

# The not-so-sweet side of sugar: Influence of the microenvironment on the processes that unleash cancer

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## ABSTRACT

The role of “aerobic glycolysis” in cancer has been examined often in the past. Results from those studies, most of which were performed on two dimensional conditions (2D, tissue culture plastic), demonstrate that aerobic glycolysis occurs as a consequence of oncogenic events. These oncogenic events often drive malignant cell growth and survival. Although 2D based experiments are useful in elucidating the molecular mechanisms of oncogenesis, they fail to take contributions of the extracellular microenvironment into account. Indeed we, and others, have shown that the cellular microenvironment is essential in regulating processes that induce and/or suppress the malignant phenotype/properties. This regulation between the cell and its microenvironment is both dynamic and reciprocal and involves the integration of cellular signaling networks in the right context. Therefore, given our previous demonstration of the effect of the microenvironment including tissue architecture and media composition on gene expression and the integration of signaling events observed in three-dimension (3D), we hypothesized that glucose uptake and metabolism must also be essential components of the tissue's signal “integration plan” – that is, if uptake and metabolism of glucose were hyperactivated, the canonical oncogenic pathways should also be similarly activated. This hypothesis, if proven true, suggests that direct inhibition of glucose metabolism in cancer cells should either suppress or revert the malignant phenotype in 3D. Here, we review the up-to-date progress that has been made towards understanding the role that glucose metabolism plays in oncogenesis and re-establishing basally polarized acini in malignant human breast cells.

## 1. Introduction

The term “aerobic glycolysis” was first presented by Otto Heinrich Warburg in 1923 [1–3]. However, the strong dependence of tumors on aerobic glycolysis and its designation as the “Warburg effect” were introduced much later [1–4]. In those early days, Warburg postulated that the main driver of oncogenesis is a dysfunctional mitochondria [2]. Irreversible mitochondrial dysfunction, as described by Warburg, is also the underlying cause of the observed metabolic switch to aerobic glycolysis and therefore the “origin of cancer” [2]. This suggested that malignant growth results from tumorigenic cells using aerobic glycolysis as their main source of energy, instead of oxidative metabolism as seen in most nonmalignant cells. Warburg regarded the fundamental differences between normal and cancerous cells to be the ratio of glycolysis to respiration [2,3]. Since the time of Warburg's observations, there has been a better understanding of the human genome and subsequent identification of “oncogenes” and “tumor suppressors”. This has generated a widely accepted central dogma that cancer is caused by mutations and altered gene expression resulting in unrestrained cellular

growth [5–10]. If this dogma holds true, then the significant metabolic changes described by Warburg would only occur in cells that have already adapted to hypoxic (reduced oxygen levels) conditions within solid tumors [11]. These alterations in glucose metabolism were presumed to result largely from the same mutations in tumor suppressor and oncogenes that also caused other observed abnormalities in cancer cells [7–10,12–18]. Therefore, it was concluded that the metabolic changes observed by Warburg are just one of the characteristic effects of oncogenes and not so much the “root cause of cancer”. If genetic mutations and dysregulation of tumor suppressors and/or oncogenes are sufficient to induce the malignant phenotype (properties characteristic of malignant neoplasms including rapid growth, decreased differentiation, invasion and metastasis) then why don't we get more cancers? Could it be that this supposition stems from the fact that many cancer and metabolism studies were performed in 2D culture rather than under more physiologically relevant culture conditions? Here we address these two questions in conjunction with the mechanisms of the Warburg effect in cancer progression.

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1) Why don't we get more cancer? Considering the trillions of cells in the human body, the number of possible mutations that occur, and the ensuing genomic instability, makes this question almost impossible to answer without taking the cellular microenvironment and phenotype into account. Since the late 1900s, we hypothesized that the microenvironment is dominant over the genotype of cells within a tissue and that correct signaling from the microenvironment assists in controlling processes that unleash cancer [19,20]. If the microenvironment were not dominant, then each of the ~ten trillion cells in the human body, which accumulate many mutations over time, would be without control or modulation of behavior. This would consequently result in either the formation of a uniform lump of cells with similar fate or absolute chaos. However, this is not the case. We, and others, have shown that proper signaling from the microenvironment is essential for suppressing cancer progression and can revert the malignant phenotype even when cells harbor a number of mutations [21–29]. Moreover, when proper signaling from the microenvironment is disrupted, cancer progression can occur [30–37]. Our earlier studies in this field were greatly influenced by the works of Karl Illmensee and Leroy Stevens. These researchers showed that stem cells derived from malignant teratomas (tumors comprised of several different types of differentiated cells and tissues) can differentiate normally when injected into a mouse embryo and give rise to normal chimeric mice or mice that are genetically mosaic. Alternatively, when those same stem cells were subcutaneously implanted under the skin of the mice, large teratomas were formed. Apparently, the embryonic environment made the difference in restricting tumor formation. These studies provided the first major indication that malignant transformation does not always result from genetic mutations, but from abnormalities in phenotype during the course of differentiation and hence leading to neoplastic growth [38–42]. These findings further allowed the authors to hypothesize that under appropriate environmental conditions these malignant teratoma cells can lose their tumorigenic properties [39]. Additional studies performed by Friedrich et al., showed that the maturation and experimental induction of teratocarcinogenesis in mouse genital ridges is significantly affected by temperature [43]. They observed high incidence of teratomas in genital ridges grafted to scrotal testes (32 °C), but not in those grafted to testes maintained at body temperature (37 °C) or grafted to other organs in the mice [43]. Friedrich et al., also showed that resistance or susceptibility of the genital ridge to teratocarcinogenesis was associated with morphologic changes in the ridge during cell culture [43–47]. This further demonstrates that teratoma formation is context dependent and microenvironmental factors such as changes in temperature can greatly influence tumorigenesis.

