the PPP and mitogenic polyamines all promote cellular proliferation (Morrison & Seidel, 1995). However, the expression of other PPP and polyamine enzymes [G6PD, TKT, spermidine synthase (SRM)] is reduced—a finding that requires further explanation (Fig 2B) (Atkinson et al., 2002; Rudarakanchana et al., 2002; Long et al., 2006; Fessel et al., 2012). In addition, ECs isolated from EC-specific BMPR2 mutant mice show similarly increased expression of PGK1, indicating altogether that alterations in glycolysis as well as PPP likely underlie PAH (Majka et al., 2011).

In addition to alterations in glycolysis, idiopathic PAH ECs have fewer mitochondria and decreased mitochondrial metabolic activity (Xu et al., 2007). BMPR2 mutant ECs have reduced quantities of TCA cycle intermediates, reduced fatty acid oxidation and transcriptional reduction of several enzymes involved in fatty acid metabolism, including the rate-limiting enzyme of fatty acid oxidation carnitine palmitoyltransferase 1 (CPT1) (Fig 2) (Fessel et al., 2012). Together, these findings suggest reduced oxidative metabolism. Indeed, pharmacological inhibition of hyper-activated pyruvate dehydrogenase kinase (PDK), an enzyme that shunts glucose-derived pyruvate away from oxidative TCA metabolism, has shown therapeutic efficacy. However, whether these effects are mediated via ECs specifically remains to be determined (McMurtry et al., 2004). For unexplained reasons, PAH patients also show increased isocitrate dehydrogenase (IDH)-1 and IDH-2 serum activity, a finding that corroborates with the increased IDH activity observed in BMPR2 mutant ECs (Fessel et al., 2012). Still, the mechanisms that alter metabolic pathways in PAH ECs and the importance of some of these metabolic adaptations in the pathogenesis of PAH remain unclear.

Reduced nitric oxide (NO) levels are another hallmark of PAH ECs (Fijalkowska et al., 2010). Low NO levels may be related to the reduced levels of the mitochondrial antioxidant manganese superoxide dismutase (MnSOD) (Fijalkowska et al., 2010). Indeed, MnSOD increases NO availability by clearing superoxide anion, which inactivates NO to form peroxynitrite (Fig 2) (Masri et al., 2008). However, other factors likely contribute to the low NO levels in PAH ECs (Xu et al., 2004). Indeed, human PAH ECs express high levels of arginase II, which competes with endothelial nitric oxide synthetase (eNOS) for their common substrate L-arginine (Fig 2) (Xu et al., 2004). Inhibition of endothelial arginase II increases NO production in vitro, suggesting that arginase II can be targeted to prevent EC hyperproliferation and restore NO availability (Krotova et al., 2010). While the mechanisms that induce abnormal metabolic activity in PAH ECs are understudied, restoring NO may provide dual benefits in preventing excessive EC proliferation as well as restoring EC vasoactivity.

The metabolic adaptations in PAH (high glycolytic rates and reduced oxidative metabolism) are partly reminiscent of the metabolic profile of angiogenic ECs. It would be thus interesting to determine if reducing glycolysis by pharmacological blockade of PFKFB3 can reduce the hyperproliferative rate in PAH ECs. Alternatively, the beneficial effects of PDK inhibition in PAH to induce oxidative metabolism could also be beneficial to block angiogenesis by preventing the glycolytic switch in ECs. Indeed, PDK blockade with dichloroacetate inhibits angiogenesis in glioblastoma patients (Michelakis et al., 2010).

EC metabolism in diseases characterized by EC dysfunction
Diabetes
Diabetes is characterized by high blood glucose levels that affect EC metabolism and cause dysfunction (Fig 3A) (Blake & Trounce, 2013). Hyperglycemia induces peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC-1α), an important regulator of metabolic gene expression and mitochondrial biogenesis (Puigserver et al., 1998; Herzig et al., 2001; Lin et al., 2002). PGC-1α increases angiogenesis when expressed in heart and muscle cells (Arany et al., 2008; Patten et al., 2012). In contrast, diabetes-induced PGC-1α expression in ECs renders them less responsive to angiogenic factors and blunts angiogenesis (Sawada et al., 2014).

In addition to affecting gene expression, high glucose levels alter metabolism to induce the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which might be mediators of EC dysfunction (Fig 3) (Blake & Trounce, 2013). High glucose levels cause ECs to produce ROS via activation of NADPH-dependent oxidases (Inoguchi et al., 2003). In addition, hyperglycemia inhibits PPP flux by down-regulation of G6PD, the rate-limiting enzyme of the PPP. The PPP is an important source...
of intracellular NADPH, which is necessary to convert oxidized glutathione (GSSH) into reduced GSH, a critical ROS scavenger (Fig 3A) (Leopold et al., 2003; Zhang et al., 2012). Therefore, by reducing PPP flux, high glucose depletes NADPH levels and contributes to ROS accumulation (Goldin et al., 2006). Interestingly, G6PD overexpression restores redox homeostasis in high glucose cultured ECs (Leopold et al., 2003; Zhang et al., 2012). Some studies suggest that high glucose shifts the normally glycolytic EC metabolism toward oxidative metabolism and increased mitochondrial respiration (Fig 3). However, these results appear contextual, as other studies did not report such an induction of oxidative metabolism (Nishikawa et al., 2000; Koziel et al., 2012; Pangare & Makino, 2012; Dymkowska et al., 2014). While the precise effects on mitochondrial respiration require further study, hyperglycemia-induced mitochondrial ROS induces DNA breaks and thereby activates polyADP-ribose polymerase (PARP-1) (Du et al., 2000, 2003; Nishikawa et al., 2000; Giacco & Brownlee, 2010; Blake & Trounce, 2013). PolyADP-ribosylation by PARP-1 inactivates GAPDH and stalls glycolysis, allowing accumulation of glycolytic metabolites (Du et al., 2003).

Accumulation of F6P increases the flux through the hexosamine biosynthesis pathway (HB), which produces UDP-GlcNac, an important precursor of glycosylation reactions (Fig 3A) (Brownlee, 2001). While glycosylation is important for physiological EC function, hyperglycemia-induced protein glycosylation inhibits angiogenic functions (Du et al., 2001; Federici et al., 2002; Luo et al., 2008). Other glycolytic intermediates are diverted into the polyol and methylglyoxal pathways that produce damaging agents such as ROS and advanced glycation end products (AGEs) (Fig 3A) (Goldin et al., 2006). AGEs induce vascular dysfunction by altering extracellular matrix protein function and dysregulating cytokine expression (Yan et al., 2008). In addition, receptor of AGE (RAGE) binding by AGEs in vascular cells causes inflammation and reduced NO availability associated with vascular complications in
diabetic patients (Bucaia et al., 1991; Vlassara et al., 1995; Min et al., 1999; Wautier & Schmidt, 2004; Goldin et al., 2006; Manigrasso et al., 2014).

Excess glucose that cannot be metabolized by glycolysis enters the polyol pathway when converted into sorbitol by aldose reductase (AR) at the expense of NADPH, increasing ROS. Sorbitol is subsequently converted into fructose and the highly reactive 3-deoxyglucosone (3DG), which promotes the formation of AGEs (Fig 3A) (Kashiwagi et al., 1994; Oyama et al., 2006; Giacco & Brownlee, 2010; Sena et al., 2012; Yoshida et al., 2012). Transgenic overexpression of human AR in the endothelium of diabetic mice accelerates atherosclerosis formation and inhibition of endothelial AR reduces intracellular ROS, EC migration and proliferation (Obrosova et al., 2003; Tamnami et al., 2011; Vedantham et al., 2011; Yadav et al., 2012).Methylglyoxal is another AGE precursor and produced from the glycolytic intermediates glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP). Methylglyoxal is detoxified by conversion into pyruvate via the multi-enzyme glyoxalase system, of which glyoxalase-I (Glol) is rate-limiting (Fig 3A) (Thornalley, 1993). Glyoxalase-I overexpression reverses hyperglycemia-induced angiogenesis defects in vitro and transgenic overexpression of glyoxalase-I in rats reduces vascular AGE formation and improves vasoreactivity (Brouwers et al., 2010, 2014) (Ahmed et al., 2008). Together, these observations indicate that targeting AR and glyoxalase might confer a therapeutic benefit in diabetic patients.

