Published in final edited form as:
Free Radic Biol Med. 2015 February ; 0: 269–280. doi:10.1016/j.freeradbiomed.2014.11.020.

Tumor Mechanics and Metabolic Dysfunction

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Abstract

Desmosplasia is a characteristic of most solid tumors and leads to fibrosis through abnormal extracellular matrix (ECM) deposition, remodeling and post translational modifications. The resulting stiff tumor stroma not only compromises vascular integrity to induce hypoxia and impede drug delivery, but also promotes aggressiveness by potentiating the activity of key growth, invasion, and survival pathways. Intriguingly, many of the pro-tumorigenic signaling pathways which are mechanically activated by ECM stiffness also promote glucose uptake and aerobic glycolysis, and an altered metabolism is a recognized hallmark of cancer. Indeed, emerging evidence suggests that metabolic alterations and an abnormal ECM may cooperatively drive cancer cell aggression and treatment resistance. Accordingly, improved methods to monitor tissue

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Competing Financial Interests: The authors declare no competing financial interests.

mechanics and metabolism promise to improve diagnostics and treatments to ameliorate ECM stiffening and elevated mechanosignaling may improve patient outcome. Here we discuss the interplay between ECM mechanics and metabolism in tumor biology and suggest that monitoring these processes and targeting their regulatory pathways may improve diagnostics, therapy, and the prevention of malignant transformation.

Introduction

Over the past several decades, an appreciation for the importance of cell and tissue mechanics in cancer initiation, progression, and metastasis has emerged (Sung, Hsieh et al. 2007; Mbeunkui and Johann 2009; Castano, Tracy et al. 2011; Sautes-Fridman, Cherfils-Vicini et al. 2011; Spano and Zollo 2012; Quail and Joyce 2013). The pro-tumorigenic cell and tissue mechano-phenotype manifests as both an intrinsic alteration of cell and tissue structure and mechanics, as well as changes in the biophysical properties of the tumor microenvironment (i.e. mechanics, geometry, and topology of the extracellular matrix) (Lelievre, Weaver et al. 1998; Nelson and Bissell 2005; Paszek, Boettiger et al. 2009). In particular, the noncellular ECM component of the tumor microenvironment plays a critical role in promoting invasion and metastasis (Butcher, Alliston et al. 2009). What is now emerging is that interactions between the cell and its associated ECM create a dynamic mechanical relationship mediated by a balance of the cell's contractility and the physical state (i.e. elastic vs. rigid) of the ECM microenvironment. This biophysical equilibrium serves to regulate a variety of critical cellular processes, including cell differentiation, proliferation, and motility. Not surprisingly, perturbation in the biophysical dynamics between the epithelium and the ECM potentiates the activity of key signaling pathways that regulate tumor growth, invasion, and survival (Lu, Weaver et al. 2012). Interestingly, many of the signaling pathways that promote the aggressive behavior of cancer cells also regulate glucose uptake and glycolysis. Deregulated cellular energetics is an emerging hallmark of aggressive cancers and reflects the metabolic reprogramming event that has been closely linked to tumor cell proliferation, in the hostile conditions of the tumor microenvironment (Cairns, Harris et al. 2011; Hanahan and Weinberg 2011). In this review, we provide a brief overview of the independent roles played by an altered tissue metabolism and an aberrant, stiffened ECM in cancer initiation and progression and highlight emerging data suggesting a regulatory connection between these two critical cancer regulators. We argue that novel tractable biomarkers and efficacious prevention and therapy programs may be developed by targeting the reciprocal feedback loop between cancer metabolism and the aberrant, mechanically-modified ECM.

Altered ECM dynamics, increased collagen cross-linking and mechanical signaling in the tumor microenvironment

ECM stiffening as a hallmark of cancer

Tumors are typically fibrotic and are often characterized by increased and abnormal deposition, turnover, and post translational modifications of the ECM that progressively stiffen the stroma (Levental, Yu et al. 2009; Egeblad, Rasch et al. 2010; Lopez, Kang et al. 2011; Mouw, Yui et al. 2014). Accordingly, the architecture and physical properties of

tumor-associated ECM are fundamentally different from that of the normal tissue stroma. In particular, collagens are often deposited in greater abundance early during cancer development (Zhu, Risteli et al. 1995; Kauppila, Stenback et al. 1998; Egeblad, Rasch et al. 2010; Conklin, Eickhoff et al. 2011) (Santala, Simojoki et al. 1999) and are major contributors to the tissue stiffness that stimulate mechanosignaling in stromal fibroblasts and the cancerous epithelium (Provenzano, Eliceiri et al. 2006; Levental, Yu et al. 2009; Samuel, Lopez et al. 2011). The extracellular processing of collagen is mediated through cleavage of collagen pro-peptides by specific proteinases. Thereafter, enzymes such as lysyl oxidases and lysyl hydroxylases, catalyze covalent intermolecular cross links between collagens and elastin, modifying their elasticity and tensile strength, profoundly changing various cellular behaviors The collagen fibers surrounding the normal epithelial structures of soft tissues, such as the mammary gland and lung, are typically curly and anisotropic (Egeblad, Rasch et al. 2010). For example, in the breast, collagen I levels are tightly regulated with oriented type I collagen fibers hugging in parallel the epithelial tubules and relaxed non-oriented fibrils surrounding the terminal ductal lobular regions. By contrast, transformed epithelial tissue is characterized by an abundance of highly linearized type I collagen bundles that are often oriented perpendicularly to the bulk of the noninvading tumor epithelial mass, and along which tumor cells can be seen migrating on thick fibers projecting into the parenchyma (Ingman, Wyckoff et al. 2006; Provenzano, Eliceiri et al. 2006; Levental, Yu et al. 2009). Consistently, malignancy is linked to expression of ECM remodeling enzymes, including heparanases, 6-O-sulfatases, cysteine cathepsins, and matrix metalloproteases (Ilan, Elkin et al. 2006; Kessenbrock, Plaks et al. 2010). Breast tumors, as well as head and neck cancer, also show elevated levels of collagen processing enzymes, including lysyl oxidases and lysyl hydroxylases, and chronic activation of lysyl oxidase, and is a poor prognostic marker (Le, Harris et al. 2009; Barker, Chang et al. 2011) Elevated lysyl oxidase-mediated cross-linking also stiffens the tissue ECM through collagen fiber stabilization and enhancement of the mechanical properties, leading to invasion, metastasis and poor patient survival (Erler and Giaccia 2006; Levental, Yu et al. 2009; Barker, Chang et al. 2011). As a result of this altered architecture and deregulated remodeling, tumor stroma is typically stiffer than normal stroma. Indeed, the ECM associated with some breast cancer tissue is robustly stiffer than normal (Levental, Yu et al. 2009; Lopez, Kang et al. 2011; Plodinec, Loparic et al. 2012). Interestingly, collagen architecture has also been linked with the reduction in breast cancer risk associated with early-age pregnancy(Maller, Hansen et al. 2013). Despite an increased abundance of collagen I in parous stroma, the matrix is actually less linearized and associated with a decrease in tissue stiffness. It is these aspects of extracellular collagen processing that serve to inhibit the invasive phenotype, despite elevated deposition.

Tumor progression is linked to a dynamic and reciprocal remodeling of the local ECM by the resident cancer cells, stromal fibroblasts, and infiltrating endothelium and immune cells (Lu, Weaver et al. 2012).For instance, a local stiffening of the ECM stimulates actomyosin cell contractility, which through elevated localized traction forces simultaneously modify the adjacent ECM and promotes cell growth, invasion, and survival by fostering focal adhesion maturation and enhancing growth factor receptor and GPCR signaling (O'Brien, Jou et al. 2001; Croft, Sahai et al. 2004). Indeed, the increased tumor cell and fibroblast contractility

initiates tension-dependent matrix remodeling and promotes the linearization of collagen fibrils surrounding the invasive front of the tumor. In addition to influencing tissue mechanics, these linearized collagen fibrils also facilitate tumor cell invasion and metastasis. For example, migrating transformed mammary epithelial cells have been observed traveling along these linear bundles of collagen (Ingman, Wyckoff et al. 2006; Wyckoff, Wang et al. 2007; Provenzano, Inman et al. 2008). Consistently, stiffening the ECM *in vitro* and *in vivo* promotes the malignant progression of oncogenically-modified, pre-malignant mammary epithelial cells (Levental, Yu et al. 2009). Furthermore, inhibiting tissue fibrosis and stiffening by preventing collagen cross-linking enhanced the latency of tumor formation in Her2/Neu mice and significantly reduced tumor incidence and grade (Levental, Yu et al. 2009). Inhibition of lysyl oxidase activity has also been shown to suppress tumor cell metastasis, and can modulate the phenotypic characteristics of basal cells (Pickup, Laklai et al. 2013)Clearly, altered ECM dynamics are not merely a feature of the tumor microenvironment, but actively contribute to tumor cell aggressiveness.