Subsequent studies performed in our laboratory revealed the inability of Rous sarcoma virus (RSV) to form sarcomas in chicken embryos [30]. While injection of RSV into the wing web of newly hatched chicks induced formation of rapidly growing sarcomatous tumors, this did not hold true when early chicken embryos were infected with RSV. In fact, the virus not only replicated in these RSV infected embryos it also expressed an active oncogene [30,48] but no tumors formed. Once tissues obtained from the limbs of chicks, infected with RSV as embryos, were disrupted and placed in culture the malignant phenotype of the RSV infected cells was restored [30]. Furthermore, cultures of chick embryo derived fibroblasts infected with RSV became rapidly transformed in culture [30,49]. We also showed that RSV induced tumors in chickens remained localized at the injection site even in viremic animals [31]. Since then, tumors have been shown to be inducible at other sites, but only if wound is inflicted or if the administration of tumors caused tissue injury [30,33,49–53]. These latter findings indicate that local wounding plays a role in the tumorigenicity of RSV and perhaps other oncoviruses. Further studies performed by Sieweke et al., showed

that wound-related RSV tumorigenesis is mediated by recombinant transforming growth factor-beta (TGF-beta). In those studies, immunohistochemical staining showed that TGF-beta is locally present shortly after wounding and that it could completely substitute for wounding in tumor induction. Subsequent studies have confirmed that TGF-beta release during wound healing creates a conducive environment for RSV tumorigenesis by acting as a major cofactor for transformation in this system. Lastly, the recent works of Thomas Seyfried also argue that cancer is not driven primarily by genetics and mutations, but is a metabolic/phenotypic disease [54–61]. To prove this, Seyfried hypothesized that “if nuclear somatic mutations are the origins of cancer, then the hallmark phenotype, *dysregulated cell proliferation*, should occur following the transfer of a tumor nucleus into a normal cytoplasm” [55,57]. Nevertheless, the delivery of a tumor cell nucleus into a normal cytoplasm produced normal cells, despite the presence of tumor-associated genomic abnormalities. The reverse was also shown to hold true, wherein the delivery of a normal cell nucleus into a tumor cell cytoplasm produced either dead or tumor cells [55,57]. These results provide additional evidence to show that tumors do not always arise from genomic defects and that correct cellular signaling and context matter. Because tissue context is a major driving force behind maintaining a nonmalignant state, this raises the following question:

2) Why are most gene expression and metabolism studies performed under 2D cell culture conditions? Cell culture can be an ideal system for studying gene regulation, because the environment is more easily controlled. In practice, however, the standard 2D cell culture techniques do not accurately represent the *in vivo* cellular microenvironment. Furthermore, quality and quantity of cellular function are dramatically altered when cells are placed in 2D culture. The very thing to be studied is altered or lost by the act of placing cells on the stiff surface of a culture dish [19]. For instance, when primary mouse epithelial cells are cultured on 2D plastic, their ability to synthesize and produce milk proteins such as beta-casein is lost [62–69]. This occurs even in the presence of lactogenic hormones. Whereas, cells cultured on reconstituted basement membrane or “floating” type I collagen gels significantly increased the expression and secretion rates of milk producing proteins [32,36,62–71]. In addition, the 2D cell culture system itself presents a context that resembles a wounding environment that is stiff like a fibrosis and upregulates tumor promoting factors such as TGF-beta. High TGF-beta expression has been shown in cells on plastic, but significantly decreased when cells are cultured on a reconstituted basement matrix. Therefore, culture in 2D promotes tumor-like characteristics in normal cells [31,33,34]. One reason for this is because for cells to function correctly they have to appropriately sense their external environment. If cells are unable to sense signals from their environment or the extracellular signals being received are incorrect, this leads to tumors *in vivo*. In the case of cultured cells growing on a stiff surface, without the correct signaling from ECM proteins, this results in those cells overgrowing the monolayer [72]. Many researchers, however, do not focus on determining factors that control proliferation and differentiation, but rather prioritize cell growth and reproducibility. In normal 2D culture conditions, cells are plated at low density and then allowed to reach confluence (or at least close to confluency) at which point they are passaged (trypsinized, diluted and re-plated). If the cells do not grow well or fast enough, often more serum or growth factors are added until the desired outcomes are obtained. If reproducible results are obtained from one passage to the next, then they are acceptable to the researcher. Nonetheless, researchers have been frustrated by the low level of cellular differentiation in cell lines cultured under 2D conditions for a long period of time and over several passages. This issue discouraged many from pursuing studies that elucidate mechanisms that regulate the differentiated phenotype and function.

Indeed, despite the fact that regulation of metabolism is cell type and organ specific *in vivo*, all cells become metabolically similar (increasing glycolytic fluxes) in culture [11,73–76]. In many respects, “normal” cell lines that are cultured under 2D conditions have metabolic signatures and gene expression profiles that are closer to transformed cells than they are to the normal tissues from which they were derived [19]. That said, modulation of function in culture may be the best tool available to study gene expression in higher organisms [77]. This is because establishing a cause and effect relationship between a mutation in the DNA, the function of the gene in question and contributions of the microenvironment, *in vivo*, is difficult. Moreover, events that accompany loss of function in culture could be advantageous in studying the regulation of normal physiology [78,79]. It also allows for a more accurate interpretation of results from *in vivo* studies. In the study of gene regulation and metabolism the most useful, perhaps, is when function can be successfully modulated. It is also evident that not all functional changes in culture are due to somatic mutations, some occur from phenotypic alterations [80,81]. Therefore, it is important to create defined culture conditions where cellular function is restored. Over the years we developed 3D (organoid) cell culture assays that allow for successful integration of extracellular signaling with canonical intercellular networks. (Fig. 1) [37]. Culture in our 3D system initiates and maintains cellular quiescence/differentiation in both nonmalignant human breast epithelial cells and mouse mammary cells. It also restores milk production in mouse mammary cells [36,62,67,68,71,82–97]. Nonmalignant (which form polarized and quiescent acinus-like structures) and malignant (which form disorganized and overgrown tumor-like structures) human breast cells can also be distinguished using our 3D culture (Fig. 2A and B). The abovementioned characteristics of our 3D cell culture assays (architecture/morphology, cellular differentiation, and milk protein synthesis and secretion to evaluate tissue specific differentiation) are all included in the criteria by which ideal culture conditions for primary mammary epithelial cells are judged. With increasingly stronger evidence to demonstrate the effect of the microenvironment, including tissue architecture and media composition, on gene expression and signal integration events (also observed in our 3D culture models), we reasoned that glucose uptake and metabolism should also be essential components of the tissue’s “signal integration plan” – that is, if uptake and glucose metabolism were hyperactivated, the canonical oncogenic pathways should also be activated in a similar manner (Fig. 2C and D) [98].

