Extracellular Matrix Regulation of Metabolism and Implications for Tumorigenesis

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Attachment to extracellular matrix (ECM) is required for the survival and proliferation of normal epithelial cells. Epithelial tumor cells, however, often acquire "anchorage independence," a property that may contribute to their ability to invade and grow in foreign environments. Although apoptosis is the most rapid and effective mechanism that causes the death of matrix-detached cells, it has become apparent that detachment from matrix alters other aspects of cell physiology prior to commitment to cell death and that some of these alterations can lead to cell death under conditions where apoptosis is suppressed. This report provides an overview of death processes that contribute to the death of matrix-detached normal cells and describes mechanisms that confer anchorage independence, with a focus on ECM regulation of cell metabolism. Loss of matrix attachment leads to metabolic stress characterized by reduced nutrient uptake, decreased ATP production, and increased levels of reactive oxygen species (ROS). The decrease in ATP levels is prevented by either constitutive activation of the PI3K/Akt pathway or exogenous antioxidants. Additionally, decreased Erk signaling in matrix-detached cells causes a disproportionate decrease in flux through pyruvate dehydrogenase (PDH), leading to decreased entry of glucose carbons into the citric acid cycle. Interestingly, forced overexpression of a PDH inhibitor suppresses de novo lipogenesis and proliferation, highlighting the importance of mitochondrial metabolism in supplying intermediates for biosynthetic processes required for proliferation. Thus, ECM attachment is a key regulator of cellular metabolism, and alterations in metabolism owing to changes or loss of ECM engagement during tumorigenesis may serve important tumor-suppressive functions.

Cellular specialization and tissue formation are inexorably linked with a necessity for appropriate tissue architecture. This process begins during early stages of embryogenesis when the first asymmetrical cell division occurs and continues throughout the life of the organism. Individual cells within a multicellular organism must receive and respond to external cues providing both functional and positional directions; stem cells are instructed when to divide and differentiate, and the differentiated daughter cells are directed to their appropriate location within the organism. Regulation of cellular proliferation and cell death is essential for maintenance of tissue integrity and function. One external signal that can contribute to the control of cell growth and survival is attachment to the extracellular matrix (ECM). This has been strongly supported by studies of mammary epithelial three-dimensional (3D) cultures using reconstituted basement membranes (Bissell et al. 2002; Debnath and Brugge 2005) and in vivo studies that either demonstrate the requirement for ECM receptors in generation of intact mammary gland structures or show a strong correlation between apoptotic clearance and the absence of ECM attachment (Li et al. 2005; Mailleux et al. 2007).

Tumorigenesis occurs when individual cells fail to respond to the signals and needs of the entire organism; tumor cells grow, divide, and take up nutrients independently of proper cues. However, it is the ability of tumor cells to invade into surrounding tissue that distinguishes benign from malignant tumor growth, and the capability of tumors to colonize and impair the function of vital organs necessitates survival in foreign environments. The vast majority of cancer deaths are in fact due to metastatic disease, and thus the restriction of both growth and survival outside the normal microenvironment serves an important tumor-suppressive function.

To gain further understanding of the alterations that allow for tumor cell survival in inappropriate microenvironments, we have focused our studies on cell–matrix interactions. Specifically, our laboratory has studied why normal epithelial cells depend on attachment to the ECM for survival and proliferation and how oncogenic events allow tumor cells to circumvent these requirements. Here, we discuss new mechanistic insight as to how cell–matrix interactions regulate intracellular growth factor signaling and cellular metabolism as well as how these processes affect cell survival and proliferation.

ANCHORAGE INDEPENDENCE IN TUMOR GROWTH

Normal epithelial cells undergo a caspase-dependent, apoptotic death termed "anoikis" if forced to grow without matrix attachment, whereas many epithelial tumor cells can survive in the absence of ECM contact. The ability of tumor cells to survive without ECM attachment or in an inappropriate matrix environment (termed "anchorage

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independence") is believed to play an important role in tumor initiation and growth (Hanahan and Weinberg 2011; Nagaprashantha et al. 2011). During early stages of tumorigenesis, excess proliferation displaces cells from their normal microenvironment; for example, in ductal carcinoma in situ, a noninvasive early form of breast cancer, cells proliferate into the hollow lumen of the ducts of the mammary gland (Harris et al. 1999). Because ECM proteins are absent from the lumen of the ducts, these pretumor cells must survive without endogenous ECM. Likewise, during later stages of tumorigenesis, tumor cells are again challenged to survive in microenvironments that are either deficient in ECM or have altered ECM composition during invasion, intravasation, and colonization. Therefore, epithelial tumor cells must survive and proliferate independent of appropriate ECM attachment. Because the majority of deaths owing to cancer are a result of metastatic disease, inhibiting the outgrowth of disseminated tumor cells (i.e., by preventing their ability to survive in inappropriate microenvironments) may greatly improve survival rates.