Because altered ECM dynamics are a fundamental characteristic of cancer, new approaches that exploit these aberrations are of great clinical value. Tumor-associated collagen signatures (TACS) aim to define the procession of changes with respect to collagen that has been observed and classified as markers of mammary carcinoma progression. TACS-1, when mammary tumors exhibiting a localized increase in the deposition of collagen near the tumor lesion, occurs very early in tumor formation. As tumors increase in size, the collagen fibers aligned parallel to the tumor boundary straight and are denoted TACS-2. The highest-risk signature, TACS-3, occurs when collagen fibers are bundled and aligned perpendicular to the tumor border. This signature has the highest stiffness and predicts poor survival in women with invasive breast cancer. In preclinical models and human breast cancer, when TACS-3 is present, breast cancer cells preferentially invade along the stiff collagen fibers into the adjacent stroma with TACS-3 collagen fibers identifying focal sites of breast cancer cell microinvasion (Schedin and Borges 2009; Schedin and Keely 2011; Maller, Hansen et al. 2013). These studies highlight the clinical importance of altered ECM dynamics in defining and understanding cancer aggressiveness.

Mechanosignaling and cellular tension drive cancer aggressiveness

Cells sense and convert exogenous forces into signaling pathways through a mechanism termed mechanotransduction (Hamill and Martinac 2001). Through mechanotransduction, cancer cells respond to mechanical changes in their microenvironment including increased compression, hydrostatic pressure, shear stress, and elevated ECM stiffness or cell-cell tension. While tissue compression and shear stress are tissue-level forces, the increased ECM stiffness that cancer cells and their associated stromal cells experience begins at the nanoscale and progresses to the tissue level to gradually promote malignant transformation (Levental, Yu et al. 2009; Plodinec, Loparic et al. 2012). A shift in the amount or composition of the ECM, an increase in the level of cross-linked matrix, or the reorientation of matrix fibrils cooperate to stiffen the tumor tissue, thereby enhancing the activity of key signaling pathways that stimulate cell growth and survival and promote invasion and migration (Paszek, Zahir et al. 2005; Provenzano, Inman et al. 2009; Mouw, Yui et al. 2014).

Cells sense, process, and respond to mechanical and other biophysical cues from the ECM using a complex web of systems that include specialized mechano-sensitive ion channels and adhesion receptors (e.g., integrins) and their associated adhesion plaque proteins which propagate signals deep into the cell through an interconnected cytoskeletal - molecular motor network. This orchestrated system allows cells to modulate their shape, nuclear architecture, and tune their actomyosin-generated cellular tension that ultimately remodels their local ECM (Janmey 1998; Giancotti and Ruoslahti 1999; Bershadsky, Balaban et al. 2003; Katsumi, Orr et al. 2004; Lele, Sero et al. 2007; Ingber 2008). The best understood matrix-directed mechanotransduction mechanism is initiated through ECM-dependent integrin activation and clustering, resulting in talin recruitment, and through vinculin activation promotes focal adhesion assembly, FAK and paxillin phosphorylation, subsequent Rho-ROCK-dependent actin remodeling, and reciprocal actomyosin-mediated cell contractility (Figure 1a) (Paszek, Zahir et al. 2005; Provenzano, Inman et al. 2008; Geiger, Spatz et al. 2009; Samuel, Lopez et al. 2011). Focal adhesions thereafter collaborate with G protein coupled receptors and transmembrane receptor tyrosine kinases to enhance cell growth, survival, migration, and invasion through ERK, JNK and phosphoinositide 3-kinase (PI3K) signaling (Figure 1b) (Wozniak, Desai et al. 2003; Paszek, Zahir et al. 2005; Levental, Yu et al. 2009). In particular, PI3K regulates cell growth, invasion, and survival and has been implicated in tumor aggression and treatment resistance (Gershtein, Shatskaya et al. 2000; Gershtein, Scherbakov et al. 2007). Not surprisingly, ECM stiffness and elevated epithelial mechanosignaling enhance growth factor receptor-dependent PI3K signaling to foster malignant cell behavior (Mouw, Yui et al. 2014).

The phosphatase and tensin homolog (PTEN) protein, a tumor suppressor frequently altered in human cancers, is the master negative regulator of the phosphoinositide 3-kinase (PI3K) signaling pathway, and therefore plays a critical role in cell growth, proliferation, and survival (Maehama and Dixon 1998; Leevers, Vanhaesebroeck et al. 1999; Hollander, Blumenthal et al. 2011). Not surprisingly, even a modest reduction in PTEN is sufficient to permit the malignant transformation of a tissue and can accelerate tumor progression and treatment resistance (Alimonti, Carracedo et al. 2010). Indeed, the exquisite cellular sensitivity to PTEN levels and function is reflected by the diverse regulatory mechanisms controlling its expression. For instance, although normal human mammary cells express high levels of PTEN protein, nonmalignant mammary epithelial cells (MECs) propagated *in vitro* on conventional tissue culture plastic express low levels of PTEN. However, PTEN protein expression is restored and presumably stabilized when MECs are grown within a three dimensional reconstituted basement membrane (3D rBM) to assemble the “differentiated” acini observed in normal breast tissue *in vivo* (Liu, Radisky et al. 2004; Fournier, Fata et al. 2009). Intriguingly, the elastic modulus of tissue culture substrates is super-physiologic, whereas the material properties of rBM hydrogels are remarkably similar to that of the normal human breast (Paszek, Zahir et al. 2005; Johnson, Leight et al. 2007). Indeed, the ECM within a human breast tumor is stiffer than normal tissue, implying matrix compliance may stabilize PTEN protein. Consistently, we recently showed that a stiff ECM increases levels of miR-18a, which directly targets PTEN mRNA, both directly and by indirectly decreasing levels of HOXA9, to regulate malignant transformation and tumor metastasis in culture and *in vivo* by increasing PI3K/AKT-signaling (Mouw, Yui et al.

2014). These findings underscore a direct relationship between PTEN, the biophysical properties of the ECM, and PI3K-dependent malignancy.

Compared to their nonmalignant counterparts, transformed epithelial cells show striking differences in their mechano-responsiveness that we and others showed can facilitate their growth, invasion, and survival in response to the increased stiffness found in primary tumors (Wang, Dembo et al. 2000; Paszek, Zahir et al. 2005; Tilghman, Cowan et al. 2010). Many tumor cells exhibit differential intermediate filament profiles and cytoskeletal architectures that modify their migratory behavior. The replacement of a keratin-based cytoskeleton with that of a vimentin-based cytoskeleton is a hallmark of epithelial-to-mesenchymal transition (EMT) in mammary tissue and contributes significantly to invasive potential (Pagan, Martin et al. 1996; Willipinski-Stapelfeldt, Riethdorf et al. 2005; Kokkinos, Wafai et al. 2007). Cancer cells are also frequently highly contractile and this elevated cytoskeletal tension contributes to their ability to grow, invade, and survive in the hostile tumor microenvironment. For instance, transformed cells plated on compliant substrates spread rapidly, grow, and survive, whereas their normal counterparts do not (Wang, Dembo et al. 2000). Tumor cells also generate high traction forces that disrupt cell-cell junctions, compromise tissue polarity, promote anchorage-independent survival, and enhance invasion (Figure 1c)(Paszek, Zahir et al. 2005). High cellular tension also increases Rho/ROCK activity, which via engagement of its downstream effectors, promotes tumor cell proliferation, migration, and survival (Samuel, Lopez et al. 2011). These findings demonstrate the reciprocal relationship between ECM micromechanical signals and cell structural control, how these signals propagate through signal transduction networks, and the subsequent modulation of key behaviors connected with tumorigenesis, invasion, and metastasis.