In the following, we provide an overview of the progress that has been made towards understanding the role that glucose metabolism plays in oncogenesis. In addition, we discuss how the glycolytic pathway has been manipulated to revert the malignant phenotype and re-establish basally polarized breast acini, under 3D culture conditions. We conclude by highlighting the possible implications of targeting cellular glucose regulation for the treatment of glandular cancers.

## 2. Glucose metabolism activates oncogenesis: true or false?

Glycolysis is the metabolic pathway that converts glucose to into either pyruvate (when oxygen is present), or lactate (in the absence of oxygen) for energy production. In normal cells, free energy released

from aerobic cellular respiration forms high energy ATP molecules that are produced in the mitochondria. However, when mitochondrial function is disrupted, as Warburg postulated, the metabolic switch to aerobic glycolysis (diverting glucose carbons to lactic acid even in the presence of oxygen) occurs leading to tumor formation [2,3]. Until recently, a direct correlation between glucose metabolism and oncogenesis had not been proven. This stems from the fact that almost all studies on aerobic glycolysis in cancer cells has been performed on tissue culture plastic (2D conditions). Together, these 2D cell culture studies show that oncogenic pathways precede glucose metabolism increases. For instance, it is well characterized that hypoxic regions within solid tumors initiate the metabolic switch from oxidative metabolism towards aerobic glycolysis. This process can be initiated by either environmental or genetic factors [11,73–75,99].

On a more molecular level, Semenza et al., showed that the transcription factor hypoxia-inducible factor-1 $\alpha$  (HIF1 $\alpha$ ) is the key regulator of the glycolytic response [100–102]. This transcription factor mediates a response to hypoxic stress by inducing the expression of survival genes including glucose transporters, hematopoietic and angiogenic growth factors. In some systems, increased HIF1 $\alpha$  levels were associated with high rates of glucose consumption, particularly in the case of the renal cell carcinoma RCC4 cell line [103–106]. HIF1 $\alpha$  is constitutively active in this cell line, because of a mutation in the enzyme that targets it for degradation (von Hippel-Lindau - VHL - ubiquitin ligase) [10,103,107,108]. Reinsertion of the VHL transgene restored HIF1 $\alpha$  levels to normalcy and significantly reduced aerobic glucose consumption rates. Further studies performed by Moeller et al., showed that hypoxia-deoxygenation injury can also lead to stabilization of HIF1 $\alpha$ , among many other non-VHL related factors [109,110]. This indicates that constitutive upregulation of HIF1 $\alpha$  may result from acute hypoxia that occurs in pre-malignant tumors. The absence of HIF1 $\alpha$  also significantly reduces cellular survival under hypoxic conditions [101,102,110–113].

Alternative glucose metabolism activating pathways have also been identified in cells with normal HIF levels. For instance, cellular redox changes by the Sp1 transcription-factor complex were shown to induce the expression of glycolytic enzymes [114,115]. Glucose transporter 1 (GLUT1) expression can be upregulated directly by MYC or indirectly by KRAS. Mutant p53 can also transcriptionally activate Hexokinase II [116,117]. Interestingly, when fibroblasts were transfected with H<sup>+</sup>-ATPase or Na<sup>+</sup>-H<sup>+</sup> exchange proteins their intracellular pH values were raised, thus leading to increases in tumorigenicity and glycolysis [118]. In addition, increased glucose transport was shown to push chick embryo fibroblasts towards a pattern of glucose metabolism that resembles RSV transformed cells [119,120]. These alternative systems for upregulating glycolysis suggest that the glycolytic phenotype is directly selected for, because it provides a growth advantage rather than just a consequence of carcinogenesis or oncogene activation.

Additional mathematical models and empirical observation indicate that the advantages conferred by constitutive upregulation of glycolysis and resistance to microenvironmental acidosis induced by increased glycolysis are both sufficient and necessary to promote unconstrained tumor growth [121,122]. Indeed, we directly showed that increased glucose uptake/metabolism activates known oncogenic pathways to induce the malignant phenotype [98]. In these studies, we used our 3D

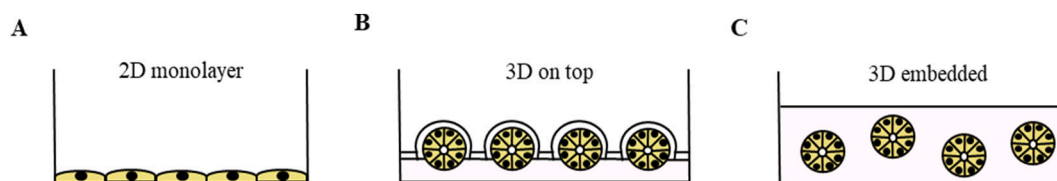
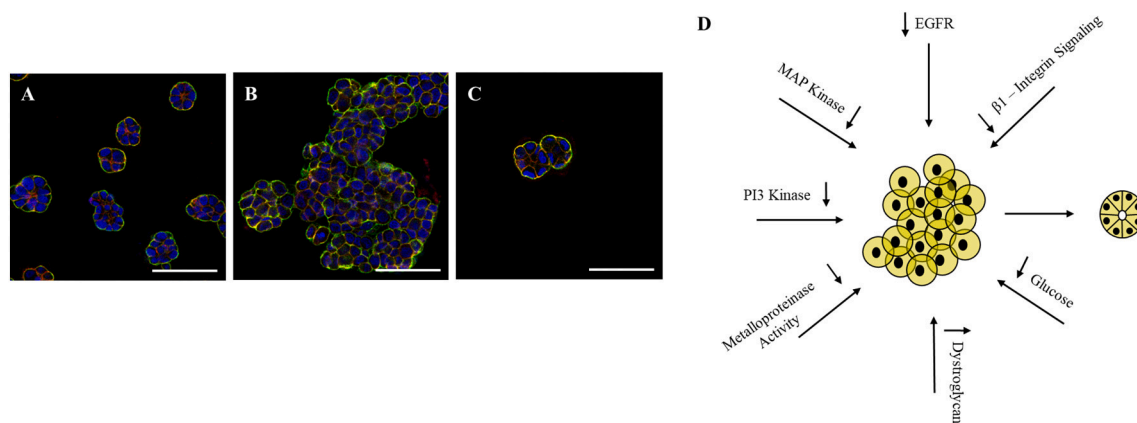