Cells interact with the ECM through a family of proteins called integrins that bind to ECM proteins such as laminin, collagen, and fibronectin. There are two subtypes of integrins, α integrins and β integrins, that form 24 different heterodimers, each with variable affinities toward the different ECM proteins (Hynes 2009; Nagaprashantha et al. 2011). The specificity of the integrin–ECM interactions helps to maintain tissue integrity and contributes to restricting the survival and proliferation of cells to the appropriate environments. Upon integrin ligation, protein adaptors are recruited to the intracellular domain of the integrins, leading to activation of downstream signaling pathways such as Src, PI3K/Akt, and Mek/Erk, which promote survival and proliferation (Cabodi et al. 2010). Studies suggest that phenotypes induced by ECM detachment are in large part due to loss of integrin engagement; for example, death of ECM-detached mammary epithelial cells can be prevented by addition of reconstituted basement membrane, in a manner dependent on integrin ligation (Reginato et al. 2003).

The requirement for integrin ligation to maintain cell survival and proliferation is probably due in large part to integrin regulation of growth factor signaling. In fact, a major consequence of loss of attachment to ECM is a dramatic decrease in growth factor signaling through pathways including PI3K/Akt and Mek/Erk (Fig. 1) (Chiarugi and Giannoni 2008; Schafer et al. 2009; Desgrosellier and Cheresh 2010; Ivaska and Heino 2010; Grassian et al. 2011a). Many reports have shown that maximal growth factor signaling requires integrin activation and that integrin ligation modulates signaling downstream from several growth factor receptors, including the epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), and platelet-derived growth factor receptor (PDGFR; Assoian and Schwartz 2001; Desgrosellier and Cheresh 2010; Grassian et al. 2011a; Nagaprashantha et al. 2011). For example, integrin ligation was found to be required for maximal

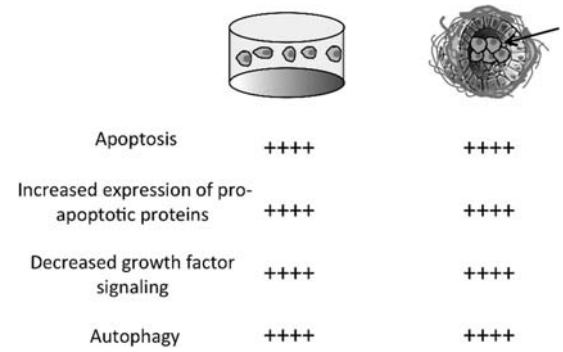

Figure 1. Similarities of suspension cells and inner acinar cells. This image shows shared properties of mammary epithelial cells under conditions where they are deprived of matrix contact by either culture in suspension or localization in the center of 3D acini.

EGF-induced activation of EGFR and downstream signaling through Mek/Erk and PI3K/Akt (Cabodi et al. 2004). Integrin ligation also regulates expression of EGFR, and EGFR levels are dramatically decreased after matrix detachment of mammary epithelial cells owing to the loss of Erk signaling and increased lysosomal degradation (Reginato et al. 2003, 2005; Grassian et al. 2011a). In addition, integrin ligation can itself induce growth factor signaling via activation of the Src family of kinases or focal adhesion kinase (Fak), and integrin activation can also stimulate ligand-independent activation of many growth factor receptors, including VEGFR, PDGFR, and EGFR (Miranti and Brugge 2002; Chiarugi and Giannoni 2008; Cabodi et al. 2010). These examples highlight the importance of coordinated signaling from both integrins and growth factor receptors for epithelial cell survival and proliferation, thereby ensuring that cells proliferate both at the correct time and in the correct microenvironment (Bill et al. 2004; Cabodi et al. 2010; Ivaska and Heino 2010).