**Atherosclerosis**

Atherosclerosis is a chronic inflammatory process in the blood vessel wall leading to luminal narrowing and subsequent cardiovascular events (Hopkins, 2013). Systemic metabolic perturbations are among the most important risk factors of atherosclerosis. However, metabolic flux changes have not been studied in ECs isolated from atherosclerotic lesions, and the effects of atherosclerosis on central metabolism of ECs thus remains to be characterized. Nonetheless, EC metabolism is strongly associated with a key pathophysiological feature of atherosclerosis: reduced and uncoupled eNOS activity resulting in low NO bioavailability and high ROS production (Fig 3B) (Kawashima & Yokoyama, 2004). eNOS activity critically depends on the availability of L-arginine, co-factor tetrahydrobiopterin (BH4) (Fig 3B) and possibly co-enzyme Q10 (CoQ10) (Gorren et al., 2000; Crabtree et al., 2009a; Mugoni et al., 2013). If L-arginine, BH4 or CoQ10 become limited, eNOS no longer oxidizes L-arginine to form citrulline and NO, but instead produces ROS (a condition termed eNOS uncoupling) (Fig 3B) (Stroes et al., 1998; Mugoni et al., 2013). Targeting L-arginine and BH4 metabolism to increase eNOS activity in patients with cardiovascular disease is potentially beneficial, but available evidence indicates that the picture is more complex than initially anticipated.

Small-scale clinical trials indicate that administration of L-arginine to patients with coronary heart disease improves vasoresponsive-ness, possibly by increasing NO production by eNOS (Lerman et al., 1998). Interestingly, however, intracellular and plasma arginine levels are sufficiently high to support NO biosynthesis via eNOS. Therefore, the benefits of L-arginine supplementation on elevating NO levels are not readily explained by increasing the supply of L-arginine; however, it is possible that L-arginine is compartmentalized in poorly interchangeable pools. Another possible explanation of the beneficial effects of L-arginine is competition with asymmetric methylated arginines, which bind and inhibit eNOS (Fig 3B) (Boger, 2004; Chen et al., 2013). More in detail, post-translational methylation of arginine residues in proteins by protein arginine methyltransferase (PRMT) results in the addition of up to two methyl groups to arginine. Protein turnover releases these post-translationally modified amino acids as asymmetric dimethyl-arginine (ADMA) and symmetric dimethylarginine (SDMA). The asymmetric dimethylarginines bind and uncouple eNOS resulting in increased ROS production and reduced NO availability (Fig 3B) (Dhillon et al., 2003; Leiper & Nandi, 2011). Hence by competing with ADMAs, supplemented L-arginine could maintain eNOS activity to produce NO (Bede-Boger et al., 2003). Additional potential interventions to reduce eNOS inhibition by ADMA include PRMT inhibition (to reduce arginine methylation) and activation of methyl-arginine catabolism by dimethylarginine dimethylaminohydrolase (DDAH) (Fig 3B) (Leiper & Nandi, 2011). Interestingly, DDAH is predominantly expressed in ECs and EC-specific deletion attenuates NO production and induces hypertension, indicating that ADMA scavenging by ECs is important to maintain homeostasis (Hu et al., 2009).

Because L-arginine is a substrate for both eNOS and arginase (Wu & Meininger, 1995), NO production depends on the relative expression levels of each enzyme (Fig 3) (Chang et al, 1998; Ming et al., 2004; Ryoo et al., 2008). Endothelial arginase expression is induced by many risk factors for cardiovascular disease, while reducing arginase expression restores NO production in vitro and improves vasodilatation in vivo (Ryoo et al, 2006, 2008; Chengchaisri et al, 2006; Romero et al., 2008). The activity of eNOS and arginase is regulated by the RhoA/ROCK signaling cascade. RhoA and ROCK decrease eNOS expression, while RhoA also increases arginase activity (Fig 3B) (Laufs et al., 1998; Takemoto et al., 2002). For proper activation and localization to the cell membrane, RhoA must be prenylated (more specifically, geranylgeranylated) by geranylgeranyltransferase (GGT) using geranylgeranyl pyrophosphate (GGPP) as a substrate (Laufs & Liao, 1998). This isoprenoid is an intermediate of the mevalonate pathway, which produces cholesterol from acetyl-coA (Fig 3B). Blocking the mevalonate pathway by inhibiting HMG-coA reductase using statins lowers cholesterol synthesis and is clinically approved to prevent cardiovascular events in dyslipidemia patients. In addition, HMG-coA blockade also decreases geranylgeranyl production, which reduces RhoA activity and restores a more beneficial eNOS/arginase balance (Goldstein & Brown, 1990; Liao & Laufs, 2005). Interestingly, UBIAD1 was recently identified as a novel prenyltransferase that produces non-mitochondrial CoQ10 from farnesy1 pyrophosphate (PPP), another isoprenoid produced in the mevalonate pathway (Fig 3) (Mugoni et al., 2013). CoQ10 is an important anti-oxidant with beneficial effects on EC function and hypothesized to be a novel co-factor required for eNOS coupling (Gao et al., 2012; Mugoni et al., 2013). Hence, in contrast to the above-mentioned beneficial effects, HMG-coA reductase inhibition might thus also have a less favorable effect by increasing ROS levels through reducing CoQ10 synthesis (Fig 3) (Mugoni et al., 2013).

In addition to CoQ10, eNOS requires BH4 as a co-factor. Reduced BH4 availability is found in patients at risk of
atherosclerosis and promotes ROS production through eNOS uncoupling (Fig 3B) (Pieper, 1997; Stroes et al., 1997; Heitzer et al., 2000). Endothelial BH4 levels are maintained by de novo biosynthesis via the rate-limiting enzyme guanosine triphosphate cyclohydrolase I (GTPCH) and by a salvage pathway from dihydrobipterin (BH2) via dihydrofolate reductase (DHFR) (Fig 3B) (Bendall et al., 2014). Insufficient levels of GTPCH and DHFR, important enzymes in GTP and folate metabolism, respectively, have been associated with reduced BH4 availability, endothelial dysfunction and cardiovascular disease in several preclinical models (Chalupska & Cai, 2005; Crabtree et al., 2009b, 2011; Sugiyama et al., 2009; Kidokoro et al., 2013). Interestingly, DHFR not only regenerates active BH4 from oxidized inactive BH2 but is also a key enzyme in folate and one-carbon metabolism, intermediates of which in turn regulate BH4 biosynthesis and are associated with cardiovascular disease (Humphrey et al., 2008).

One-carbon (1C) metabolism centers around the ability of folate-derived co-enzymes to carry activated 1C units (Fig 3) (Tibbetts & Appling, 2010). DHFR catalyzes the formation of tetrahydrofolate (THF) from folate fueling 1C metabolism. THF accepts 1C units from serine to produce 5,10-methylene-THF (meTHF) and glycine. MeTHF is reduced to 5-methyl-THF (mTHF) by methylenetetrahydrofolate reductase (MTHFR) (Fig 3).