Fig. 1. Cell culture systems used to study breast cancer. (A) Conventional 2D monolayer cell cultures. 3D cultures, where cells are either (B) growing on top of laminin-rich extracellular matrix (lrECM) overlaid with a dilute solution layer of lrECM or (C) embedded in lrECM.



**Fig. 2.** Modulation of specific pathways “phenotypically reverts” the malignant phenotype in breast cancer cells cultured under 3D conditions. Cells were cultured in laminin-rich extracellular matrix (lrECM), to create a 3D microenvironment for 5 days after which immunofluorescence was performed in (A) Nonmalignant S1 cells; (B) malignant T4-2 cells; (C) phenotypically reverted T4-2 cells (also treated with a MAP Kinase inhibitor for the duration of the experiment to induce phenotypic reversion). Blue: nuclei, Green:  $\alpha$ -integrin and Red:  $\beta$ -catenin. Scale bars, 20  $\mu$ m. (D) Schematic diagram indicating how the modulation of a single oncogenic pathway leads to the phenotypic reversion of malignant cells.

(organoid) cell culture system in which both malignant and non-malignant breast epithelial cells behave phenotypically analogous to what is seen *in vivo* [26,37,94,123–129]. The findings, published in Onodera et al., in 2014, fill an important gap in understanding if and how glucose metabolism is involved in cancer [98]. Specifically, we demonstrated that overexpression of a glucose transporter (GLUT3) in nonmalignant human breast cells upregulated canonical oncogenic signaling. This led to the disruption of cell polarity and formation of disorganized tumor-like structures in 3D cultures. Pathways/mechanisms that play significant roles in the crosstalk between aerobic glycolysis and canonical oncogene signaling were also discovered [98]. First, the glycolytic pathway between GAPDH and LDH was shown to be required for increased  $\beta$ 1 integrin (encoded by *ITGB1*) expression and downstream AKT activity. The second crucial pathway that was discovered linking glucose metabolism and canonical oncogenic signaling was the hexosamine biosynthetic pathway (HBP). Inhibition of glycolysis and/or HBP was sufficient to induce suppression of all oncogenic signaling measured, including EGFR and  $\beta$ 1 integrin pathways. We also showed that both the glycolytic and HBP pathways were involved in reciprocal signaling - but only in a 3D structure [98]. Studies performed by Seyfried et al., showed that the *in vivo* growth of experimental astrocytoma was directly correlated to circulating glucose levels [130]. It was clear from their results that the higher the glucose levels, the faster the tumor grows. Alternatively, as glucose levels decrease, tumor size (weight) and growth also falls [130]. In summary, these findings strongly suggest that increased glucose levels/uptake and metabolism in nonmalignant/premalignant cells could indeed be an oncogenic event. This event would be analogous to activation of EGFR,  $\beta$ 1 integrin, PI3K-AKT, or MEK-ERK.

### 3. Canonical metabolic signaling pathways in 2D do not induce the malignant phenotype in 3D cultures

When nonmalignant cells are cultured in two dimensional conditions (2D, tissue culture plastic) they lose their tissue-specific structure and function and become exceedingly glycolytic. This led to the notion that growth rather than malignancy is the reason behind aerobic glycolysis in tumors, which will be further explored in this section. These 2D studies have revealed that the opposing actions of AMPK and mammalian target of rapamycin (mTOR) pathways are affected by intracellular fuel and energy status [131]. The mTOR pathway promotes anabolic processes under nutrient-and-energy-rich conditions, whereas AMPK redirects cell metabolism towards a catabolic process when nutrient and energy levels are low. Studies performed by Sun et al.,

showed mTOR as a central activator of the Warburg effect in cancer cells by inducing pyruvate kinase M2 (PKM2, rate limiting glycolytic enzyme) under normoxic (normal oxygen levels) conditions [132]. Disruption of hyperactive mTOR signaling, using rapamycin, suppressed cell proliferation and tumorigenesis. Conversely, AMPK is considered a tumor suppressor and it has been shown to inhibit mTOR signaling by directly phosphorylating one of the molecules of the mTOR complex [131,133–135]. This action then leads to the inhibition of many growth and proliferation related pathways. AMPK is also hypothesized to negatively regulate the expression of genes associated with metabolism by localizing to the nuclei of many cells. Other corroborating studies have directly linked AMPK signaling to increases in mitochondrial biogenesis and oxidative phosphorylation capacity in cancer cells, under glucose limiting conditions [136–138].

Likewise, HIF-1 $\alpha$  and HIF-2 $\alpha$  levels have been shown to regulate the expression of glycolytic enzymes and glucose transporters in many studies performed in 2D [139]. Therefore, activation of the HIF metabolic signaling pathway is also considered as one of the principal mechanisms of aerobic glycolysis. HIFs are a family of transcription factors that are activated by hypoxia, oncogenic, inflammatory, metabolic and oxidative stress pathways [100–103,105,111,112,139–146]. The HIFs form heterodimers consisting of oxygen-independent  $\beta$ -subunits (HIF- $\beta$ ) and oxygen-labile  $\alpha$ -subunits (HIF- $\alpha$ ). The downstream effects of HIF activity are determined by which  $\alpha$  subunit is involved. Under normal oxygen (normoxia) and 2D culture conditions, the action of prolyl hydroxylases (PHDs) and VHL ubiquitin ligases degrade the  $\alpha$ -subunits [145]. This prevents the stabilization and thus activation of HIF. In hypoxic cells, PHD-mediated hydroxylation of HIF- $\alpha$  is restricted by the low availability of molecular oxygen. This results in an accumulation of HIF- $\alpha$  subunits, which are then able to form active dimers with HIF- $\beta$  and drive transcription of hypoxia response genes [145]. Further 2D cell culture studies have revealed that HIF-1 can be transcriptionally induced by mTOR under normoxic conditions through 4 EB1 and Stat3 [142,147,148]. mTOR-mediated activation of HIF-1 mimics the effect of hypoxia and thus leads to increases in glycolysis and decreases in oxidative phosphorylation [149].