Given the importance of ECM attachment in regulation of cell survival and growth, both in vitro and in vivo models have been developed to study anchorage independence (Fig. 2). A common in vitro assay to monitor anchorage independence is an assay that monitors colony formation in a semisolid growth media, such as soft agar, which deprives cells of attachment to a solid substrate (Macpherson and Montagnier 1964; Kahn and Shin 1979). The ability to form colonies under these conditions is strongly correlated with tumorigenicity (Jones et al. 1976; Barrett et al. 1979). Additionally, a suspension culture system can also be employed to assay anchorage dependence. In this assay, tissue-culture plates are coated with a nonadhesive material such as poly(2-hydroxy methacrylate; poly-HEMA), thus preventing cells from engaging integrin signaling and forcing them to grow in suspension (Folkman and Moscona 1978). Both the soft agar and suspension assay systems are well suited for studies in which genetic manipulations or pharmacological inhibitors are employed to assess the effects

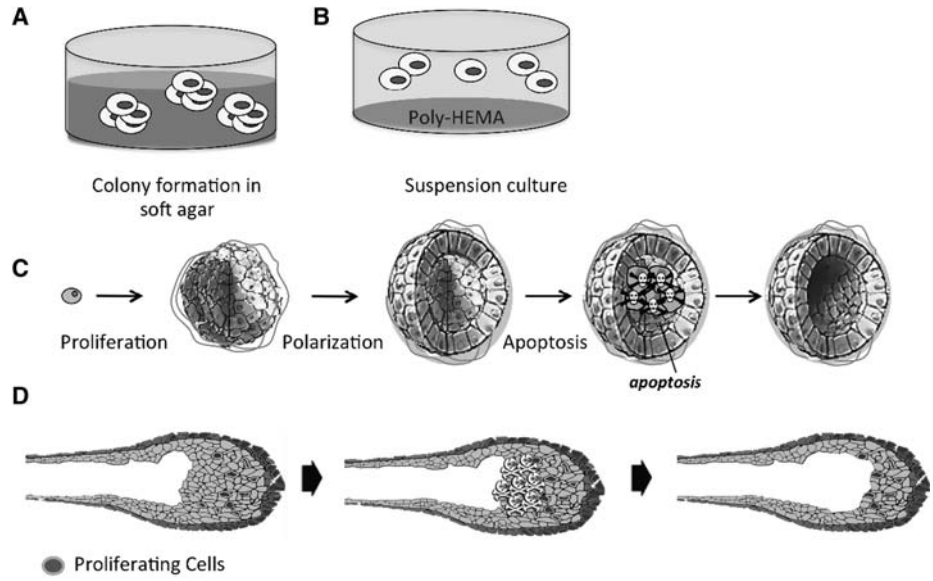

Figure 2. Models to study anchorage independence. (*A*) Colony formation in soft agar assay. (*B*) Suspension culture with poly-HEMA-coated tissue culture plates. (*C*) Morphogenesis of MCF-10A cells in 3D culture. Cells undergo an initial period of proliferation, followed by growth arrest, and death of the inner, extracellular matrix (ECM)–deprived cells. (Adapted, with permission, from Debnath and Brugge 2005.) (*D*) Processes associated with lumen formation during pubertal expansion of mouse mammary gland. Proliferation at the tips of the growing ducts leads to the formation of bulbous structures. Cells that lack matrix contact are cleared by apoptosis, leading to the formation of a hollow lumen. (Adapted, with permission, from Mailleux et al. 2008.)

of loss of matrix or the contribution of specific genes or pathways to anchorage independence. The suspension assay is particularly well suited for biochemical studies that are difficult to perform on cells embedded in soft agar or in vivo.

Three-dimensional cell culture systems provide a more physiologically relevant in vitro system for studying matrix regulation of cell survival and growth (Debnath et al. 2002; Nelson and Bissell 2005). Our laboratory has extensively utilized 3D cultures of the immortalized, nontumorigenic mammary epithelial cell line, MCF-10A (Soule et al. 1990; Debnath et al. 2003). When grown in 3D culture in the presence of reconstituted basement membrane, these cells undergo a spatially and temporally regulated morphogenesis program (Debnath et al. 2002). Upon seeding, MCF-10A cells proliferate to form small cell masses. Shortly thereafter, the structures undergo growth arrest and develop an axis of apicobasal polarity associated with basal secretion of matrix components and apical orientation of the Golgi. Interestingly, the inner cells fail to secrete matrix after the outer cells polarize. The matrix-deprived inner cells of the structures subsequently become unresponsive to proliferative signals and a lumen is formed by cavitation, involving the removal of centrally localized cells via apoptosis (Figs. 1 and 2). Cells that proliferate aberrantly after lumen formation undergo cell death if they populate the matrix-deprived lumen. Thus, the MCF-10A 3D tissue culture model facilitates analysis of cellular responses to deprivation of ECM in a context that better recapitulates aspects of glandular architecture that are relevant for early-stage tumorigenesis.