Experimental methods to evaluate ECM stiffness and tissue mechanics

The degree of tumor stiffness, which is typically determined by measuring its elastic modulus, has been exploited as a means to detect malignant lesions in an otherwise normal tissue. The elastic modulus is a measure of how easily a material deforms and is calculated by dividing stress by strain; the greater the elastic modulus, the stiffer the material. Thus, the elastic modulus offers a quantitative manner for determining mechanical differences between tissues.

Multiple techniques have been utilized to characterize tissue level changes in elastic modulus in tumors with varying degrees of success. Unconfined compression and shear rheology testing have both been used to quantify incremental stiffening of the mammary gland as it transitions from normal to premalignant to invasive cancer, and demonstrated that the stromal tissue adjacent to the invading epithelium is substantially stiffer than normal (Paszek, Zahir et al. 2005; Levental, Yu et al. 2009). Shear rheology testing has also been used to monitor changes in tissue stiffness during the progression of liver fibrosis (Georges, Hui et al. 2007; Perepelyuk, Terajima et al. 2013).These techniques lend themselves to gross tissue level assays where resolution and sensitivity are less demanding. However, recent findings that stress the functional implications of ECM stiffness and an altered cell tension on transformation potential and treatment response demand more precise measurements.

Thus, while unconfined compression and shear rheology provide valuable quantitative measurements of tissue elastic modulus, they are limited by their millimeter resolution capabilities.

Atomic force microscopy (AFM) is one imaging modality that permits higher resolution imaging of tissue elastic modulus. AFM measures the interaction force between a sample surface, such as a living cell, and a microscale to nanoscale spring-like cantilever. Tip-sample interactions generate a force that deflects the cantilever, which can then be optically tracked via a photodiode and converted to an interaction force using the spring constant of the cantilever. The force measurement capability of AFM has been used to measure the regional tissue elastic modulus of cultured brain slices (Elkin, Azeloglu et al. 2007), pancreatic ductal adenocarcinoma tissue (Ozdemir, Pentcheva-Hoang et al. 2014), and excised mammary tissue (Lopez, Kang et al. 2011; Maller, Hansen et al. 2013; Pickup, Laklai et al. 2013; Mouw, Yui et al. 2014). These measurements yielded high resolution force heat maps that demonstrate the existence of stiffness tracts that register with regions of collagen fiber enlargement and linearization. While AFM has effectively characterized changes in tissue elastic modulus at the microscale, the clinical applicability of the technique is severely limited due to the requirement of unprocessed samples.

Over the last decade, elastography, which can employ either ultrasound or magnetic resonance imaging (MRI) techniques, has been utilized clinically to map tissue elastic modulus. This technique has found utility in tumor diagnosis (Khaled, Reichling et al. 2006), as well as intraoperative localization of tumor tissue during resection of gliomas (Selbekk, Bang et al. 2005; Unsgaard, Rygh et al. 2006). Despite its widespread usage, elastrography is limited by its low resolution compared with the length scales relevant to many diseases (hundreds of microns for ultrasound elastography, and millimeters for MRI elastography), the long acquisition times associated with MRI elastography, and issues with tissue penetrance and user variability with ultrasound elastrography. Because of the limitations described above, new imaging techniques must be developed in order to measure tissue elastic modulus as a means of precisely and accurately diagnosing and monitoring cancer.

Metabolism of cancer

Oncogenesis and glycolysis

Transformed cells exhibit aberrant growth rates that demand elevated levels of ATP to support the biogenesis of the cells' four major macromolecules: carbohydrates, proteins, lipids, and nucleic acids. The Physician Scientist Otto Warburg hypothesized early on that the tumor cells must adjust their metabolic machinery to accommodate these energy requirements. Warburg found that tumor cells transition from the conventional ATP-producing system, oxidative phosphorylation (OXPHOS), to glycolysis in the presence of adequate oxygen. The oddity of this observation, known as the Warburg Effect or aerobic glycolysis, is that glycolysis is usually reserved for anaerobic ATP production, and per until of glucose is a much less efficient producer of ATP than OXPHOS (Warburg, Wind et al. 1927; Warburg 1956). Although apparently counterintuitive glycolysis allows much faster, on-demand, ATP production than OXPHOS, but at the demand of more glucose uptake.

Despite having major implications in basic tumor cell biology as well as clinical diagnostics and therapy, tumor metabolism was largely ignored for decades until recently when transforming oncogenes were found to have both direct and indirect effects on the cells' central metabolism.

At present a growing list of oncogenes and tumor suppressor genes have been functionally linked to cell metabolism (For-an-excellent-comprehensive-review-see (For an excellent comprehensive review, see(Cairns, Harris et al. 2011). Ras and Src were the first oncogenes associated with the Warburg Effect owing to their induction of glucose transporter (Glut genes) expression(Flier, Mueckler et al. 1987). Similarly PI3K/AKT upregulation leads to increased GLUT1 and GLUT4 expression, thus allowing more uptake of glucose to fuel glycolysis but also activates (phosphorylates) a number of metabolic enzymes including hexokinase and phosphofructokinase 2, the former which catalyzes glucose to glucose-6-phosphate, the first step of glycolysis. Additionally, by activating mTOR, AKT activity leads to increased mRNA translation and protein biogenesis (Laplante and Sabatini 2012). Another common oncoprotein, Myc, drives aerobic glycolysis directly through the upregulation of glucose transporters and phosphofructokinase-2 (Osthus, Shim et al. 2000), and cooperatively with HIF1 to induce hexokinase 2 and pyruvate dehydrogenase (Kim, Gao et al. 2007). Not surprisingly loss of function of tumor suppressors can mimic the metabolic phenotype driven by oncogene expression. Through TIGAR (TP53-inducible glycolysis and apoptosis regulator) p53 reduces glycolysis and enhances OXPHOS through SCO2 (cytochrome c oxidase 2) (Bensaad, Tsuruta et al. 2006; Matoba, Kang et al. 2006) P53 also maintains expression of PTEN, which is the major regulatory brake on the PI3K/AKT/mTOR pathway (Stambolic, MacPherson et al. 2001). Further, PTEN loss or AKT activation lead to increased glycolysis through depletion of ATP stores (Fang, Shen et al. 2010). These findings highlight the pleotropic regulation of metabolism by classical oncogenic and tumor suppressive signaling networks (Figure 2).

Tumor Redox

A major component of the tumor microenvironment and a poor predictor of outcome is hypoxia, which occurs due to the combination of the enhanced metabolic and replicative state of tumor cells with a compromised vasculature (Vaupel, Mayer et al. 2004; Vaupel 2009). Through activation of HIF1, hypoxia acts to reinforce glycolysis and sustained hypoxia leads to mitochondrial dysfunction (Papandreou, Cairns et al. 2006). Collectively, this results in an abnormal cellular redox status. Redox, which is the balance of the reduced and oxidized state of a cell, is governed by the levels of reactive oxygen and nitrogen species (ROS and RNS) with antioxidant molecules. As a consequence of increased ATP production and macromolecule biogenesis, tumor cells accumulate abnormally high levels of ROS (Bae, Kang et al. 1997) (Szatrowski and Nathan 1991; Toyokuni, Okamoto et al. 1995). Through posttranslational modifications, these oxidative molecules contribute to tumor progression by augmenting the activity of oncogenic proteins such as PI3K, MAPK, and Src while further inhibiting tumor suppressors such as PTEN and PTP (Thomas, Witting et al. 2008)). Further, ROS supports the transcription of Hif1a and GLUT1, thus creating a feed forward mechanism that enhances the Warburg Effect in tumor cells (Bell, Klimova et al. 2007). Although increased ROS production is usually thought of as pro-tumorigenic,

certain thresholds of oxidative damage to proteins and nucleic acids can trigger cell death through apoptosis (Takahashi, Ohtani et al. 2006; Cairns, Harris et al. 2011), thus revealing another potential therapeutic opportunity in the treatment of tumors.