Although majority of 2D studies invariably show the involvement of AMPK, mTOR and HIFs in oncogenic signaling, these results differ from those obtained when more physiologically relevant 3D cell culture models were used. In 2014, Onodera et al., showed that these canonical metabolic pathways were not important players in inducing oncogenic signaling by increased glucose uptake and metabolism in breast cells [98]. For instance, treatment with 2-deoxy-D-glucose (2DG, a hexokinase inhibitor) in 3D cultures did not result in phosphorylation of



**Table 1**  
KEGG pathways identified in STING network show differentially expressed proteins between 2D and 3D cultured cells. Information was adapted from Kim et al., count: the number of differentially expressed proteins [158].

Pathway ID	Pathway Description	Count	FDR
Pathways up regulated in 3D relative to 2D cell culture			
110	Metabolic pathways	44	4.99E-20
1200	Carbon metabolism	18	5.40E-19
10	Glycolysis	12	3.53E-13
1230	Biosynthesis of amino acids	12	1.91E-12
4540	Gap junction	9	1.98E-07
30	Pentose phosphate pathway	6	6.67E-07
4145	Phagosome	8	1.33E-04
230	Purine metabolism	8	3.11E-04
4066	HIF-1 signaling pathway	4	4.89E-02
Pathways down regulated in 3D relative to 2D cell culture			
3040	Spliceosome	28	1.5E-25
190	Oxidative phosphorylation	8	1.2E-02

AMPK or acetyl-CoA carboxylase (ACC) its major target. Rather, phosphorylation and total ACC levels were already appreciably high in malignant breast cells. However, both molecules were phosphorylated by 2DG treatment in 2D culture conditions [98]. These data indicate that the AMPK pathway is differentially regulated in 2D versus 3D contexts. In addition, findings by Reidi et al., in 2017 showed that both AKT and mTOR signaling pathways were drastically reduced in 3D spheroids when compared to 2D cultures [150]. This held true even though receptor tyrosine kinase (RTK) signaling upstream of AKT-mTOR is specifically induced in malignant cells. Of note is an observed reduction of AKT phosphorylation in 3D but not 2D described by Weigelt et al., in breast cancer cells and Luca et al., in colon carcinoma cells (CRC) [151–153]. Phosphorylation levels of S6K and 4EBP1, two major targets of mTOR complex 1 (mTORC1), were also shown by Onodera et al., to only slightly differ between nonmalignant and malignant breast cells [98]. This indicates that the mTOR pathway can only be modestly influenced by inhibition of glucose metabolism in 3D. Yet, commonly used mTOR inhibitors, such as rapamycin had little to no effect on the phenotype of malignant cells, despite reducing AKT phosphorylation and cellular proliferation [98]. These results give additional credence to our contention that phenotypic reversion is not simply a matter of growth inhibition.

Similarly, expression of HIF-1 $\alpha$  and HIF-2 $\alpha$  (hypoxic response) which have been shown to regulate expression of glycolytic enzymes and glucose transporters in many 2D cell culture studies did not correlate with the expression levels of glycolytic enzymes or the malignant phenotype in 3D [98,154]. In one of those studies, knockdown of HIF-1 $\alpha$  and/or HIF-2 $\alpha$ , alone or in combination, did not suppress activities of the canonical oncogenic pathways, nor did they induce phenotypic reversion [98]. Studies performed by DelNero et al., utilized alginate-based, oxygen-controlled 3D tumor models to show the interdependence of culture context, hypoxia response and angiogenesis [154]. In it, the authors showed that although hypoxia, and resulting oxidative stress, is necessary to activate pro-inflammatory pathways (specifically IL-8/NF- $\kappa$ B) in 2D culture, these signaling axis are constitutively active in 3D cultures [154]. Previous studies performed by the same group also showed that hypoxia upregulates secretion of VEGF and IL-8 in 2D, while caused an opposite effect in 3D culture [155]. Further analysis of glucose transporter expression by microarray and western blot showed a dramatic upregulation of GLUT3 in malignant breast cells, whereas GLUT1 (another glucose transporter known to be upregulated in many tumor types) remained unchanged. More importantly, the mechanism of reciprocity shown in 3D-specific treatments with metabolic inhibitors that revert the malignant phenotype failed to suppress key oncogenic signaling in 2D cultures [98]. For instance, overexpression of GLUT3, which led to induction of the