One in vivo model of lumen formation is the developmental process that takes place during expansion of the murine mammary gland associated with pubertal outgrowth (Fig. 2D; Mailleux et al. 2007). Proliferation of the terminal end buds (TEBs) at the tips of the growing ducts leads to the formation of bulbous structures that over time hollow out to form a collection of tubes and ducts that serve to store and transport milk. The inner cells of the TEB are cleared by an apoptotic death, creating a hollow lumen (Mailleux et al. 2008). Whereas the surviving outer cells of the TEBs are surrounded by a laminin-rich basal lamina, no laminin is detected in the inner cell mass that undergoes apoptosis. The inner cells also fail to secrete fibronectin or collagen IV. As such, lumen formation in the bulbous TEBs resembles aspects of the lumen formation in the 3D MCF-10A model. Thus, we speculate that apoptosis of the inner TEB cells is at least in part due to lack of matrix attachment. Underscoring the importance of proper ECM attachment in mammary gland morphogenesis, blocking antibodies against β1 integrin or laminin, inducible knockout of β1 integrin, or expression of a dominant inhibitory β1 integrin prevents the formation of the TEBs and ductal network in vivo (Klinowska et al. 1999; Faraldo et al. 2000; Li et al. 2005).

Using these model systems, we and other investigators have characterized the consequences of ECM detachment as well as how oncogenes can promote anchorage independence (Fig. 3). Anoikis was first described by Steven Frisch and Martin Schwartz (Frisch and Screaton 2001; Nagaprashantha et al. 2011). Because of its importance in both normal developmental processes and pathological conditions such as cancer, it is the best-characterized

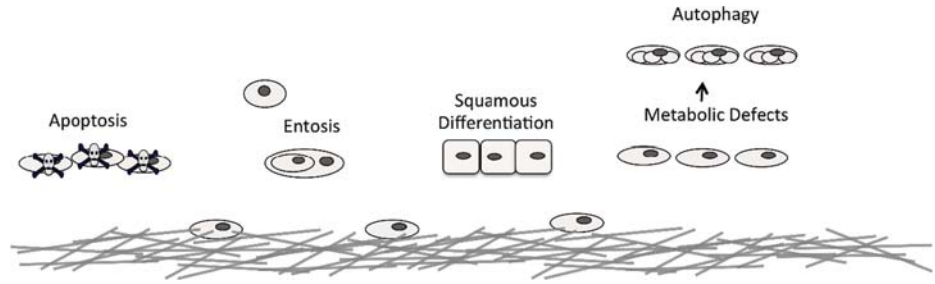

Figure 3. Phenotypes of ECM-detached mammary epithelial cells. Mammary epithelial cells in suspension can undergo apoptosis (anoikis), entosis (cell-in-cell invasion), squamous cell differentiation, autophagy, and metabolic defects.

consequence of ECM detachment (Meredith et al. 1993; Frisch and Francis 1994; Cabodi et al. 2010; Nagaprashantha et al. 2011). Importantly, however, other phenotypes of ECM-detached epithelial cells that can lead to cell death have also been described. One example is entosis, a nonapoptotic cell death characterized by cell-in-cell invasion induced by ECM detachment (Overholtzer et al. 2007). Cells that invade other cells are encased in a vacuole that is eventually eliminated following fusion of lysosomes with the vacuole membrane. This process can be tumor suppressive by eliminating matrix-detached cells; however, it may also contribute to tumorigenesis by promoting aneuloidy owing to failed cytokinesis of the "host" cell (Overholtzer et al. 2007; Krajcovic et al. 2011). Additionally, ECM-detached mammary epithelial cells undergo squamous differentiation both in vitro and in vivo (Mailleux et al. 2007).