Therapeutic targeting of tumor metabolism

An exciting development in tumor therapy is the exploitation of abnormal tumor metabolism (Galluzzi, Kepp et al. 2013). Metformin, is an agonist of AMPK (AMP-activated protein kinase), a finely tuned sensor of the cellular AMP/ATP ratio. At high ratios, AMPK is activated and negatively regulates mTOR, thus reducing protein production and proliferation. Secondarily AMPK activity reduces insulin production and gluconeogenesis (Shackelford and Shaw 2009). Interestingly, before the mechanism of action of Metformin was studied, it was already found to improve insulin sensitivity and reduce blood glucose in diabetics. Today this is the most common drug in the treatment of type 2 diabetes(Dowling, Goodwin et al. 2011). From decades of diabetic patient data, large meta-analyses were performed showing that those patients on Metformin have significantly reduced risk of cancer incidence and cancer-associated death (Evans, Donnelly et al. 2005; Bowker, Majumdar et al. 2006), which has led to clinical trials in several solid tumors, including colorectal, endometrial, prostate, breast, and brain (Recently reviewed by (Kasznicki, Sliwinska et al. 2014)). While mouse tumor models and cell culture experiments show that Metformin induces mTOR inhibition, growth arrest, and apoptosis of tumor cells (Isakovic, Harhaji et al. 2007; Liu, Fan et al. 2009), patient data are quite limited. In breast cancer patients, Metformin reduces mTOR transcription and Ki67 staining proliferative index (Hadad, Dewar et al. 2010) and also improve treatment responses rates (Jiralerspong, Giordano et al. 2008). Metformin treatment also reduces the rate of colorectal cancer formation and levels of prostate cancer death (Hosono, Endo et al. 2010; Patel, Hruby et al. 2010). Many ongoing clinical trials will elucidate the efficacy of Metformin as a first-line of treatment, in combination therapy, and in tumor prevention studies. In addition to Metformin, multiple drugs which target various aspects of metabolism are in preclinical and clinical trials (Galluzzi, Kepp et al. 2013).

Because Metformin negatively regulates pathways downstream of AKT (Gwinn, Shackelford et al. 2008), which are important in tumor ECM remodeling and mechanosignaling, it is possible that use of this drug may help to reduce tissue fibrosis and thereby temper the malignant effects of a stiffened ECM. Exciting recent data in support of this hypothesis include findings that Metformin treatment in heart failure-prone rats reduced perivascular fibrosis and prevented heart failure. Additionally, in a mouse model of cardiac fibrosis, Metformin treatment led to reduced levels of TGFbeta and activated Smad3, thus decreasing collagen deposition and fibrosis (Xiao, Ma et al. 2010; Cittadini, Napoli et al. 2012). These studies hint at connections between altered tumor mechanics and metabolism, which are discussed below.

Clinical metabolic imaging techniques

Links between dysfunctional metabolism and malignancy have fostered the development of a number of noninvasive clinically-tractable approaches to image tissue metabolism *in situ.* By delivering radioactively tagged glucose to patients, tumors can be detected by their

abnormally high uptake of the metabolite. [F18]-Fluorodeoxyglucose (F18-FDG) is taken up through Glut transporters and phosphoralyated by hexokinase. Owing to the lack of a 2′-hydroxyl group, this radiolabelled analogue of glucose is not metabolized further, allowing for the identification of metabolically active tissue with positron emission (PET) imaging. Clinically, FDG-PET has been used successfully in diagnosis, staging, monitoring response to therapy, and predicting outcome in tumors of the breast, colon, and lung (Gambhir, Czernin et al. 2001; Jadvar, Alavi et al. 2009). Clinical histopathology and experimental xenograft studies have shown that FDG uptake is correlated with increased HIF1a and Glut expression (Ai, Pan et al. 2007; Kaira, Murakami et al. 2013)as well as Ki67 proliferative status (Han, Lin et al. 2009).

MRI and magnetic resonance spectroscopy (MRS) allow quantitative analysis of metabolites in a tissue. This technique has been particularly useful in the diagnosis and prognosis of brain tumors (McKnight, Lamborn et al. 2007). An adaptation of MRS is hyperpolarized 13C-MRS, which allows tracing of the fate of [13C]-radiolabeled metabolites. Although not yet FDA approved this technique is safe in patients (Nelson, Kurhanewicz et al. 2013) and has been used in cell culture and mouse models to study glutaminase activity in hepatocellular carcinoma cells (Gallagher, Kettunen et al. 2008) and the abnormal metabolism of alpha ketogluterate in IDH1 mutant gliomas (Chaumeil, Larson et al. 2014). Current advances in this technology and reduction in 13C reagent costs will likely push this technique into the clinic (Kurhanewicz, Vigneron et al. 2011). Metabolic changes occur early during tumorigenesis thus advancements in FDG-PET and MRS metabolic imaging have the potential to help diagnose various tumor types at much earlier stages.

Another approach which is useful in the metabolic analysis of archived biopsy tissue is the fluorescent imaging of NADH/NADPH and FAD. These ubiquitous metabolic cofactors, which play a key role in glycolysis, OXPHOS, and ROS scavenging, are autofluorescent and readily imaged due to their relatively high concentration within the cell. Biochemical means have uncovered changes in the redox state of these molecules in tumor cells during early stages of carcinoma and when tumors progress (Zipfel, Williams et al. 2003; Villette, Pigaglio-Deshayes et al. 2006; Skala, Riching et al. 2007). In addition to intensity, the fluorescent lifetime imaging (FLIM) of NADPH and FAD serves as a readout of metabolic state, as each of these molecules has a unique FLIM value depending on whether it is free in the cytosol or bound to protein (Figure 3). Metabolic imaging of NADPH and FAD by FLIM matches metabolic perturbations in culture, and this technology is able to distinguish basal cell skin sarcomas from surrounding normal skin, as well as glioma from normal brain (Bird, Yan et al. 2005; Leppert, Krajewski et al. 2006; De Beule, Dunsby et al. 2007; Galletly, McGinty et al. 2008; Walsh, Cook et al. 2013). FLIM of NADPH and FAD discerns early carcinoma *in situ* of the mammary gland in mouse (Conklin, Provenzano et al. 2009), suggesting these approaches may be useful for *in situ* determinations of early changes associated with aerobic glycolysis. Consistent with this finding, our recent data demonstrate that changes in NADPH fluorescent lifetime values may serve as an early biomarker for biochemical changes in tissue that is deemed morphologically normal by standard pathological assessment. The clinical relevance of these approaches is further highlighted by the finding that metabolic imaging by FLIM corresponds to tamoxifen responsiveness in

both breast xenographs and human head and neck squamous cell carcinoma (Walsh, Cook et al. 2013; Shah, Demory Beckler et al. 2014).

A proposed role for tissue mechanics-mediated regulation of tumor cell metabolism and cancer progression

Physical forces influence cellular metabolism

A stiffened ECM is becoming appreciated as a critical component of the tumor microenvironment and data are accumulating to show that mechanics and cell adhesion regulate metabolism. Upon integrin-mediated ECM-adhesion, cells exhibit a spike of ROS which is necessary for subsequent integrin signaling and Src activation (Chiarugi, Pani et al. 2003) and quenching of ROS reduces vSrc-driven tumorigenicity and invasion (Giannoni, Buricchi et al. 2005). In addition to adhesion, the compliance of ECM has marked effects on cellular growth and proliferation (Paszek, Zahir et al. 2005; Tilghman, Cowan et al. 2010) processes which demand increased metabolic flux. Interestingly, EMT, a process heavily implicated in invasion, is favored on stiffer substrates (Tilghman, Cowan et al. 2010; Lee, Chen et al. 2012) and mesenchymal cell types exhibit increased aerobic glycolysis through increased Zeb1-induced expression of glucose transporters (Masin, Vazquez et al. 2014)and Snail-induced methylation of fructose-1,6-bisphosphate (Dong, Yuan et al. 2013). Moreover, stiffness-induced Rac1b facilitates formation of the NADPH oxidase complex, which drives ROS production and enhances the EMT phenotype in breast epithelial cells(Lee, Chen et al. 2012).