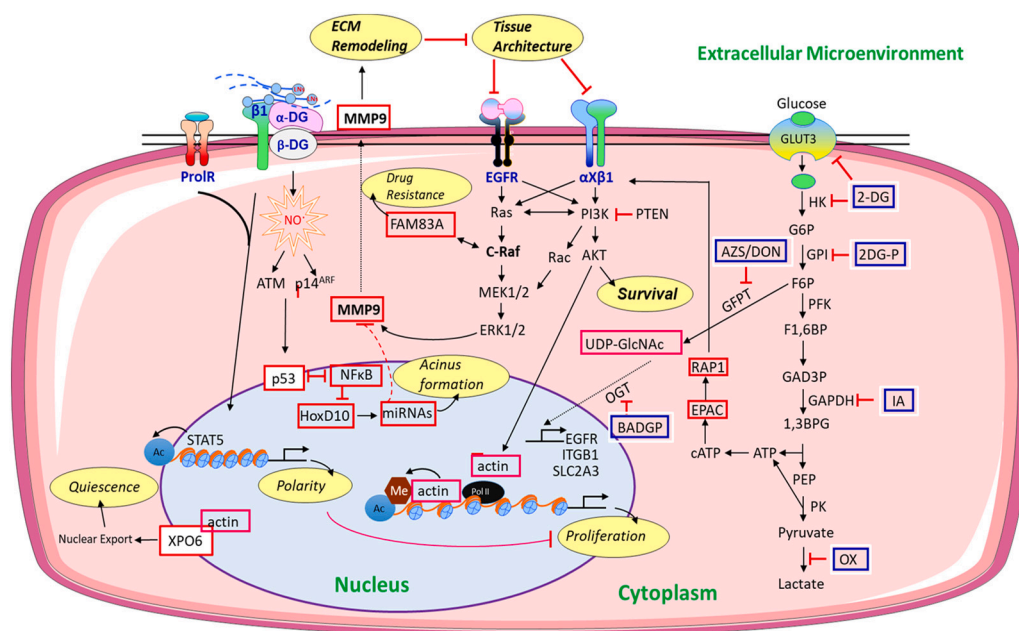
malignant phenotype in nonmalignant cells in 3D, did not significantly change signaling activity or morphology under 2D culture conditions. In esophageal adenocarcinomas, GLUT3 expression was observed to be increased in patients undergoing radiotherapy or surgery, which indicates clonal selection [156]. Additional clinical studies performed in laryngeal carcinoma patients showed an association with GLUT3 expression from tumor biopsy with poor survival [157]. Most surprising, however, was the finding that despite significantly higher levels of glucose transporters in malignant cells when compared to their non-malignant counterparts, even in 2D, no significant difference in glucose uptake was observed by Onodera et al. [98]. Unlike the glycolytic intermediates, relative oxygen consumption rates remained unchanged between nonmalignant and malignant cells, under 3D conditions. Thus, mitochondrial respiration is not essentially altered even with increased aerobic glycolysis. Lastly, quantitative proteomic studies of 2D and 3D cultured colorectal cancer cells performed by Kim et al., identified 136/247 differentially expressed proteins (up/down regulated in 3D compared to 2D) [158]. The observed up/down regulated proteins were mainly involved in energy metabolism, cell growth and cell to cell interaction. We have summarized the most enriched pathways shown in this study in Table 1.

On the basis of these and related findings, it can be concluded that glucose metabolic pathways require 3D contexts in order to retain their physiological/pathological function *in situ*. The interdependence of form and function in regulating glucose metabolism parallels our previous findings for other oncogenic signaling in which integration occurs in the context of 3D architecture, but rarely in 2D monolayers.

**4. Manipulation of glucose metabolism to revert the malignant phenotype in 3D breast cancer cell cultures**

Signaling pathways that function in cells cultured on 2D plastic become reciprocally integrated or reprogrammed when placed in a 3D microenvironment. With primary epithelial cells, the substratum these cells sit on determines which functions are expressed. In the case of mouse primary mammary epithelial cells cultured in monolayers (2D tissue culture plastic), these cells lose the ability to functionally differentiate even in the presence of lactogenic hormones [69]. When the same cells are transplanted into gland-free mice fat pads, they are able to respond to the correct hormonal cues and form tubular structures. From these studies, it was evident that either the cellular micro-environment surrounding epithelial cells plays an essential role in driving their functional differentiation or systemic factors not present in 2D culture are operating *in vivo*. In contrast to the 2D studies, mouse primary mammary epithelial cells cultured on floating collagen membranes, in the presence of lactogenic hormones, differentiate and secrete casein into the medium as they do *in vivo* and in organ cultures [66,69,159].

Numerous studies performed in our laboratory have used a laminin-rich extracellular matrix (lrECM) gel to create 3D microenvironments, which induce differentiation and reestablishment of cellular structure and function, in nonmalignant human cells [26,126,129]. In addition to the more commonly used breast cancer cell lines, we utilized the HMT3522 breast tumor progression series that was derived from reduction mammoplasty of a healthy woman [37]. These human breast epithelial cell lines span from non-malignant (S1), pre-malignant (S2 and S3), to malignant cells that form tumors in mice (T4-2). When cultured in 3D lrECM, this series can be used as a model for studying acquisition, maintenance, disruption, and reacquisition of tissue polarity [37]. In particular, nonmalignant S1 cells form polarized, quiescent colonies with hollow lumen (resembling normal breast ‘acini’), whereas tumorigenic T4-2s form disorganized colonies that continue to proliferate (Fig. 2). By studying these cells in 3D lrECM, we observed that the malignant phenotype of T4-2 cells could be reverted, to resemble their nonmalignant counterparts, despite still containing the cancer genome [21,22,24,29,39]. This phenotypic reversion is achieved



**Fig. 3.** Schematic relating the cellular microenvironment, particularly components of the extracellular matrix (ECM), to intracellular signal transduction networks leading to cellular quiescence and acinus formation. Disruption of this tight regulation is a fundamental step in tumor formation and disease progression. The schematic also illustrates how inhibition of intracellular signaling pathways, including glucose metabolism under 3D conditions, induces the formation of acinus-like structures in malignant cells.

by interfering with a number of pathways (Figs. 2 and 3). The inhibition of  $\beta 1$ -integrin or EGFR, or of different components of MAPK, PI3K and glycolytic pathways, or of Raf-induced MMP9 in the T4-2 tumor-like colonies leads to functional and morphological reversion of these malignant cells to growth-arrested acinar-like structures. Moreover, the attenuation of specific aberrant signaling pathways in cancer cells grown in 3D lrECM leads to their normalization to levels similar to those observed in nonmalignant S1 cells. This feedback inhibition does not occur in 2D cell cultures. For instance, the  $\beta 1$ -integrin and EGFR signaling pathways were shown to be coupled and bidirectional when cells are grown in a 3D lrECM environment, but not in 2D. Other more aggressive breast cancer cell lines, such as metastatic MDA-MB-231, can also either be phenotypically reverted or killed with a combination of inhibitors that simultaneously target multiple signaling pathways in 3D [125,151,160,161].