Importantly, inactivating mutations in the MTHFR gene result in hyperhomocysteinemia, which decreases GTPCH and DHFR levels and may subsequently reduce BH4 levels (Bendall et al., 2014). Indeed, MTHFR mutations have been associated with cardiovascular disease, but the exact association is still controversial (Kelly et al., 2002; Klerk et al., 2002; Frederiksen et al., 2004; Yang et al., 2012). mTHF produced by MTHFR activity is required as a methyl donor in the methionine synthase (MS) catalyzed reaction that converts mTHF into THF (completing the folate cycle) and forms methionine (MET) from homocysteine (hCYS) (Fig 3B) (Locasale, 2013). Methionine is used to generate S-adenosylmethionine (SAM), which is an important methyl donor and plays a pivotal role in methylation of lysine and arginine residues in proteins (Fig 3B) (Leiper & Nandi, 2011). As discussed above, methylated arginine residues are emerging as important mediators of EC dysfunction. Moreover, SAM-mediated protein methylation produces S-adenosylhomocysteine, which is converted back into homocysteine. Homocysteine decreases the bioavailability of BH4 possibly through downregulation of GTPCH and DHFR, while BH4 supplementation alleviates homocysteine-induced EC dysfunction (Dhillon et al., 2003; Topal et al., 2004). Together, these findings suggest that dysregulation of endothelial 1C metabolism is involved in the pathogenesis of cardiovascular disease, but the
exact mechanisms remain to be elucidated. Nonetheless, early clinical and preclinical studies have found that therapeutic targeting of 1C metabolism, for example, via folate supplementation lowers levels of homocysteineemia and increases BH4 regeneration from BH2 (Verhaar et al., 2002). However, large-scale clinical trials failed to show benefits of folate or BH4 supplementation to prevent cardiovascular disease (Clarke et al., 2010; Cunnington et al., 2012; Marti-Carvajal et al., 2013). These clinical and preclinical findings suggest that while L-arginine, folate, methionine, COQ10 and homocysteine metabolism are potential therapeutic targets, a more detailed understanding of how these pathways cause dysfunction is required to design more rational therapeutic agents.

**EC metabolism in the pathogenesis of other diseases**

EC metabolism is best characterized in the diseases discussed above. However, these represent only a minor fraction of the disorders in which pathological EC responses are presumably involved. Indeed, it is highly likely that EC metabolic alterations are also involved in the pathogenesis of other diseases such as ischemia, pre-eclampsia, vasculitis, vascular neoplasms and others although this has hardly been studied.

On the other hand, many of the EC metabolic alterations that lead to EC dysfunction are likely induced by cardiovascular risk factors such as those that characterize metabolic syndrome, hyperhomocysteineemia and hyperuricemia. For example, elevated serum uric acid (a breakdown product of purine nucleotides generated by xanthine oxidase with potent anti-oxidant activity) is common in patients with hypertension and may even be a root cause of EC dysfunction leading to cardiovascular disease (Feig et al., 2008). Interestingly, while uric acid has been described as major anti-oxidant in human plasma, ECs exposed to uric acid display increased ROS production creating a paradox that has not been resolved (Lippi et al., 2008; Sautin & Johnson, 2008). Regardless, in cardiovascular disease models uric acid reduces mitochondrial content, intracellular ATP and arginase activity (Zharikov et al., 2008; Sanchez-Lozada et al., 2012). In addition, uric acid inhibits NO production in ECs in vitro, and in vivo levels of serum nitrates (an indicator of NO production) are inversely proportional to serum uric acid concentrations (Khosla et al., 2005). Interestingly, ECs exposed to uric acid increase expression of AR and alter expression of several other proteins linked to metabolism (Zhang et al., 2014). These studies suggest that hyperuricemia induces EC dysfunction through metabolic alterations. Whether the same is true for other cardiovascular risk factors remains in question.

A broader characterization of EC metabolism in the future might reveal novel therapeutic targets in metabolic pathways that are generally not considered to be important in pathological EC function. Recent findings that endothelial cholesterol efflux to high-density lipoprotein regulates angiogenesis (Fang et al., 2013), and that EC-specific insulin receptor knock-out accelerates atherosclerotic plaque formation (Gage et al., 2013) point to a key role for EC metabolism in the pathogenesis of disease and indicate that many more yet to be identified non-traditional but potentially druggable metabolic enzymes, transporters and pathways may play a role in vascular disease.

**Therapeutic targeting of EC metabolism**

Overall, it is clear that pathological blood vessel responses are associated with metabolic alterations in ECs. These metabolic adaptations are not just innocent bystanders, but in many cases mediate important aspects of disease. Increased EC glucose metabolism is emerging as a key feature of angiogenic and hyper-proliferative ECs. Targeting EC glucose metabolism has recently been shown as a viable strategy to curb pathological angiogenesis, but is still in its infancy (Schoors et al., 2014b). Recent technical and conceptual advances, however, now make it possible to perform comprehensive metabolic studies. These technical breakthroughs have led to a resurgent interest in targeting cell metabolism for therapeutic gains. As a proof of concept, targeting EC metabolism by pharmacological inhibition of the glycolytic enzyme PFKFB3 has shown recent success in inhibiting pathological angiogenesis (Fig 4) (De Bock et al., 2013b; Schoors et al., 2014b; Xu et al., 2014). These results, together with the observation that EC metabolism is altered in many diseases, suggest that EC metabolism is an attractive and viable but understudied therapeutic target.

**For more information**

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Conflict of interest
The authors declare that they have no conflict of interest.

References


Targeting EC metabolism in disease


transporter MCT1 in endothelial cells inhibits lactate-induced HIF-1 activation and tumor angiogenesis. PLoS ONE 7: e33418


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The heart has been recognized as an endocrine organ for over 30 years (de Bold, 2011); however, little is known about how the heart communicates with other organs in the body, and even less is known about this process in the diseased heart. In this issue of EMBO Molecular Medicine, Magida and Leinwand (2014) introduce the concept that a primary genetic defect in the heart results in aberrant hepatic lipid metabolism, which consequently exacerbates hypertrophic cardiomyopathy (HCM). This study provides evidence in support of the hypothesis that crosstalk occurs between the heart and liver, and that this becomes disrupted in the diseased state.

See also: JA Magida & LA Leinwand (April 2014)

Hypertrophic cardiomyopathy (HCM) is an inherited cardiovascular disease primarily caused by mutations in genes encoding proteins in the sarcomere, the contractile apparatus of cardiac myocytes. HCM is characterized by increased heart mass and abnormal cardiac function with susceptibility to arrhythmias and sudden cardiac death. Histological manifestations of the disease include cardiac myocyte hypertrophy, myocardial fibrosis, extracellular matrix disorganization, and myocyte disarray. While many affected individuals are asymptomatic and remain undiagnosed, HCM is the most frequent cause of sudden death in young athletes (Seidman & Seidman, 2011; Maron & Maron, 2013).

To date, 13 genes containing over 900 distinct mutations have been identified as genetic causes of HCM. Most of these genes encode for proteins found within the thick and thin filaments of sarcomeres, such as β-myosin heavy chain (MYH7) and tropomysin T (TTNT2). Mutations in MYH7 increase force generation and actin-myosin sliding velocity within sarcomeres. These findings indicate that genetic mutations in HCM patients are the primary cause of cardiac hypertrophy (Wang et al., 2010).

Numerous animal models have been generated to investigate HCM (Maass & Leinwand, 2000), and much focus has been given to an R403Q mutation in MYH7, which causes an especially severe clinical phenotype (Seidman & Seidman, 2011). While the various animal models of R403Q highlight different aspects of HCM, they share common traits of HCM including cardiac hypertrophy and fibrosis (Maass & Leinwand, 2000). An interesting, and poorly understood characteristic of hypertrophic cardiomyopathy, as opposed to other types of cardiomyopathies, is that systemic metabolic alterations occur secondary to the cardiomyopathy (Maron & Maron, 2013). This is recapitulated in the R403Q model used in the study published by Magida and Leinwand (2014).