The interplay between physical forces and metabolism is perhaps best understood in endothelial cells. Analogous to the regulation of homeostasis by ECM compliance, physiologic (regular pulsatile or laminar flow) shear stress helps regulate gene expression and redox of endothelial cells, however under conditions of elevated or irregular (turbulent or oscillatory) shear stress, these cells upregulate Rac1 activity which fuels ROS production, driving $NF\kappa B$ activation, and facilitating atherosclerosis(Tzima, Del Pozo et al. 2002). Further, irregular shear stress in ischemia leads to PI3K/AKT activation and ROS production in a feed-forward mechanism (Chatterjee, Browning et al. 2012). Clear connections between cell adhesion, mechanical signaling, and metabolism exist, however more studies are needed to understand the molecular mechanism by which these processes influence one another.

Tissue stiffness regulates metabolism and drives cancer progression: a developing direction

Both altered metabolism and deregulated ECM dynamics clearly act as fundamental drivers of cancer progression. However, emerging evidence suggests that these two characteristic features of cancer may also be interconnected, driving the progression of cancer via an synergistic, cooperative network. The PI3K signaling pathway is central for regulation of glucose metabolism. In fact, activation of PI3K/AKT pathway renders cells dependent on high levels of glucose flux, a classic biochemical phenomenon of cancer cells (Buzzai, Bauer et al. 2005). In addition to activation of mTOR mediated signaling, the PI3K pathway also regulates glucose uptake and utilization. In insulin-independent tissues, PI3K signaling

through AKT regulates the expression of glucose transporters, GLUT1 and GLUT4 (Kohn, Summers et al. 1996), via hexokinase, thereby stimulating phosphofructokinase activity and increasing glucose uptake (Rathmell, Fox et al. 2003; Biswas, Lunec et al. 2012), an action critical for enhanced aerobic glycolysis. However, PI3K activation also occurs in the context of increased matrix stiffness via integrin-mediated activation of FAK and downstream pathways (Provenzano, Inman et al. 2008; Provenzano and Keely 2009) (Xia, Nho et al. 2004; Kallergi, Agelaki et al. 2007; Guan 2010). Thus, regulation of PIP3 by PI3K and subsequent activation of AKT and mTOR represent mechanistic means by which matrix stiffness and increased ECM deposition may influence cellular metabolism, thereby promoting both cell proliferation and survival (Wozniak, Desai et al. 2003; Paszek, Zahir et al. 2005), as well as facilitating oncogenic transformation and tumor metastasis (Levental, Yu et al. 2009). Matrix stiffness may also regulate metabolism through effects on p53, as AKT drives p53 degradation via MDM2 (Hu, Feng et al. 2012). Moreover, p53 is further regulated by reduction of cysteine residues, and may become inactivated as the redox balance of the cell shifts (Forsberg and Di Giovanni 2014).

Matrix stiffness-mediated PI3K activation of AKT is also reinforced through β-catenin and myc-dependent matrix stiffness-induced expression of miR18a, which inhibits PTEN expression and stimulates cancer progression (Mouw, Yui et al. 2014). Consistent with these findings, knock out of FAK in mammary tumors down-regulates the activity of PI3K and AKT, while enhancing PTEN expression and disrupting tumor growth and metastasis (Provenzano, Inman et al. 2008). Activation of PI3K by growth factors alone has also been shown to be insufficient to induce accumulation of PIP3, and oxidation-mediated inactivation of PTEN is required to increase the concentration of PIP3 sufficiently to trigger downstream signaling events (Kwon, Lee et al. 2004). Thus, oxidation- and stiffness-mediated inactivation of PTEN may work cooperatively to trigger the PIP3 downstream signaling events that drive cancer progression.

Evidence also suggests a dynamic relationship between cell attachment to the ECM and metabolic activity regulation. Detachment of mammary epithelial cells from ECM causes an ATP deficiency due to loss of glucose transport. However, this deficiency can be rescued via overexpression of ErbB2, which restores glucose uptake through stabilization of EGFR and PI3K activation via glucose-stimulated flux through the antioxidant-generating pentose phosphate pathway. Interestingly, ATP deficiency can also be rescued by antioxidant treatment without rescue of glucose uptake, which is dependent on stimulation of fatty acid oxidation, a process inhibited by detachment-induced reactive oxygen species (Schafer, Grassian et al. 2009). These findings demonstrate that cell attachment to the ECM directly regulates metabolic activity, providing a means for improving cell survival in the disrupted ECM tumor microenvironment through antioxidant restoration of ATP generation.

Intriguing microarray data generated from culturing normal mouse mammary gland cells in dense versus compliant 3D collagen matrices suggests an enhanced oxidative phosphorylation signature in stiffer environments (Provenzano, Inman et al. 2009). While this may reflect the difference between normal and transformed cells, there is increasing evidence that oxidative phosphorylation is also increased in tumors (Solaini, Sgarbi et al. 2011). Increased oxidative phosphorylation in the context of increased aerobic glycolysis

allows cells a robust metabolism that may be necessary for the substantial energy demands of proliferation, migration, and metastasis. Indeed, AKT/mTOR activation of 4EBP1 leads to increased mitochondrial biogenesis (Goo, Lim et al. 2012), and may represent an additional means by which matrix stiffness regulates metabolism through a FAK/PI3K/AKT pathway.

Enhanced ECM deposition, aligned collagen, collagen cross-linking and fibrosis are all key aspects of breast cancer progression. As described here, not only is there solid data suggesting that ECM stiffness stimulates aberrant cell growth, survival, invasion, and metastasis through modulation of the PI3K/AKT network, but ECM stiffness may also contribute to altered cellular metabolism.

Conclusions and future perspectives

Increased deposition, remodeling, and cross-linking of ECM within the breast, and the resulting increased tensile forces, are characteristic of breast cancer progression and also serve to activate signaling pathways critical for proliferation, survival, invasion, and metastasis. Emerging evidence also shows that some of these same ECM-activated signaling pathways promote aerobic glycolysis and glucose uptake. Here we provide evidence that tissue mechanosignaling activates signaling networks that simultaneously promote breast carcinogenesis and metabolic reprogramming. Both altered ECM dynamics and metabolic reprogramming are critical drivers of cancer aggressiveness. Thus, research focusing on their interconnected feedback loop may be a promising direction for biomarker discovery and drug development.

Emerging clinical data has begun to link stiffer matrices with increased cancer aggressiveness in human patients, emphasizing how matrix stiffness and deregulated mechanosignaling components could provide useful strategies for clinical intervention and to identify novel biomarkers (Mouw, Yui et al. 2014). In basal-like or luminal B samples, breast ECM stiffness and miR-18a expression were found to be significantly higher as compared to levels measured in the normal and luminal A tumor biopsies. Due to this mechanically-mediated miR-18a expression profile, PTEN expression was found to be expressed in appreciable quantities in the more differentiated luminal B cancers and normal breast tissue, whereas less differentiated luminal B, basal-like, and $HER2^{+}$ tumors showed reduced expression of PTEN, along with higher levels of activated AKT (Figure 4). miR-18a levels also distinguished luminal A from luminal B tumors and predict clinical outcome in patients with luminal type breast cancer. These findings suggest that macro-level tumor stiffness may parallel future disease aggression, with tumor size, grade, and subtype as independent factors influencing tissue mechanics. Furthermore, ECM stiffness acts as a biophysical parameter that can distinguish luminal A from luminal B tumors, with miR-18a and PTEN presenting as tractable biomarkers. Unfortunately, imaging techniques that characterize tissue stiffness in the clinical setting are severely limited. However, due to the interconnected relationship of metabolism and tissue stiffness, the identification of surrogate metabolic markers for tissue stiffness may serve to accurately monitor cancer progression.