Interestingly, we have found that inhibition of apoptosis is not sufficient to maintain the viability of epithelial cells without ECM attachment; matrix-deprived cells in suspension culture, 3D acini, and TEBs in vivo undergo a caspase-independent death after a significant delay (Fig. 4A). Under these conditions, the self-digestive process of autophagy is observed (Debnath et al. 2002; Fung

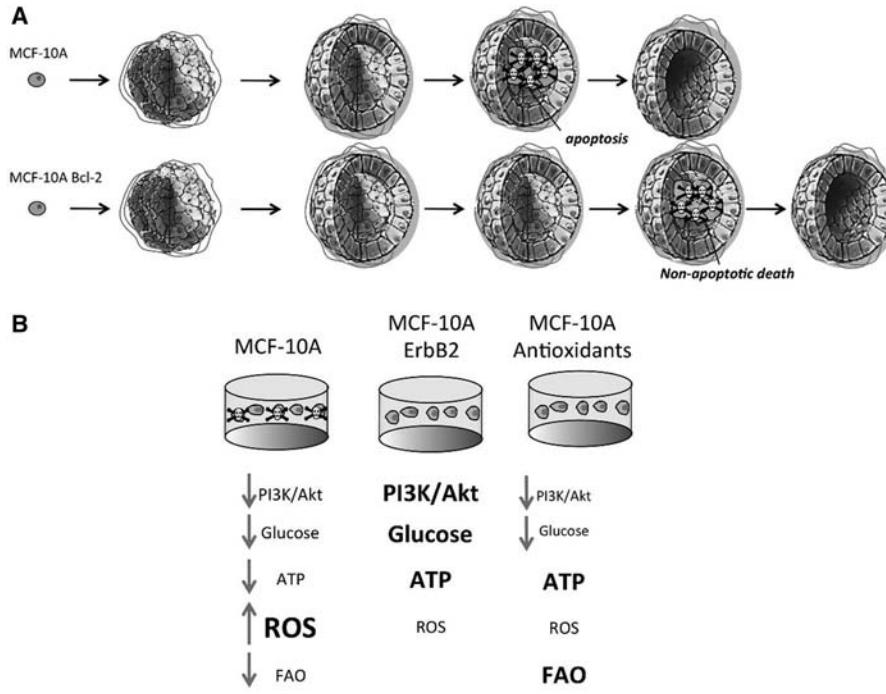

Figure 4. Nonapoptotic death and metabolic impairments of ECM-detached cells. (*A*) (*Top*) Control MCF-10A acini develop a hollow lumen owing to selective apoptosis of the inner, matrix-deprived cells. (*Bottom*) Inhibition of apoptosis delays but does not prevent the death of the inner cells, which are cleared by a caspase-independent mechanism. (*B*) Summary of metabolic defects of ECM-detached MCF-10A cells and rescue of the drop in ATP by ErbB2 overexpression or exogenous antioxidants. In suspended cells, loss of PI3K/Akt signaling leads to decreased glucose uptake, decreased ATP levels, and increased reactive oxygen species (ROS) levels. ErbB2 overexpression maintains PI3K signaling, promotes glucose uptake and ATP levels, and suppresses the increase in ROS. Addition of exogenous antioxidants neutralizes the increased ROS and prevents ROS-mediated inhibition of fatty acid oxidation. Arrows and text size indicate change relative to attached cells.

et al. 2008). Because autophagy is often induced as a response to metabolic stress, this suggests that matrix detachment compromises normal metabolic activity and has led us to analyze how metabolism in epithelial cells is altered after matrix detachment.

ECM REGULATION OF AUTOPHAGY

ECM attachment maintains low levels of autophagy, and matrix-detached mammary epithelial cells as well as the inner cells of the 3D structures display increased autophagy (Debnath et al. 2002; Lock and Debnath 2008). Up-regulation of autophagy is a stress response stimulated by hypoxia, decreased nutrients, and growth factor withdrawal; whereas excessive or unrestricted autophagy may lead to cell death, its primary role appears to be promotion of cell viability, especially under stress conditions (Jin and White 2007; White and DiPaola 2009). The prosurvival function of autophagy is probably due both to the degradation and removal of damaged proteins and organelles as well as the supplementation of oxidizable carbon sources during periods of metabolic crisis (Boya et al. 2005; Jin and White 2007). Although the role of autophagy appears to be highly context dependent, both in vitro and in vivo findings clearly indicate that autophagy can promote the survival of tumor cells (Jin and White 2007; Rosenfeldt and Ryan 2009; White and DiPaola 2009). Thus, although prolonged and excessive autophagy may promote cell death, regulated autophagy can function to increase cell viability.

Combined inhibition of apoptosis and autophagy is unable to prevent the death of ECM-detached cells, and consistent with the prosurvival function of autophagy, inhibition of autophagy accelerates ECM detachment-induced cell death and decreases replating ability (Fung et al. 2008). Autophagy is also important for the tumorigenic activity of oncogenes; for example, constitutively active Ras increases cell proliferation and survival of ECM-detached cells dependent on an induction of autophagy (Lock et al. 2011), and survival of Ras-transformed cells during nutrient starvation requires autophagy (Guo et al. 2011). Thus, under ECM-detached conditions, autophagy can promote cell viability.