Clinical studies have revealed that HCM patients harboring mutations in sarcomeric genes present with deficient cardiac energetics (Crilley et al., 2003). In the present study, the authors demonstrate that the R403Q HCM mouse model has diminished cardiac ATP levels and impaired lipid utilization in the heart, assessed by decreased cardiac triglycerides and fatty acid content, and decreased expression of fatty acid translocase (CD36), lipoprotein lipase (LPL), and very low density lipoprotein receptor (VLDLR). Notably, they observed an approximate two-fold reduction in active CD36 protein at the plasma membrane, coupled with a similarly decreased level of nonesterified fatty acid (NEFA) released from VLDL by the left ventricle. The authors suggest that this decreased lipid uptake in the heart leads to the observed lipid elevation in the plasma, ultimately resulting in hepatic lipid accumulation and pathologically enhanced gluconeogenesis. The authors propose that this elevation in hepatic glucose production creates a vicious cycle between the heart and the liver in which the spillover of VLDL triglyceride and oleic acid from the heart insults the liver via elevated protein kinase C signaling. The liver responds by increasing blood glucose levels leading to exacerbation of the primary cardiac disease (summarized in Fig 1). Importantly, features of the diseased state can be rescued either by restoring the energetic deficit at the level of the cardiomyocyte via AMPK agonism, or by blocking the deleterious elevation in hepatic glucose output using the phosphoeno- pyruvate carboxykinase (PEPCK) inhibitor 3-MPA (Magida & Leinwand, 2014).

“...These findings raise the interesting concept that the lack of use of a specific metabolic substrate by one tissue directly affects another...”

These findings raise the interesting concept that the lack of use of a specific metabolic substrate by one tissue directly affects another, perhaps revealing an inter-tissue homeostatic feedback mechanism. Namely,
that the heart signals to the liver to elevate glucose production by selectively excluding uptake and use of oleic acid and triglyceride in VLDL particles. Indeed, an emerging theme in homeostatic feedback is the recognition of metabolites as signaling effectors between tissues as means of physiologic integration within the body [see (Blad et al., 2012; Liu et al., 2013; Roberts et al., 2014) for examples]. However, in the setting of an HCM genotype, the current work suggests this relationship is injurious.

Many metabolic diseases, such as diabetes and obesity, are ultimately detrimental to cardiac function, but the reverse has yet to be investigated. There is a clear relationship between cardiac metabolism and cardiac function, but diminished cardiac function, per se, has thus far not been reported to negatively influence systemic metabolism. There is a clear link between liver dysfunction, specifically non-alcoholic fatty liver disease, and cardiac dysfunction (Bhatia et al., 2012), but new evidence reported in this issue of EMBO Molecular Medicine suggests the reverse is also true.

While the link between cardiac dysfunction, specifically the alteration of cardiac metabolism, and deregulated hepatic lipid metabolism is interesting, the mechanisms regulating this crosstalk are not resolved by the work of Magida and Leinwand (2014). Further studies are required to clarify whether HCM-induced metabolic abnormalities are the primary cause of liver dysfunction. It remains unclear whether hepatic lipid accumulation in this mouse model results from decreased fatty acid uptake in the heart alone. Certainly, the relationship between the heart and liver is not monogamous, and other tissues such as skeletal muscle, pancreas, and adipose are likely to be directly affected by elevated circulating oleic acid and VLDL triglyceride. Indeed it is likely that lipid uptake, utilization, or storage in each of these tissues contributes to the metabolic phenotype described by Magida and Leinwand (2014) and would be influenced by systemic agonism of AMPK. Further, PEPCK inhibition not only affects glucose production by the liver, kidney, and intestine, but also glyceroegenesis in adipocytes. Additionally, it would be interesting to know if other sarcomeric mutations also decrease liver function in end-stage disease, and if so, if a similar mechanism is involved.

Other aspects of HCM can also be explored in the R403Q HCM mouse model within the framework of metabolic abnormalities. For example, what role does calcium homeostasis play in the development of cardiac and metabolic dysfunction? Calcium is an important regulator of energy metabolism and calcium levels and homeostasis are altered in human HCM patients (Wang et al., 2010). Perhaps restoring calcium homeostasis in the heart could restore metabolism in this mouse as well? Moreover, what is the basis for the phenotypic gender differences in HCM? Is there likely a protective role for estrogen at the level of cardiac energetics as well as liver metabolism in the HCM patient? Estrogen certainly has a role both as it relates to AMPK and hepatic lipid metabolism (D’Eon et al., 2005; Bryzgalova et al., 2008), properties which could be therapeutically exploited.

The studies of Magida and Leinwand (2014) add to a growing number of examples in which the heart modulates energy homeostasis and metabolism in non-cardiac tissues. In this regard, the cardiac natriuretic peptides, ANP and BNP, have been shown to improve metabolic parameters by inducing the “browning” of white adipocytes (Bordicchia et al., 2012). While the thermogenic action of ANP is restricted to human, but not rodent adipocytes (Bordicchia et al., 2012), ANP was shown to induce glyceroegenesis in rat hepatocytes (Rashed et al., 1992). Therefore, it is curious that ANP expression is dramatically enhanced in HCM, but this mechanism for hepatic glucose output was left unexplored in these studies. Similarly, elevated expression of the Mediator subunit MED13 in the heart confers metabolic benefits in mice. MED13 is negatively regulated by a cardiac specific microRNA, miR-208, which plays a key role in cardiac hypertrophy (Grueter et al., 2012). Whether the miR-208/MED13 axis influences the metabolic consequences associated with HCM is an interesting question for the future. Perhaps a miR-208 inhibitor can remedy the metabolic defects observed in HCM by activating cardiac MED13, thus enhancing systemic metabolism, and reversing or preventing liver steatosis.

In summary, the work of Magida and Leinwand (2014) highlights the inextricable
relationship between sarcomeric structural integrity and metabolically-derived energy at the organismal level, and opens up many more avenues for future investigation.

Conflict of interest
The authors declare that they have no conflict of interest.

References

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Mitochondrial diseases can arise from mutations either in mitochondrial DNA or in nuclear DNA encoding mitochondrially destined proteins. Currently, there is no cure for these diseases although treatments to ameliorate a subset of the symptoms are being developed. In this issue of *EMBO Molecular Medicine*, Khan *et al* (2014) use a mouse model to test the efficacy of a simple dietary supplement of nicotinamide riboside to treat and prevent mitochondrial myopathies.

See also: NA Khan *et al* (June 2014)

Getting the right levels of vitamins is essential for health. Those of us of a certain age will remember in junior school being taught about the consequences of vitamin deficiency and having to memorise those consequences. For example, one deficiency, exotically named *pellagra*, resulted in a combination of dermatitis, diarrhoea and dementia. The underlying cause was identified as a lack of nicotinic acid or nicotinamide (vitamin B3). Indeed, the defect was exacerbated by a dietary lack of tryptophan. This is now understood, as all three components are important building blocks for the production of nicotinamide adenine dinucleotide, NAD, a redox-active coenzyme and enzyme substrate. This molecule is well known as a key player in metabolism, being the primary electron donor in the mitochondrial respiratory chain. It is also utilised and broken-down by a variety of proteins in other subcellular compartments, such as the family of protein deacetylases (sirtuins), the poly (ADP ribose)-phosphorylases (PARPs) and NAD glycohydrolases. *De novo* synthesis from tryptophan is a complex 8-step enzymatic process, so there are likely to be recycling pathways that utilise NAD synthesis intermediates as substrates. This is where nicotinamide and nicotinic acid feature. Both are intermediates in NAD biosynthesis, requiring enzymatic pathways of only 2 or 3 steps respectively to generate NAD (Bogan & Brenner, 2008). An additional salvage pathway has been identified in eubacteria and eukaryotes that is distinct from these nicotinic acid or nicotinamide recycling (or salvaging) pathways; in a two-step process, nicotinamide riboside (NR) can be first phosphorylated and then adenylylated to form NAD⁺ (Bieganowski & Brenner, 2004; see Fig 1). Those of us who remember memorising those vitamin deficiency diseases at school, probably also remember the compulsory bottle of milk to be drunk at break time. Although we did not realise it then, this was a good source of nicotinamide riboside, which in addition to being a normal metabolite in the body is also present in cow’s milk.