Altered metabolism and tissue elastic modulus have typically been identified as independent features of cancer. In fact, activation of PI3K/AKT, excess metabolic energy, and glycolysis are all associated with poor prognosis in triple-negative breast cancer (Rose, Haffner et al. 2007; Phipps, Chlebowski et al. 2011; Kim, Kim et al. 2014). Interestingly, these factors appear to influence clinical outcome through similar and overlapping pathways. In response to increased mechanical stiffness, integrin clustering, ERK activation, cytoskeletal remodeling, and Rho GTPase-dependent contractility are engaged, and foster the assembly of focal adhesions, which in turn collaborate with G protein coupled receptors and transmembrane receptor tyrosine kinases to alter PI3K signaling and downstream AKT signaling. Mutations in the key component PI3K lead to subsequent down-stream activation of AKT and predict poor survival and risk of recurrence (Gonzalez-Angulo, Ferrer-Lozano et al. 2011; Ocana, Vera-Badillo et al. 2014). Genetic defects in mitochondrial respiration and increase in glycolysis also activate AKT signaling via increases in metabolic potential mediated by NADH, which promotes recruitment of GLUTs to the cell surface and increased glucose uptake and glycolysis (Manning and Cantley 2007). These high glucose levels have also been shown to promote the aggressive behavior of triple-negative breast cancer including proliferation, activation of oncogenic signaling, apoptosis-resistance and reduced drug efficacy (Wahdan-Alaswad, Fan et al. 2013). Interestingly, women at high risk for developing triple-negative breast cancer show altered metabolic state (Ibarra-Drendall, Dietze et al. 2011). Random peri-areolar fine needle aspiration samples taken from high risk patients were shown to express high levels of activated proteins representing RTK/AKT/mTOR, RTK/AKT/ERK, and mitochondrial apoptosis, suggesting significant metabolic dysfunction exists long before tumors can be detected in these patients. The strong correlation between ECM stiffness and AKT suggests that these high risk women may also have altered ECM, making them prime candidates for elastography imaging studies. PET imaging is also now being used to determine glucose uptake in these high-risk patients (Figure 5). Many of these high risk patients are also involved in chemotherapy prevention trials that include metformin. It will be interesting to see whether this early state of metabolic dysfunction is reversible and whether early targeting of altered metabolism is an effective preventative strategy. Taken together, these studies highlight the central role of AKT and redox activation in defining the aggressive behavior of triple-negative breast cancer. Given the key role that matrix stiffness plays in regulating PI3K/AKT-signaling, these studies also provide evidence for the convergence of mechanosignaling and redox activation in aggressive cancers. Since AKT-network signaling is active in premalignant tissue from women at high-risk for breast cancer prior to the development of an invasive phenotype (Pilie, Ibarra-Drendall et al. 2011; Ibarra-Drendall, Troch et al. 2012), this opens the possibility that combination therapies which target both cellular metabolism and tissue stiffness may serve to more effectively halt disease progression.

Altered metabolism and ECM stiffening are two chief component of the tumor microenvironment that profoundly impact cancer progression. Although new evidence suggest that both are intricately linked through their downstream signaling pathways, additional studies will need to be conducted to fully investigate their complex relationship. Nevertheless, this newly developing area of cancer research may provide fresh avenues to pursue for the development of tractable biomarkers and novel cancer therapeutics.

Acknowledgments

This work was supported by: National Institutes of Health grants NHLBI T32 HL007544 to JCT, and F32 CA174319 to JMB as well as R01 CA155664, R01 CA158668, and R01CA170851 to VLS and R01CA136590, R01 CA114462, R01 CA142833, to PJK and NIBIB R21 EB008811 to PJK, and U54CA163155-01, U54CA143836, CA138818-01A1, R01 CA174929, R33 CA183685-01, and U01 ES019458 to VMW. Support for the review was also provided by the Susan G. Komen Breast Awards KG091020 to VLS and KG110560PP to VMW and; a V-Foundation Award. [http://www.jimmyv.org/] to VLS; and CDMRP Breast Cancer Research Program grants BC074970 to PJK and Era of Hope Scholar Expansion W81XWH-13-1-0216 to VMW.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Highlights

- ECM stiffness drives signal transduction networks that lead to subsequent modulation of key cell behaviors that influence cancer aggressiveness
- Oncogenes commonly activated in cancer are linked to metabolic reprogramming
- Deregulated ECM dynamics and altered metabolism may form an interconnected feedback loop that cooperatively drives cancer progression
- Targeting the interwoven network of these two critical cancer regulators is a promising avenue for new clinical applications

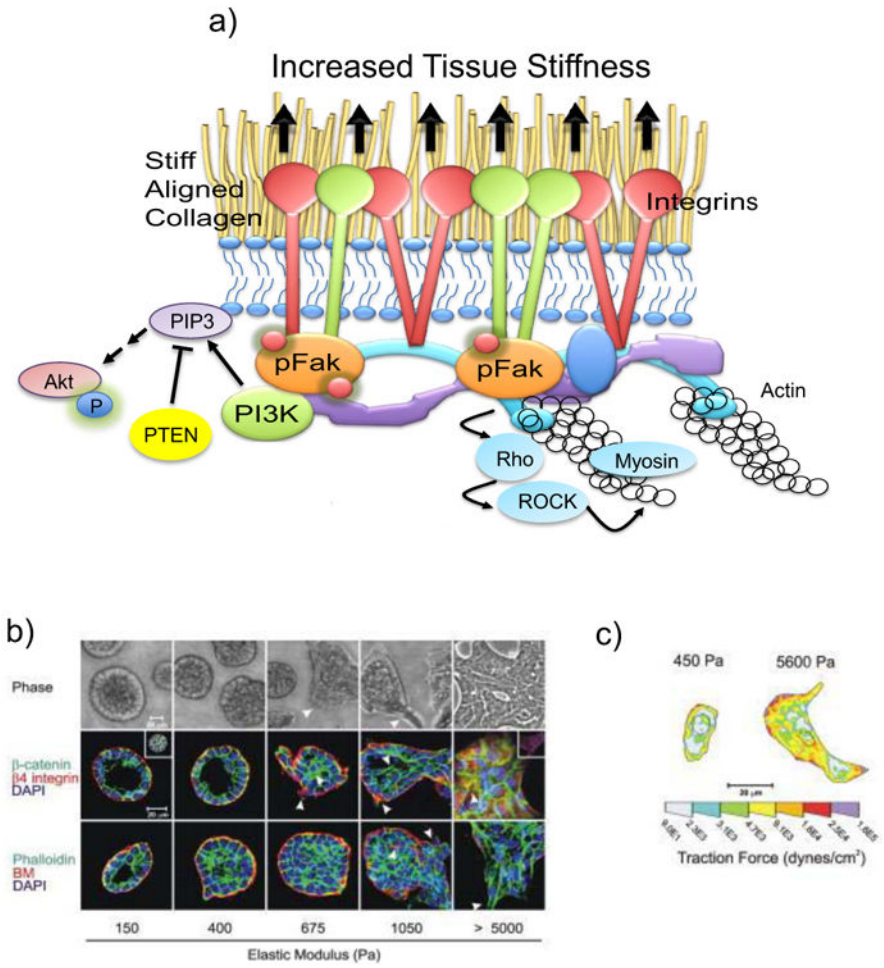

Figure 1.

Mechanosignaling and cellular tension drive cancer aggressiveness. A) Both normal and malignant cells sense and respond to mechanical cues from the ECM via adhesion receptors (e.g., integrins), intracellular focal adhesions, cytoskeletal networks, and molecular motors. Cell adhesion and focal adhesion in turn collaborate with G protein coupled receptors and transmembrane receptor tyrosine kinases to enhance cell growth and survival and invasion by altering phophoinositide 3-kinase (PI3K) signaling and downstream p-AKT. B) In compliant 3D gels with material properties similar to that measured in the normal mammary gland, non-malignant human mammary epithelial cells form growth-arrested, polarized acini analogous to the terminal ductual lobular units observed at the end buds of the differentiated breast. Incremental stiffening of the basement membrane gel progressively compromises tissue morphogenesis and alters EGF-dependent growth of these cells. Thus, colony size progressively increases, lumen formation is compromised, cell-cell junctions are disrupted, and tissue polarity is inhibited. Arrows indicate loss of endogenous basement membrane and disruption of basal polarity. C) Non-malignant human epithelial cells grown on stiff substrates show greater cell contractility, measured via traction force microscopy, compared to the same cells grown of softer substrates. Reprinted from Cancer Cell, 8/3, Paszek et al, Tensional homeostasis and the malignant phenotype, 241-54, copyright 2004, with permission from Elsevier.