For all reversion studies, the inhibitors were added to the 3D lrECM culture early during the single cell stage [21,22,24,29,39]. To make a more clinically relevant cell culture assay, we allowed the S1, S2 or other malignant cell lines to form their respective structural phenotypes before adding inhibitors. The inhibitors were then added either by removing individual colonies that formed inside the lrECM gels, without disrupting the 3D structures and placing them onto other matrix surfaces or testing the inhibitors directly onto the 3D structures that formed on top of the lrECM. Combined results from these studies showed that 1) resistance to cell killing by clinically used apoptotic agents directly correlated with colony/tissue polarity, rather than the malignant phenotype, 2) treatment with growth blocking or glucose metabolism targeting inhibitors leads to decreased proliferation and increased apoptosis in several breast cancer, but not the nonmalignant, cell lines and 3) growth blocking or glucose metabolism targeting inhibitor treatment induces changes in cellular structure and morphology (phenotypic reversion) in malignant cells towards the formation of growth arrested, polarized acinus-like structures that resemble those of nonmalignant S1 cells [23,25,162–167]. Taken together these studies provide evidence that our 3D reversion model can be adopted for evaluation of drug response and efficacy of pharmacological candidates allowing us to better predict therapeutic outcomes (resistance and/or effective cell killing).

More specifically, Onodera et al., showed in our 3D culture model that inhibition of glucose metabolism using 2DG induced phenotypic reversion in malignant T4-2 cells. The observed phenotypic changes

included cellular growth arrest, reestablishment of basally polarized acini and decreased activity of canonical oncogenic pathways [98]. A dramatic downregulation of GLUT3 and other glycolytic enzymes were also observed. Whereas glucose uptake and lactate release were suppressed, PDC components, among others, were elevated while oxygen consumption rate remained unaffected [98]. These phenotypically reverted T4-2 cells form growth-arrested, polarized structures that resemble nonmalignant cell colonies. Treatment of nonmalignant S1 cells with the same 2DG concentrations neither altered growth rate nor acinar morphology. Comparable to the nonmalignant S1 cells, MCF10A nonmalignant human mammary epithelial cells cultured in 3D also lead to the formation of acini-like structures [98]. Moreover, introduction of oncogenes and/or activated growth factor receptors in nonmalignant HMT3522 or MCF10A cells disrupts their morphogenesis and elicits distinct morphological phenotypes of cancer progression [168]. Expression of signaling pathway effectors such as a conditionally active variant of AKT in MCF10A cells have also been shown to elicit large, misshapen structures as a result of enhanced proliferation and increased cell size. However, when transmembrane receptors upstream of AKT are hyperactivated the formation of tumor-like structures is observed. These properties of hyperactivated AKT have been directly linked to increased rates of glucose metabolism in cancer cells *versus* normal/nonmalignant cells.

## 5. Implications for glucose metabolism targeted drug discovery and development for cancer treatment

The development of effective strategies to combat cancer require an in-depth mechanistic understanding of the influence of cellular microenvironment on therapeutic response. Manipulation of the tumor microenvironment has the potential to prevent cancer progression, inhibit metastatic disease and increase the efficacy of currently available drugs [169,170]. Furthermore, focusing on therapies that target specific malignant properties has significant advantages over conventional chemotherapies. One reason for this is because phenotypes such as tumor hypoxia and altered metabolism towards aerobic glycolysis, among many other, are commonly found in solid tumors [1]. Together, these distinct phenotypes significantly contribute to oncogenesis, tumor progression and malignancy. In addition, the risk of developing drug resistance is greatly reduced when changes in cellular phenotype are targeted or at least taken into account. To that extent, several classes of

drugs that target hypoxia, cellular response to hypoxia and glucose metabolism in tumors are currently under development [171]. These drugs include inhibitors that specifically target the HIF-1 $\alpha$  pathway, mTOR, glucose transporters, hexokinase, phosphofructokinases, pyruvate kinase M2, pyruvate dehydrogenase kinase and lactate dehydrogenase. However, despite all the progress that has been made towards developing drugs that specifically target the glucose metabolic pathway, none have been approved for clinical use thus far [171].

More disappointing, was the failure of 2DG as a single agent chemotherapy in the limited clinic trials performed with this drug. At high doses, 2DG was shown to induce hypoglycemia and adverse cardiac effects and at tolerable doses failed to show a significant antitumor effect in many *in vivo* experiments in both mice and humans. However, when 2DG is synergistically combined with other agents like metformin, doxorubicin, cisplatin, and anti-autophagy agents, its cytotoxic/antitumor nature (at tolerable doses) becomes more evident [171]. 2DG is a synthetic glucose analog in which the hydroxyl group at the C-2 position is replaced with a hydrogen [172]. This molecule has been extensively studied both in the scientific and clinical settings since the early 1950s. In these studies, it has been shown to target glucose metabolism and deplete cancer cells of energy through the competitive inhibition of glucose uptake [173–176]. Upon entering the cell, 2DG is phosphorylated by hexokinase (HK) to form 2-deoxy-D-glucose-6-phosphate (2DG-6-P), which accumulates in the cell and non-competitively inhibits HK and competitively inhibits phosphoglucose isomerase (PGI). Because of the upstream effects of 2DG, it was hypothesized to lead to the partial disruption of glycolysis, oxidative phosphorylation and induce decreases in ATP production [172,174]. Decreased intracellular ATP levels have also been shown to increase the AMP/ATP ratio, which activates and increases AMPK levels. This results in elevated catabolism through phosphorylation of downstream targets such as mTOR [131,133–138]. In addition, 2DG increases oxidative stress, inhibits N-linked glycosylation of proteins and induces autophagy. It has been shown to effectively slow cell growth and potentially facilitate apoptosis in specific cancer cells [172,174].