**NR can protect against mitochondrial myopathy in mice**

Defects of the mitochondrial (mt) respiratory chain constitute one of the most common forms of heritable metabolic disease. Clinical presentation varies widely, and significantly, there is no effective cure. Khan *et al* hypothesised that under conditions of respiratory chain deficiency, NADH utilisation is partially blocked leading to a decrease in the NAD⁺/NADH ratio. This constitutes a signal in the cell that is translated as indicating high nutrient availability, a condition completely at odds with the defective mitochondrial function. Therefore, by repleting levels of NAD⁺, the authors surmise that mitochondrial dysfunction could be ameliorated. To challenge their hypothesis, the authors have used their mt-Deletor mouse, a model of mitochondrial myopathy, and administered the NAD⁺ precursor, NR. The Deletor mouse carries a dominant pathogenic mutation in the major mitochondrial DNA (mtDNA) replicative helicase, Twinkle, that corresponds to a mutation found in patients (Tynnyrinen *et al*, 2005). In Deletor mice, this causes increased levels of deleted mtDNA and a subtle but chronically progressive mitochondrial myopathy. Control mice and pre- and post-symptomatic Deletor mice were dosed with large (400 mg/kg/day) amounts of NR for up to 4 months, a regime previously documented to result in increased levels of NAD⁺ in skeletal muscle of wild-type mice (Canto *et al*, 2012). Khan *et al* show that this treatment resulted in a marked increase in mitochondrial biogenesis in skeletal muscle and brown adipose tissue compared to undosed controls. A similar increase had been shown in the previous experiments following NR treatment, both of cultured cells and in various mice tissue (Canto *et al*, 2012). Crucially, however, for these new NR supplement experiments, the mt-biogenesis was concomitant with a decrease in markers of disease progression in Deletor mice, which were also protected from ultrastructural abnormalities of mitochondria. NR invoked a minor increase in overall mtDNA levels in both control and Deletor mice, but intriguingly caused a decrease in the levels of deleted mtDNA that accumulated in skeletal muscle of the Deletors. Thus, data were consistent with NR treatment and increasing NAD⁺ levels protecting against mitochondrial disease in the Deletor mice. In addition to promoting mt-biogenesis, NR also appeared to enhance...

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the mitochondrial unfolded protein response. This increase in a subset of mitochondrial chaperones and proteases is believed to be beneficial to health and promote an increased lifespan (Pellegrino et al., 2013).

Why does HR treatment promote mitochondrial biogenesis?

Previous reports have implicated increased NAD\(^+\) levels with increased sirtuin activity, most notably SIRT1 and SIRT3 (Lagouge et al., 2006; Hirschy et al., 2011). The consequence is an activation of key transcription factors including SIRT1 and SIRT3 (Canto et al., 2012), which upregulate gene products that are central to mt-biogenesis (Feige et al., 2008). In addition to enhancing oxidative metabolism in a range of tissues, SIRT1 activation has also been reported to protect against diet-induced metabolic disorders by enhancing fatty acid oxidation (Feige et al., 2008). Consistent with this, Khan et al. present data to show an NR-mediated increase in skeletal muscle mRNA levels encoding proteins that are involved in fatty acid transport or oxidation, namely CD36, ACOX1 and MCAD. Increasing mitochondrial biogenesis as a way of treating mitochondrial dysfunction is encouraging and has been previously shown to be efficacious for mouse models of mitochondrial disease (Wenz et al., 2008). However, it has been well described that mitochondrial proliferation can occur as a consequence of mtDNA disease in man. It will certainly be interesting to discover whether drug-induced mitochondrial biogenesis can also be beneficial to patients with mitochondrial dysfunction.

Why are these results so encouraging?

To date, there is no effective therapy for patients with mitochondrial myopathy. Vitamin cocktails including vitamin B3 (although at far lower doses than used here) have often been used to treat such patients for many years, with only sporadic reports of efficacy. The rationale for increasing NAD\(^+\) levels in order to increase mitochondrial mass is reasonable, and the results reported here are compelling. What is particularly exciting is that NAD\(^+\) intermediates such as NR are readily available and relatively simple drugs. If the efficacy of NR is...
strikingly high (400 mg/kg/day) compared to most commercially available supplements (60–500 mg/person/day). Whether such a large dosage is viable as a supplement needs to be established; however, it will be exciting to follow new pharmacokinetic data for this potentially therapeutic nucleoside derivative.

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Conflict of interest
The authors declare that they have no conflict of interest.

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Long-term therapeutic silencing of miR-33 increases circulating triglyceride levels and hepatic lipid accumulation in mice

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Plasma high-density lipoprotein (HDL) levels show a strong inverse correlation with atherosclerotic vascular disease. Previous studies have demonstrated that antagonism of miR-33 in vivo increases circulating HDL and reverse cholesterol transport (RCT), thereby reducing the progression and enhancing the regression of atherosclerosis. While the efficacy of short-term anti-miR-33 treatment has been previously studied, the long-term effect of miR-33 antagonism in vivo remains to be elucidated. Here, we show that long-term therapeutic silencing of miR-33 increases circulating triglyceride (TG) levels and lipid accumulation in the liver. These adverse effects were only found when mice were fed a high-fat diet (HFD). Mechanistically, we demonstrate that chronic inhibition of miR-33 increases the expression of genes involved in fatty acid synthesis such as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) in the livers of mice treated with miR-33 antisense oligonucleotides. We also report that anti-miR-33 therapy enhances the expression of nuclear transcription Y subunit gamma (NFYC), a transcriptional regulator required for DNA binding and full transcriptional activation of SREBP-responsive genes, including ACC and FAS. Taken together, these results suggest that persistent inhibition of miR-33 when mice are fed a high-fat diet (HFD) might cause deleterious effects such as moderate hepatic steatosis and hypertriglyceridemia. These unexpected findings highlight the importance of assessing the effect of chronic inhibition of miR-33 in non-human primates before we can translate this therapy to humans.

Synopsis

Although short-term anti-miR-33 therapy was reported to increase circulating HDL-cholesterol and reduce atherosclerosis, long-term adverse effects are here shown for the first time in mice fed a high-fat diet to result in hypertriglyceridemia and moderate hepatic steatosis.

- The effect of long-term inhibition of miR-33 was determined in mice fed a chow diet and high-fat diet.
- Chronic therapeutic silencing of miR-33 increased circulating triglycerides and lipid accumulation in the livers of mice fed a high-fat diet.
- miR-33 inhibition raised the expression of genes involved in fatty acid synthesis and lipid metabolism.
- Further studies are warranted to understand the complex gene regulatory network controlled by miR-33.
Fluctuations in intracellular molecule abundance can lead to distinct, coexisting phenotypes in isogenic populations. Although metabolism continuously adapts to unpredictable environmental changes, and although bistability was found in certain substrate-uptake pathways, central carbon metabolism is thought to operate deterministically. Here, we combine experiment and theory to demonstrate that a clonal Escherichia coli population splits into two stochastically generated phenotypic subpopulations after glucose-gluconeogenic substrate shifts. Most cells refrain from growth, entering a dormant persister state that manifests as a lag phase in the population growth curve. The subpopulation-generating mechanism resides at the metabolic core, overarches the metabolic and transcriptional networks, and only allows the growth of cells initially achieving sufficiently high gluconeogenic flux. Thus, central metabolism does not ensure the gluconeogenic growth of individual cells, but uses a population-level adaptation resulting in responsive diversification upon nutrient changes.
Macrophages and β-cells are responsible for CXCR2-mediated neutrophil infiltration of the pancreas during autoimmune diabetes