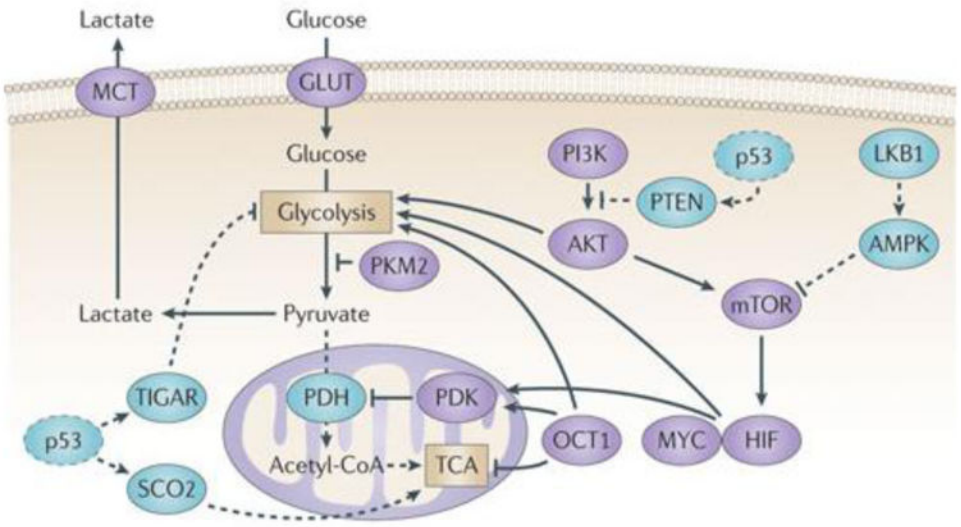

Figure 2.

Regulation of metabolism in tumor cells. Normal cells use the TCA cycle (OXPHOS) for ATP production, whereas oncogenic signaling drives a glycolytic phenotype in tumor cells. PI3K activates AKT, which stimulates glycolysis by directly regulating glycolytic enzymes and by activating mTOR. mTOR has pleotropic effects on metabolism, but facilitates the glycolytic phenotype by enhancing HIF1 activity, which engages a hypoxia-adaptive transcriptional program. HIF1 induces the expression of glucose transporters (GLUT), glycolytic enzymes and PDK1, which blocks the entry of pyruvate into the TCA cycle. The LKB1 tumor suppressor activates AMPK which opposes the glycolytic phenotype by inhibiting mTOR. MYC cooperates with HIF to induce several genes that encode glycolytic proteins, but also increases mitochondrial metabolism. The tumor suppressor p53 opposes the glycolytic phenotype by suppressing glycolysis through TIGAR, increasing mitochondrial metabolism via SCO2 and supporting expression of PTEN. OCT1 acts in an opposing manner to activate the transcription of genes that drive glycolysis and suppress oxidative phosphorylation. The switch to the PKM2 isoform affects glycolysis by slowing the pyruvate kinase reaction and diverting substrates into alternative biosynthetic and reduced NADPH-generating pathways. TCA, tricarboxylic acid; HIF1, hypoxia-inducible factor 1; LKB1, liver kinase B1; PDK1, pyruvate dehydrogenase kinase, isozyme 1; AMPK, AMP-activated protein kinase; PKM2, pyruvate kinase M2; TIGAR, TP53-induced glycolysis and apoptosis regulator; MCT, monocarboxylate transporter; PDH, pyruvate dehydrogenase. The dashed lines indicate the shift from TCA to glycolysis under the loss of p53 function. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer, 11/2, Cairns et al, Regulation of cancer cell metabolism, copyright 2011.

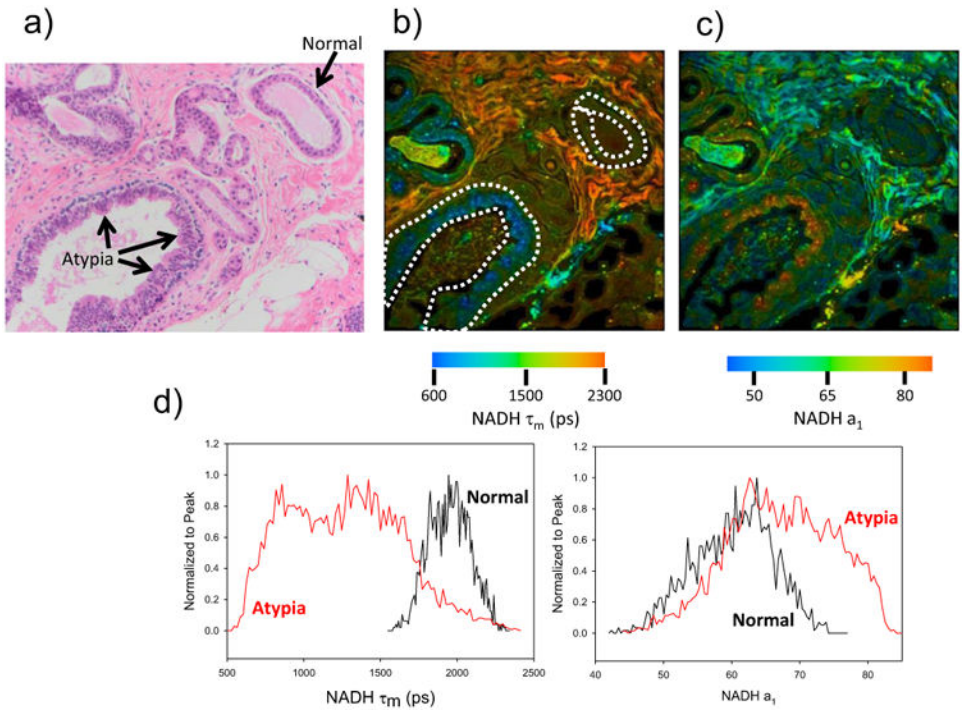

Figure 3.

Fluorescent lifetime imaging of a high risk breast biopsy. A) Standard H&E stained section with a region of atypia. B) FLIM of the unstained adjacent section in which the weighted average lifetime of NADH (τm) and C) the fraction of free NADH (a1) is shown as a color map. D) Quantitative histograms of the pixel-by-pixel data from the regions-of-interest outlined in (B). An increase in a_1, which corresponds to a decrease in τm, in epithelium displaying atypia is an indication these cells have either increased glycolysis or decreased oxidative phosphorylation.