ECM-DETACHED MAMMARY EPITHELIAL CELLS ARE METABOLICALLY COMPROMISED

The evidence that matrix-detached mammary epithelial cells die in the absence of apoptosis and significantly up-regulate autophagy led us to investigate how ECM modulates cellular metabolism (Schafer et al. 2009). When cultured in suspension, MCF-10A cells display a significant decrease in ATP levels, demonstrating that matrix detachment compromises cellular metabolism (Fig. 4B). Although biochemical analysis of metabolic pathways is not yet technically feasible in 3D culture, we took advantage of the native fluorescent properties of NAD(P)H to image the metabolic status of both inner and outer cells by two-photon microscopy. These studies indicated that the inner-matrix-deprived cells of the acinar cultures have compromised bioenergetics similar to cells grown in suspension. This data implied that ECM attachment regulates cellular metabolism in addition to proliferation and apoptosis. Furthermore, these data suggest that loss of matrix attachment in vivo might result in a metabolic defect that could prevent the survival of mammary epithelial cells even when apoptosis is inhibited.

Several recent studies have demonstrated that growth factor signaling, especially through the PI3K/Akt pathway, is an important positive regulator of nutrient uptake (Deberardinis et al. 2008; Vander Heiden et al. 2009; Levine and Puzio-Kuter 2010). Importantly, these pathways are often constitutively active in tumor cells and can promote growth factor independence (Rathmell et al. 2003; Engelman 2009). Matrix detachment of mammary epithelial cells reduces PI3K/Akt activity, resulting in decreased uptake of both glucose and glutamine and a decrease in flux through glycolysis, the pentose phosphate pathway (PPP), and the citric acid (TCA) cycle (Fig. 4B) (Schafer et al. 2009; Grassian et al. 2011b). Overexpression of oncogenes that maintain PI3K/Akt signaling after matrix detachment (e.g., ErbB2, activated PIK3CA, IGF-1 receptor, myristoylated Akt) also maintain nutrient uptake in a PI3K-dependent fashion (Schafer et al. 2009; ZT Schafer, AR Grassian, and JS Brugge, unpubl.). Consistent with the control of cellular energy levels by ECM attachment, the integrin-activated kinase, Fak, regulates the activity of mammalian target of rapamycin (mTOR), which is a key player in the regulation of cell metabolism, nutrient uptake, autophagy, and cell growth (Zoncu et al. 2011). Fak phosphorylates and inhibits tuberous sclerosis protein 2 (TSC2), a negative regulator of mTOR, leading to mTOR activation (Gan et al. 2006), and in vitro and in vivo inhibition of Fak decreases glucose uptake (Huang et al. 2006; Bisht et al. 2008). The matrix attachment and integrin regulation of critical signaling pathways known to control metabolism could help to ensure that cell proliferation occurs only in appropriate microenvironment conditions and could therefore function as a tumor suppressor.

REACTIVE OXYGEN SPECIES REGULATION OF FATTY ACID OXIDATION AND ATP PRODUCTION

In addition to ATP production, metabolism of both glucose and glutamine generates reducing equivalents that help to neutralize reactive oxygen species (ROS) (Deberardinis et al. 2008). Metabolic flux through the PPP, malic enzyme, and isocitrate dehydrogenase 1/2 produce NADPH, an important cellular reducing agent. Additionally, glutamine is also a component of the endogenous antioxidant glutathione. The decreased uptake of glucose and glutamine in the ECM-detached cells correlates with an increase in ROS levels as well as a decrease in the percentage of reduced glutathione (Schafer et al. 2009). Similarly, the inner cells of the TEB structures that are not cleared by apoptosis display an increase in oxidized

lipids and in expression of the ROS-responsive protein, psoriasin (Mailleux et al. 2007), and detachment or growth factor deprivation of MCF-10A cells also increases psoriasin levels (Enerback et al. 2002).