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Autoimmune type 1 diabetes (T1D) development results from the interaction between pancreatic β-cells, and the innate and the adaptive immune systems culminating with the destruction of the insulin-secreting β-cells by auto-reactive T cells. This diabetogenic course starts during the first postnatal weeks by the infiltration of the pancreatic islets by innate immune cells and particularly neutrophils. Here, we aim to determine the cellular and molecular mechanism leading to the recruitment of these neutrophils in the pancreatic islets of non-obese diabetic (NOD) mice. Here, we show that neutrophil recruitment in the pancreatic islets is controlled by inflammatory macrophages and β-cells themselves. Macrophages and β-cells produce the chemokines CXCL1 and CXCL2, recruiting CXCR2-expressing neutrophils from the blood to the pancreatic islets. We further show that pancreatic macrophages secrete IL-1β-inducing CXCR2 ligand production by the β-cells. Finally, the blockade of neutrophil recruitment at early ages using CXCR2 antagonist dampens the diabetogenic T-cell response and the later development of autoimmune diabetes, supporting the therapeutic potential of this approach.

Synopsis

This study reveals the interaction between inflammatory macrophages and β-cells leading to the recruitment of diabetogenic neutrophils in the pancreas of neonatal mice via CXCR2/CXCL2 ligands. Inhibition of CXCR2 reduces the diabetogenic T-cell response, insulinitis, and incidence of diabetes.

- In young NOD mice, CXCR2+ neutrophils are recruited from the blood into the pancreatic islets and not in the two non-diabetes prone C57BL/6 and BALB/c mice.
- The two CXCR2 ligands, CXCL1 and CXCL2, are secreted in the pancreatic islets from the young and not from the adult NOD mice or the two non-diabetes prone mice.
- The pancreatic β-cells are the main source of CXCL1 and CXCL2 in the pancreatic islets of young NOD mice.
- The production of CXCL1 and CXCL2 by the β-cells is induced by IL-1β-producing macrophages infiltrating the pancreatic islets of young NOD mice.
- The early blockade of neutrophil recruitment using CXCR2 antagonist reduces the insulitis, the effector activity of diabetogenic CD8+ T cells, and the development of autoimmune diabetes in NOD mice.
Deoxypyrimidine monophosphate bypass therapy for thymidine kinase 2 deficiency

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Autosomal recessive mutations in the thymidine kinase 2 gene (TK2) cause mitochondrial DNA depletion, multiple deletions, or both due to loss of TK2 enzyme activity and ensuing unbalanced deoxynucleotide triphosphate (dNTP) pools. To bypass Tk2 deficiency, we administered deoxycytidine and deoxythymidine monophosphates (dCMP + dTMP) to the Tk2 H126N (Tk2−/−) knock-in mouse model from postnatal day 4, when mutant mice are phenotypically normal, but biochemically affected. Assessment of 13-day-old Tk2−/− mice treated with dCMP+dTMP 200 mg/kg/day each (Tk2−/−200dCMP/dTMP) demonstrated that in mutant animals, the compounds raise dTTP concentrations, increase levels of mtDNA, ameliorate defects of mitochondrial respiratory chain enzymes, and significantly prolong their lifespan (34 days with treatment versus 13 days untreated). A second trial of dCMP + dTMP each at 400 mg/kg/day showed even greater phenotypic and biochemical improvements. In conclusion, dCMP/dTMP supplementation is the first effective pharmacologic treatment for Tk2 deficiency.
Screen for mitochondrial DNA copy number maintenance genes reveals essential role for ATP synthase

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The machinery of mitochondrial DNA (mtDNA) maintenance is only partially characterized and is of wide interest due to its involvement in disease. To identify novel components of this machinery, plus other cellular pathways required for mtDNA viability, we implemented a genome-wide RNAi screen in Drosophila S2 cells, assaying for loss of fluorescence of mtDNA nucleoids stained with the DNA-intercalating agent PicoGreen. In addition to previously characterized components of the mtDNA replication and transcription machineries, positives included many proteins of the cytosolic proteasome and ribosome (but not the mitoribosome), three proteins involved in vesicle transport, some other factors involved in mitochondrial biogenesis or nuclear gene expression, > 30 mainly uncharacterized proteins and most subunits of ATP synthase (but no other OXPHOS complex). ATP synthase knockdown precipitated a burst of mitochondrial ROS production, followed by copy number depletion involving increased mitochondrial turnover, not dependent on the canonical autophagy machinery. Our findings will inform future studies of the apparatus and regulation of mtDNA maintenance, and the role of mitochondrial bioenergetics and signaling in modulating mtDNA copy number.

Synopsis

An RNAi screen for genes needed in mtDNA copy number maintenance in Drosophila yielded 97 positives, including previously characterized mtDNA maintenance proteins, subunits of the cytoribosome, proteasome, and ATP synthase.

- An RNAi screen for genes needed in mtDNA copy number maintenance in Drosophila yielded 97 positives.
- These included previously characterized components of the mtDNA maintenance machinery.
- Other major classes of positives were the cytoribosome, proteasome, and ATP synthase.
- ATP synthase deficiency results in increased ROS and activation of mitochondrial turnover by pathway(s) distinct from classical autophagy.
Constitutive hippocampal cholesterol loss underlies poor cognition in old rodents

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Cognitive decline is one of the many characteristics of aging. Reduced long-term potentiation (LTP) and long-term depression (LTD) are thought to be responsible for this decline, although the precise mechanisms underlying LTP and LTD dampening in the old remain unclear. We previously showed that aging is accompanied by the loss of cholesterol from the hippocampus, which leads to PI3K/Akt phosphorylation. Given that Akt de-phosphorylation and LTD dampening in the old remain unclear. We previously showed that aging is accompanied by the loss of cholesterol in neuronal membranes may contribute to the deficits in LTD typical of aging. Here, we show that cholesterol loss triggers p-Akt accumulation, which in turn perturbs the normal cellular and molecular responses induced by LTD, such as impaired AMPA receptor internalization and its reduced lateral diffusion. Electrophysiology recordings in brain slices of old mice and in anesthetized elderly rats demonstrate that the reduced hippocampal LTD associated with age can be rescued by cholesterol perfusion. Accordingly, cholesterol replenishment in aging animals improves hippocampal-dependent learning and memory in the water maze test.

Synopsis

It is well established that cognitive deficits go hand in hand with aging. Restoring cholesterol levels in the aged hippocampus to values found in the young can rescue learning and memory in the old, linking age-dependent cholesterol decline with synaptic plasticity and neuronal function.

- A mild yet significant reduction in membrane cholesterol characterizes the aging rodent hippocampus.
- Low synaptic hippocampal cholesterol determines reduced Akt dephosphorylation after NMDA-induced LTD, together with reduced glutamate (AMPA) receptor lateral diffusion and endocytosis.
- Low synaptic hippocampal cholesterol plays a role in the poor LTD of old mice and rats, in ex-vivo and in vivo paradigms.
- Normal levels of pAkt after NMDA, proper receptor lateral diffusion, and internalization and normal (young animals-like) LTD in the old can be rescued by membrane cholesterol replenishment.
- Cholesterol replenishment in living old rats improves learning and memory.
Mouse liver metabolites were quantified by mass spectrometry and mapped by genome-wide association. Genetic factors were shown to contribute substantially to metabolite levels and adenoviral overexpression validated several of the identified loci.