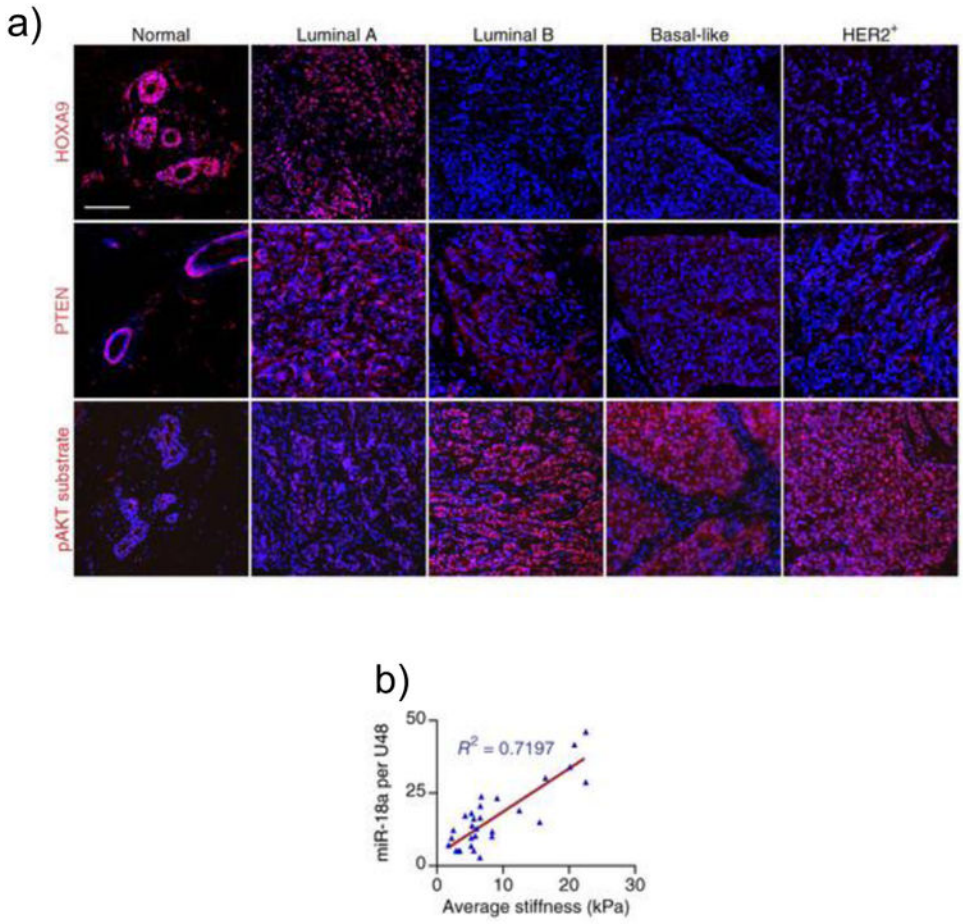

Figure 4.

Breast malignancy associates with miR-18a and reduced PTEN expression. A) HOXA9 (top, red), PTEN (middle, red), pAKT substrate (bottom, red) and DAPI (blue) for human nonmalignant breast samples and breast tumor samples. The epithelium of normal breast tissue coexpressed appreciable quantities of nuclear HOXA9 and cytoplasmic PTEN proteins, as did the more differentiated luminal A breast tumors. In contrast, the less differentiated luminal B, basal-like, and HER2+ tumors showed reduced expression of HOXA9 and PTEN and higher levels of activated AKT. Scale bar, 100 μm. B) As measured by atomic force microscopy, miR-18a expression is correlated with breast ECM elastic modulus in human patient samples from both normal and transformed breast tissue. Reprinted by permission from Macmillan Publishers Ltd: Nature Medicine, 20/4, Mouw et al, Tissue mechanics modulate microRNA-dependent PTEN expression to regulated malignant progression, copyright 2014.

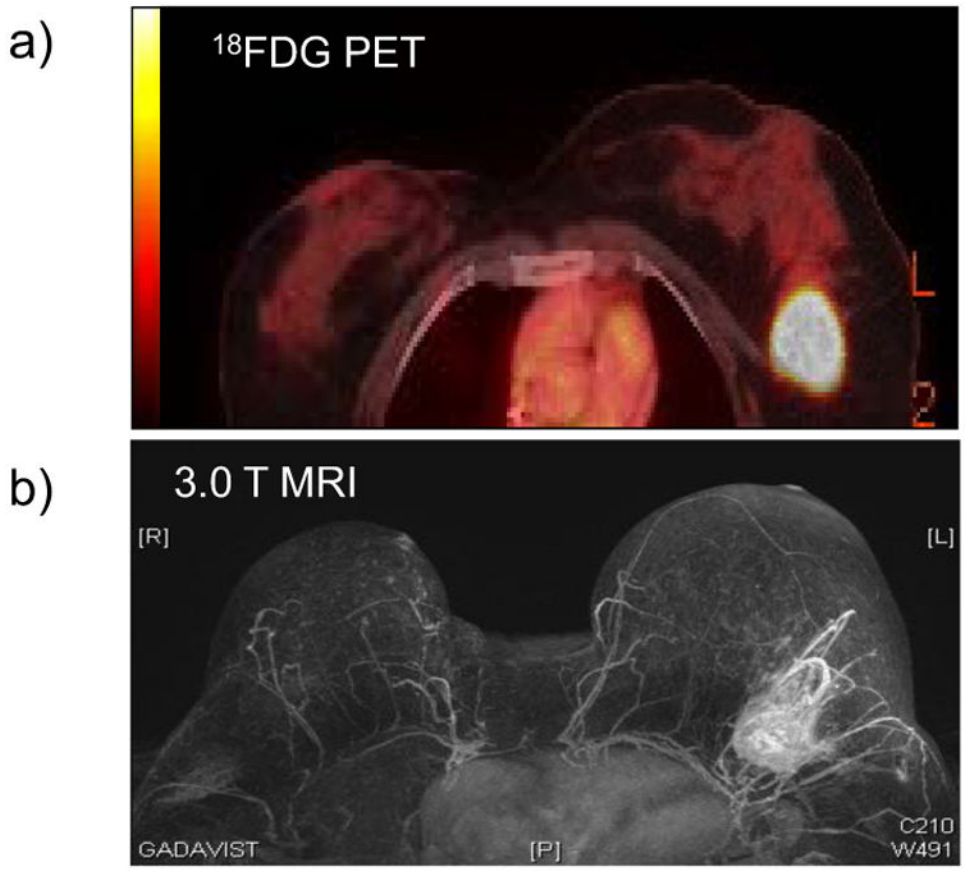

Figure 5.

Metabolically active triple-negative breast cancer from women at high-risk for breast cancer. a). High glucose uptake as measured by ^{18}Flurodeoxyglucose Positron Emission Tomography (^{18}FDG-PET). b) Timed 3.0 Tessla Magnetic Resonance Imaging (3.0 T MRI) demonstrates the vascularity of the cancer.

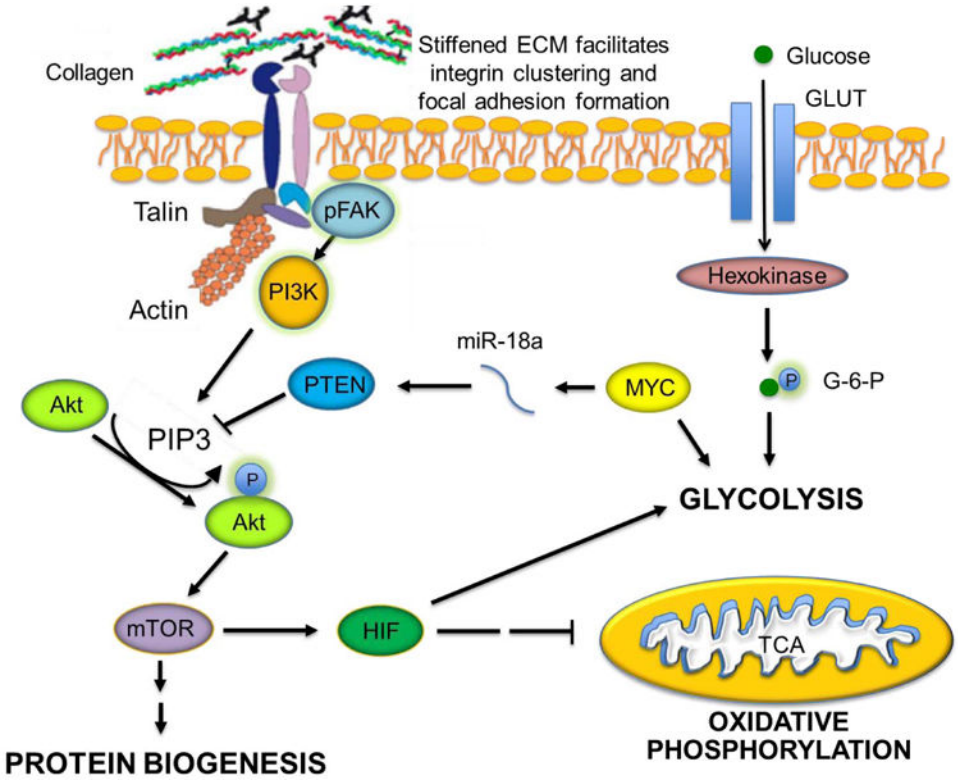

Figure 6.

Proposed model by which a stiffened ECM regulates metabolism. Through mechanical activation of pathways highlighted in Figures 1 and 2, ECM stiffness (and other perturbed forces within the tumor microenvironment) drives a glycolytic phenotype. ECM, extracellular matrix.