The increase in ROS and decrease in ATP levels after matrix detachment is prevented by either ErbB2 overexpression, which maintains glucose uptake and PPP flux, or addition of exogenous antioxidants, such as *N*-acetyl cysteine (NAC) or Trolox (a cell-permeable analog of vitamin E; Schafer et al. 2009). Interestingly, although antioxidants are able to maintain ATP levels in the matrix-detached cells, antioxidants do not rescue the defect in glucose uptake (Fig. 4B). Previous studies had shown that fatty acid oxidation (FAO) can compensate for glucose metabolism and maintain ATP levels under conditions of glucose deprivation (Buzzai et al. 2005). In the matrix-detached mammary epithelial cells, exogenous antioxidants prevent an ROS-mediated inhibition of FAO that allows the use of fatty acids as an alternative energy source under conditions of low glucose uptake (Schafer et al. 2009). Interestingly, ROS inhibition of peroxisomal FAO has been previously observed; the initial, rate-limiting step of peroxisomal FAO is fatty acyl coenzyme A (CoA) oxidase, which produces hydrogen peroxide as a feedback mechanism to suppress its own activity (Hashimoto and Hayashi 1987; Gulati et al. 1993; Sheikh et al. 1998).

In addition to increasing ATP levels, antioxidants also promote survival in ECM-detached conditions (Schafer et al. 2009). Antioxidant treatment of ErbB2-transformed cancer cells leads to increased growth in soft agar, and a greater percentage of the MCF-10A acini grown in 3D remains filled (indicative of increased survival of the inner, matrix-deprived cells). Interestingly, this data suggests that antioxidants, through maintenance of FAO and increased ATP production, might promote the survival of tumor cells that do not have constitutive nutrient uptake. Correlating with this, breast-cancer-derived brain metastases have increased expression of enzymes involved in the PPP and glutathione synthesis, both of which function to neutralize ROS (Chen et al. 2007). Additionally, expression of two enzymatic antioxidants, superoxide dismutase 2 (SOD2) and thioredoxin (TXN), shows increasing expression with an increased grade of breast cancer (Fig. 5).

MODULATION OF PDH FLUX BY ECM AND ERK1/2

Although matrix detachment results in an overall decrease in central carbon flux (i.e., glycolysis, PPP, and TCA cycle), there is a disproportionate drop in flux through PDH (Fig. 6A) (Grassian et al. 2011b). PDH regulates the entry of glucose carbons into the TCA cycle; this is a critical node in the regulation of glucose metabolism, because only by entering the TCA cycle can these carbons be utilized for certain metabolic biosynthetic processes such as lipogenesis and nucleotide synthesis. As predicted by a reduction in PDH flux, the percentage of glucose carbons that are secreted from the cell without entering the TCA cycle (i.e., lactate, pyruvate, and

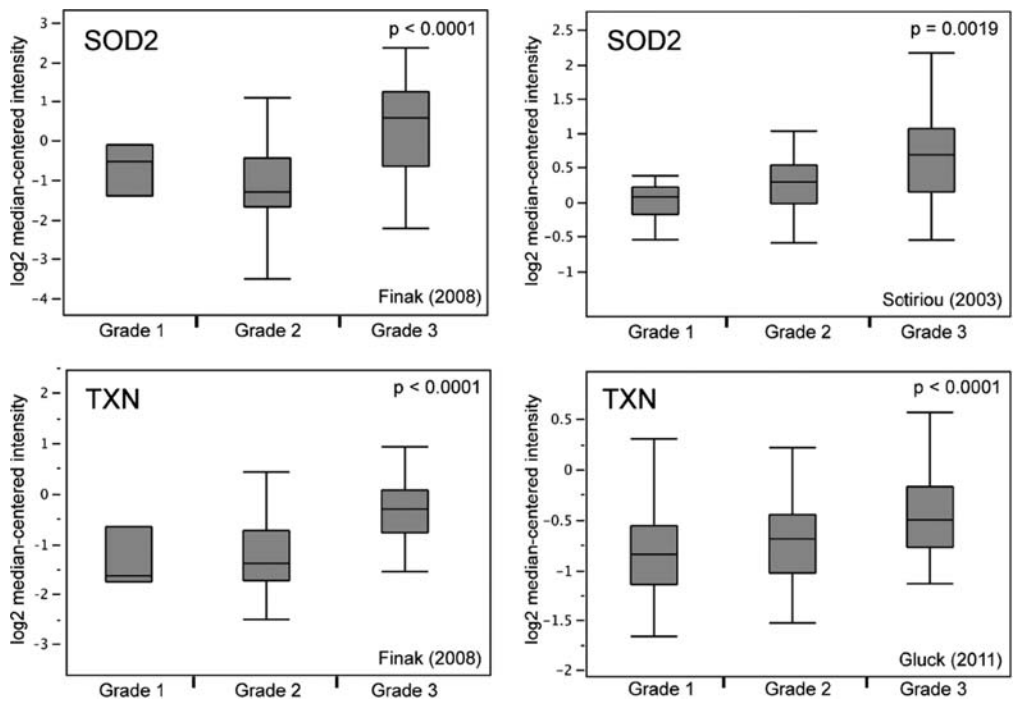

Figure 5. Correlation of tumor grade with expression of enzymatic antioxidants SOD2 and thioredoxin (TXN). Oncomine data mining reveals an increase in expression of SOD2 and TXN (Sotiriou et al. 2003; Finak et al. 2008; Gluck et al. 2011).