- Liver metabolites exhibit a wide range of variation, indicating strong genetic influences.
- Approximately 40% of metabolites are estimated to be regulated by genetic factors.
- A significant overlap was observed between genetic factors regulating mouse liver metabolites and genetic factors regulating human serum metabolites.
- Metabolite levels correlated significantly both with each other and with other phenotypes such as transcript levels and physiological traits.
Thioredoxin-interacting protein regulates protein disulfide isomerases and endoplasmic reticulum stress

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The endoplasmic reticulum (ER) is responsible for protein folding, modification, and trafficking. Accumulation of unfolded or misfolded proteins represents the condition of ER stress and triggers the unfolded protein response (UPR), a key mechanism linking supply of excess nutrients to insulin resistance and type 2 diabetes in obesity. The ER harbors proteins that participate in protein folding including protein disulfide isomerases (PDIs). Changes in PDI activity are associated with protein misfolding and ER stress. Here, we show that thioredoxin-interacting protein (Txnip), a member of the arrestin protein superfamily and one of the most strongly induced proteins in diabetic patients, regulates PDI activity and UPR signaling. We found that Txnip binds to PDIs and increases their enzymatic activity. Genetic deletion of Txnip in cells and mice led to increased protein ubiquitination and splicing of the UPR regulated transcription factor X-box-binding protein 1 (Xbp1s) at baseline as well as under ER stress. Our results reveal Txnip as a novel direct regulator of PDI activity and a feedback mechanism of UPR signaling to decrease ER stress.

Synopsis

More and more evidence implicates ER stress in diabetes. Thioredoxin-interacting protein (Txnip) is here shown to interact with and regulate protein disulfide isomerases (PDIs) activity and ER stress. This study highlights new therapeutic targets for treating diabetes.

- An unbiased proteomics approach as well as specific pulldown assays revealed an interaction of thioredoxin-interacting protein (Txnip) with protein disulfide isomerases (PDIs).
- Txnip increases PDI activity, and Txnip knockout leads to increased protein ubiquitination and increased levels of Xbp1s, a marker of ER stress.
- Increased levels of Xbp1s in Txnip-KO mice is reversed by treatment with chemical chaperones.
Metabolic shifts toward glutamine regulate tumor growth, invasion and bioenergetics in ovarian cancer

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Glutamine can play a critical role in cellular growth in multiple cancers. Glutamine-addicted cancer cells are dependent on glutamine for viability, and their metabolism is reprogrammed for glutamine utilization through the tricarboxylic acid (TCA) cycle. Here, we have uncovered a missing link between cancer invasiveness and glutamine dependence. Using iso- tope tracer and bioenergetic analysis, we found that low-invasive ovarian cancer (OVCA) cells are glutamine independent, whereas high-invasive OVCA cells are markedly glutamine dependent. Consistent with our findings, OVCA patients’ microarray data suggest that glutaminolysis correlates with poor survival. Notably, the ratio of gene expression associated with glutamine anabolism versus catabolism has emerged as a novel biomarker for patient prognosis. Significantly, we found that glutamine regulates the activation of STAT3, a mediator of signaling pathways which regulates cancer hallmarks in invasive OVCA cells. Our findings suggest that a combined approach of targeting high-invasive OVCA cells by blocking glutamine’s entry into the TCA cycle, along with targeting low-invasive OVCA cells by inhibiting glutamine synthesis and STAT3 may lead to potential therapeutic approaches for treating OVCAs.

Synopsis

Glutamine plays an important role in cellular growth in several cancers. In this study, a further link between glutamine dependency and tumor invasiveness is established in ovarian cancer. Glutamine maintains the high-invasive phenotype by regulating STAT3 signaling.

- High-invasive ovarian cancer (OVCA) cells are glutamine dependent in contrast to low-invasive cells that are glutamine independent.
- Glutamine regulates STAT3 activation in high-invasive cancer cells.
- Glutamine’s entry into TCA cycle modulates the invasive potential of high-invasive cancer cells.
- The ratio of glutamine catabolism over glutamine anabolism is associated with worse overall survival in OVCA patients.
Embelin inhibits endothelial mitochondrial respiration and impairs neoangiogenesis during tumor growth and wound healing

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In the normal quiescent vasculature, only 0.01% of endothelial cells (ECs) are proliferating. However, this proportion increases dramatically following the angiogenic switch during tumor growth or wound healing. Recent evidence suggests that this angiogenic switch is accompanied by a metabolic switch. Here, we show that proliferating ECs increasingly depend on mitochondrial oxidative phosphorylation (OxPhos) for their increased energy demand. Under growth conditions, ECs consume three times more oxygen than quiescent ECs and work close to their respiratory limit. The increased utilization of the proton motive force leads to a reduced mitochondrial membrane potential in proliferating ECs and sensitizes to mitochondrial uncoupling. The benzoquinone embelin is a weak mitochondrial uncoupler that prevents neoangiogenesis during tumor growth and wound healing by exhausting the low respiratory reserve of proliferating ECs without adversely affecting quiescent ECs. We demonstrate that this can be exploited therapeutically by attenuating tumor growth in syngenic and xenograft mouse models. This novel metabolic targeting approach might be clinically valuable in controlling pathological neoangiogenesis while sparing normal vasculature and complementing cytostatic drugs in cancer treatment.

Synopsis

Weak mitochondrial uncouplers prevent neoangiogenesis in vitro and in vivo by depleting cellular energy reserves in proliferating but not normal quiescent endothelial cells (ECs).

- New vessel formation during tumor growth requires EC proliferation and increased oxidative phosphorylation to meet the greater energy demand during angiogenesis.
- Weak mitochondrial uncouplers prevent neoangiogenesis by depleting cellular energy reserves in proliferating but not normal quiescent ECs.
- Proliferating ECs are sensitized to mitochondrial uncouplers by a reduction in membrane potential and lower respiratory reserve capacity.
- Genetic accumulation of mitochondrial DNA mutations in mitochondrial mutator mice highlights the link between reduced OxPhos activity and impaired angiogenic response.
- Weak mitochondrial uncouplers could be clinically valuable in controlling pathological neoangiogenesis while sparing normal vasculature and complementing cytostatic drugs in cancer treatment.
Mitochondrial response to nutrient availability and its role in metabolic disease

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Metabolic inflexibility is defined as an impaired capacity to switch between different energy substrates and is a hallmark of insulin resistance and type 2 diabetes mellitus (T2DM). Hence, understanding the mechanisms underlying proper metabolic flexibility is key to prevent the development of metabolic disease and physiological deterioration. An important downstream player in the effects of metabolic flexibility is the mitochondrion. The objective of this review was to describe how mitochondrial metabolism adapts to limited nutrient situations or caloric excess by changes in mitochondrial function or biogenesis, as well as to define the mechanisms propelling these changes. Altogether, this should pinpoint key regulatory points by which metabolic flexibility might be ameliorated in situations of metabolic disease.

Synopsis

In this review, the authors introduce the concept of understanding and further maintaining metabolic flexibility as a way to limit spreading of metabolic disorders. By modifying function or biogenesis, the mitochondria perfectly illustrates this point as they adapt to different stress situations.
Non-enzymatic glycolysis and pentose phosphate pathway-like reactions in a plausible Archean ocean

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The reaction sequences of central metabolism, glycolysis and the pentose phosphate pathway provide essential precursors for nucleic acids, amino acids and lipids. However, their evolutionary origins are not yet understood. Here, we provide evidence that their structure could have been fundamentally shaped by the general chemical environments in earth’s earliest oceans. We reconstructed potential scenarios for oceans of the prebiotic Archean based on the composition of early sediments. We report that the resultant reaction milieu catalyses the interconversion of metabolites that in modern organisms constitute glycolysis and the pentose phosphate pathway. The 29 observed reactions include the formation and/or interconversion of glucose, pyruvate, the nucleic acid precursor ribose-5-phosphate and the amino acid precursor erythrose-4-phosphate, antedating reactions sequences similar to that used by the metabolic pathways. Moreover, the Archean ocean mimetic increased the stability of the phosphorylated intermediates and accelerated the rate of intermediate reactions and pyruvate production. The catalytic capacity of the reconstructed ocean milieu was attributable to its metal content. The reactions were particularly sensitive to ferrous iron Fe(II), which is understood to have had high concentrations in the Archean oceans. These observations reveal that reaction sequences that constitute central carbon metabolism could have been constrained by the iron-rich oceanic environment of the early Archean. The origin of metabolism could thus date back to the prebiotic world.