With all the documented negative effects exerted by 2DG on cancer cells it makes one wonder why it failed to be efficacious as a single agent in clinical trials. One reason for this failure, apart from the reported toxicities, could be the cell culture techniques used during the preclinical phase of developing 2DG as a potential therapeutic drug for cancer treatment. Because as previously discussed, most studies on glucose metabolism are performed under 2D cell culture conditions. Performing such experiments in 2D creates a much simpler and low-cost maintenance system in terms of cell culture and with the performance of functional tests. Now more than ever, we are aware that cellular signaling pathways, gene expression, cell metabolism and morphology are all dramatically influenced by their 3D microenvironments (Fig. 3). Furthermore, traditional pharmacological assays of drug discovery, development and resistance measure tumor cell response using cell proliferation and/or cell death as readouts. These assays are also often performed under 2D conditions that do not necessarily translate to *in vivo* realities, hence the high failure rates for drugs in clinical trials. The use of phenotype-based assays could remedy this, as it may lead to the discovery of previously unknown therapeutic targets and may well better explain the source of drug resistance and relapse [170]. To that extent, we recently published a functionally robust phenotypic drug screen that also identified novel drug resistance genes/targets using 3D cell culture and results were later confirmed in mice [26,28,177]. Our 3D cell culture model also showed that 2DG induced phenotypic reversion in breast cancer cells, which is a state akin to those observed in drug resistant cancer stem cells.

Glucose metabolism specific studies performed by Kurtoglu et al., showed that energy deprivation alone does not sufficiently account for the antitumor effects of 2DG [178]. This is because ATP is necessary for apoptosis, as apoptotic cells generally have high ATP levels, thereby suggesting that 2DG may paradoxically exert a cytoprotective effect. In

addition, 2DG treatment, mimicking glucose deprivation, does not predispose some cancer cells with high oxidative phosphorylation function to death, as these cells could use alternative carbon sources to synthesize ATP under normoxic conditions. Treatments that rescue cells from 2DG induced cell death did not reverse ATP depletion [178]. The action of HIFs have also been shown to reduce the efficacy of 2DG and induce resistance by upregulating the expression of glucose transporters and several key glycolytic enzymes including GLUT1, HK, PKM and LDH [179]. Although these studies when taken together provide a plausible explanation for the lack of results obtained from clinical trials that used 2DG as a single agent, most were again performed under 2D cell culture conditions without taking the 3D cellular microenvironment into account.

Therefore, it is our hope that over time, and with more complex culture models, scientists may be able to better discriminate between the effects of specific treatments on tumor cells, the microenvironment or both to determine the relative contribution of each to any given clinical response.

## 6. Conclusion

It is remarkable how the original hypothesis proposed by Warburg “aerobic glycolysis is the origin of cancer” [1–3,180–182] still remains a point of contention. Most academic scientists that conduct cancer research would consider cancer a type of genetic disease where damage to nuclear DNA underlies the transformation of a normal cell into a possibly fatal tumor cell. Aberrations in oncogenes, which are dominantly expressed, and in tumor suppressor genes, which are recessively expressed, as the driving force of cancer have been the central dogma in the field for several decades [5,6,13–16,18]. The findings of millions of gene modifications in different cancers has also led to the opinion that cancer is not a single disease, but a collection of many different diseases [183]. Consideration of cancer as a “disease complex” rather than as a single disease has contributed to the perception that successful management of its various forms will require “individualized” or “personalized” drug treatments. These tailored therapies will be unique to the genomic defects within individual tumors and are now viewed as the future of cancer therapeutics. This personalized therapeutic approach would indeed be logical, as mentioned by Thomas Seyfield, if the nuclear somatic mutations detected in tumors were the main/only drivers of the disease [55,57,58,130]. With new and more concrete data indicating the contrary, it is questionable that all tumors arise from somatic mutations and that mutations are the only drivers of the disease. Additional complications arise from the fact that most of these studies are performed under culture conditions that do not accurately mimic the 3D microenvironment of cells *in vivo*.

To that extent, we and others have developed 3D cell culture models that allow for successful integration of extracellular signaling with canonical intercellular networks [22,37,88,124,125]. Our system achieves signal integration by taking the cellular microenvironment into account, thus making it more physiologically relevant and laying the framework for the current organoid field [97]. With this 3D cell culture model, we and numerous others, have shown significant differences in gene expression and cellular function when compared to cells cultured in 2D. Under 2D conditions important cellular structure and function is often lost. In the case of glucose metabolism, we showed that integration of increased aerobic glycolysis and oncogenic signaling in 3D cultures does not involve canonical metabolic pathways. This means that canonical metabolic signaling pathways in 2D are not determinants of the malignant phenotype in 3D. Furthermore, extracellular glucose levels alone were sufficient to induce the malignant phenotype in breast cells. Although further studies will have to be performed and these presented findings confirmed by other groups, it is our perspective that glucose metabolism/aerobic glycolysis is a cause, and not just a consequence of tumorigenesis. This we suspect will hold true even in tumors with functional mitochondria. Because



mitochondrial dysfunction need not always be present in transformed cells when there is increased aerobic glycolysis. Hypoxic micro-environments resulting from decreased/inconsistent blood supply to tumors, loss of tumor suppressors and oncogene-driven metabolic reprogramming are also initiating events of abnormal energy metabolism in cancer cells.

We have also used the 3D cell culture models to develop phenotype-based assays that could lead to the discovery of previously unknown therapeutic targets and may better explain the source of drug resistance and relapse observed in cancer patients [26,28,184,185]. This, we believe, may lead to the development of more effective drugs and enhanced therapeutic strategies that would be less toxic and prolong the lives of patients living with this fatal disease. Above all, it is our sincere hope that the best interest of cancer patients is prioritized over every other consideration. It is also important that we, as a community of scientists and clinicians, make massive efforts to discover effective combinatorial therapies that target both the tumor and its micro-environment. This is of course with the continued expectation that the side effects of each therapy alone will be minimized when drugs are used in combination, while maximizing therapeutic efficacy and reducing the incidence of drug resistance.

### CRedit authorship contribution statement

Mam Y. Mboge conceptualized and wrote the initial draft of the manuscript. Mina J. Bissell conceptualized, critically read and revised the manuscript.

### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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