Synopsis

Modern cells possess a sophisticated metabolic network, but its origins remain largely unknown. Reconstructing scenarios of the Archean ocean, we observe chemical reactions reminiscent of modern metabolic sequences, indicating that metabolism could be of prebiotic origin.

- Metabolites of glycolysis and the pentose phosphate undergo non-enzymatic interconversion reactions.
- Metal ions abundantly found in sediments of the prebiotic Archean ocean, predominantly Fe(II), catalyse additional sugar phosphate interconversion reactions.
- Reactions catalysed by the Archean ocean metals resemble enzyme-catalysed reactions found in the modern glycolytic and pentose phosphate pathways.
- The observed reactions are accelerated and gain specificity in conditions simulating the Archean ocean.
Metabolic crosstalk between the heart and liver impacts familial hypertrophic cardiomyopathy

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Familial hypertrophic cardiomyopathy (HCM) is largely caused by dominant mutations in genes encoding cardiac sarcomeric proteins, and it is etiologically distinct from secondary cardiomyopathies resulting from pressure/volume overload and neurohormonal or inflammatory stimuli. Here, we demonstrate that decreased left ventricular contractile function in male, but not female, HCM mice is associated with reduced fatty acid translocase (CD36) and AMP-activated protein kinase (AMPK) activity. As a result, the levels of myocardial ATP and triglyceride (TG) content are reduced, while the levels of oleic acid and TG in circulating very low density lipoproteins (VLDLs) and liver are increased. With time, these metabolic changes culminate in enhanced glucose production in male HCM mice. Remarkably, restoration of ventricular TG and ATP deficits via AMPK agonism as well as inhibition of gluconeogenesis improves ventricular architecture and function. These data underscore the importance of the systemic effects of a primary genetic heart disease to other organs and provide insight into potentially novel therapeutic interventions for HCM.

Synopsis

A primary cardiac myocyte defect leads to aberrant lipid accumulation and signaling in the liver. The resulting hepatic phenotype impacts cardiac function. Normalization of heart lipid delivery or inhibition of gluconeogenesis improves ventricular function.

- Genetic heart disease causes metabolic abnormalities in the liver.
- There is reduced triglyceride clearance by the HCM heart.
- Accumulating plasma triglycerides are sequestered by hepatocytes.
- Activation of gluconeogenesis exacerbates cardiac pathology.
Effective treatment of mitochondrial myopathy by nicotinamide riboside, a vitamin B3

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Nutrient availability is the major regulator of life and reproduction, and a complex cellular signaling network has evolved to adapt organisms to fasting. These sensor pathways monitor cellular energy metabolism, especially mitochondrial ATP production and NAD+/NADH ratio, as major signals for nutritional state. We hypothesized that these signals would be modified by mitochondrial respiratory chain disease, because of inefficient NADH utilization and ATP production. Oral administration of nicotinamide riboside (NR), a vitamin B3 and NAD+ precursor, was previously shown to boost NAD+ levels in mice and to induce mitochondrial biogenesis. Here, we treated mitochondrial myopathy mice with NR. This vitamin effectively delayed early- and late-stage disease progression, by robustly inducing mitochondrial biogenesis in skeletal muscle and brown adipose tissue, preventing mitochondrial ultrastructure abnormalities and mtDNA deletion formation. NR further stimulated mitochondrial unfolded protein response, suggesting its protective role in mitochondrial disease. These results indicate that NR and strategies boosting NAD+ levels are a promising treatment strategy for mitochondrial myopathy.

Synopsis

Nicotinamide riboside (vitamin B3) delays the progression of mitochondrial myopathy by preventing pathology-associated mitochondrial ultrastructure, improving mitochondrial DNA stability and further stimulating mitochondrial unfolded protein response.

- Nicotinamide riboside, vitamin B3, delays the progression of mitochondrial myopathy.
- Nicotinamide riboside cures pathology-associated mitochondrial ultrastructure.
- Nicotinamide riboside improves mitochondrial DNA stability.
- Mitochondrial disease induces mitochondrial unfolded protein response, further enhanced by nicotinamide riboside.
- Nicotinamide riboside is a promising treatment for adult-onset mitochondrial myopathy.
Identification of anticancer drugs for hepatocellular carcinoma through personalized genome-scale metabolic modeling

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Genome-scale metabolic models (GEMs) have proven useful as scaffolds for the integration of omics data for understanding the genotype–phenotype relationship in a mechanistic manner. Here, we evaluated the presence/absence of proteins encoded by 15,841 genes in 27 hepatocellular carcinoma (HCC) patients using immunohistochemistry. We used this information to reconstruct personalized GEMs for six HCC patients based on the proteomics data, HMR 2.0, and a task-driven model reconstruction algorithm (tINIT). The personalized GEMs were employed to identify anticancer drugs using the concept of antimetabolites; i.e., drugs that are structural analogs to metabolites. The toxicity of each antimetabolite was predicted by assessing the in silico functionality of 83 healthy cell type-specific GEMs, which were also reconstructed with the tINIT algorithm. We predicted 101 antimetabolites that could be effective in preventing tumor growth in all HCC patients, and 46 antimetabolites which were specific to individual patients. Twenty-two of the 101 predicted antimetabolites have already been used in different cancer treatment strategies, while the remaining antimetabolites represent new potential drugs. Finally, one of the identified targets was validated experimentally, and it was confirmed to attenuate growth of the HepG2 cell line.
Glutamine-driven oxidative phosphorylation is a major ATP source in transformed mammalian cells in both normoxia and hypoxia

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Mammalian cells can generate ATP via glycolysis or mitochondrial respiration. Oncogene activation and hypoxia promote glycolysis and lactate secretion. The significance of these metabolic changes to ATP production remains however ill defined. Here, we integrate LC-MS-based isotope tracer studies with oxygen uptake measurements in a quantitative redox-balanced metabolic flux model of mammalian cellular metabolism. We then apply this approach to assess the impact of Ras and Akt activation and hypoxia on energy metabolism. Both oncogene activation and hypoxia induce roughly a twofold increase in glycolytic flux. Ras activation and hypoxia also strongly decrease glucose oxidation. Oxidative phosphorylation, powered substantially by glutamine-driven TCA turning, however, persists and accounts for the majority of ATP production. Consistent with this, in all cases, pharmacological inhibition of oxidative phosphorylation markedly reduces energy charge, and glutamine but not glucose removal markedly lowers oxygen uptake. Thus, glutamine-driven oxidative phosphorylation is a major means of ATP production even in hypoxic cancer cells.

Synopsis

The impact of oncogene activation and hypoxia on energy metabolism is analyzed by integrating quantitative measurements into a redox-balanced metabolic flux model. Glutamine-driven oxidative phosphorylation is found to be a major ATP source even in oncogene-expressing or hypoxic cells.

- The integration of oxygen uptake measurements and LC-MS-based isotope tracer analyses in a redox-balanced metabolic flux model enabled quantitative determination of energy generation pathways in cultured cells.
- In transformed mammalian cells, even in hypoxia (1% oxygen), oxidative phosphorylation produces the majority of ATP.
- The oncogene Ras simultaneously increases glycolysis and decreases oxidative phosphorylation, thus resulting in no net increase in ATP production.
- Glutamine is the major source of high-energy electrons for oxidative phosphorylation, especially upon Ras activation.