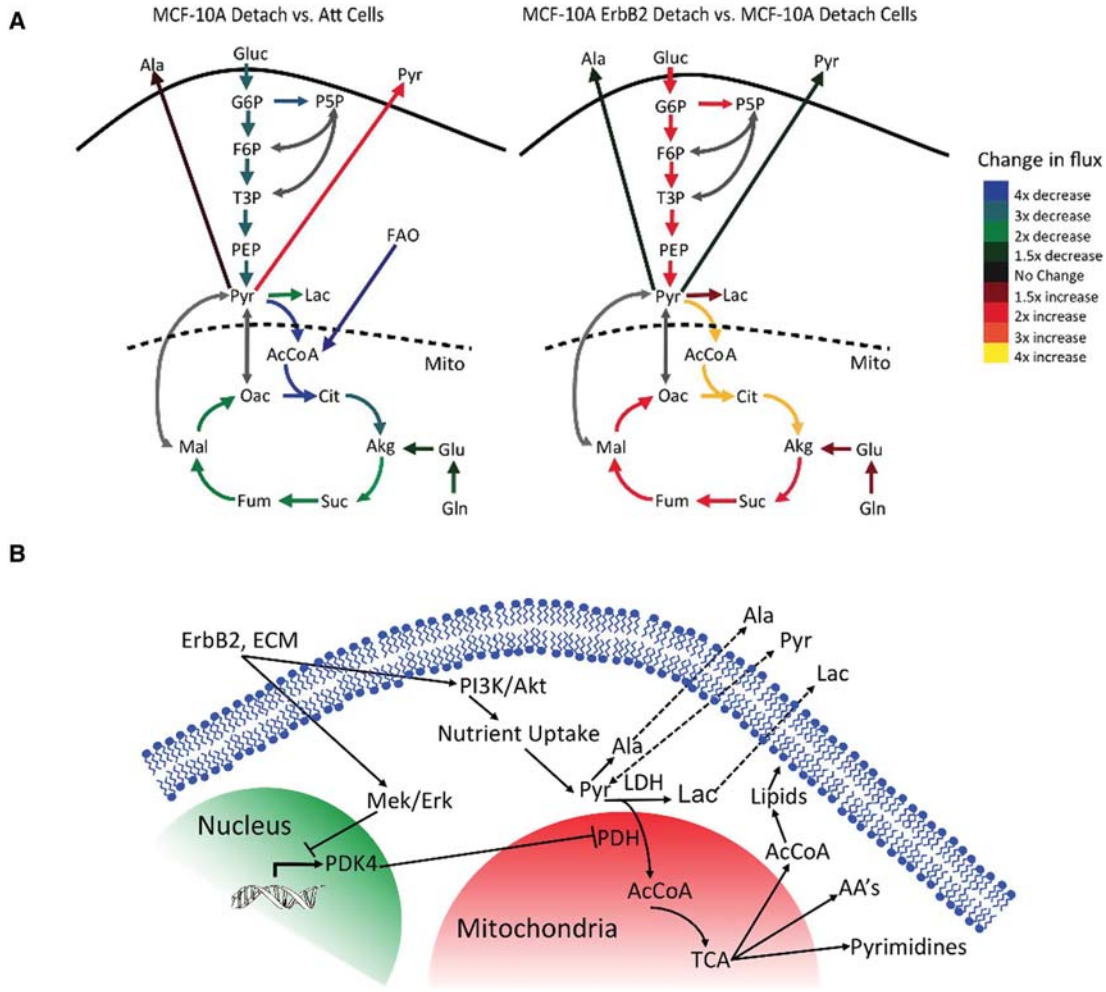

Figure 6. ECM attachment and Erk regulation of lipogenesis and proliferation via increased PDH flux. (*A*) Schematic representation of the changes in flux owing to ECM detachment of MCF-10A cells (*left*) and ErbB2 overexpression in the ECM-detached MCF-10A cells (*right*). The colors of the arrows represent the fold differences in MCF-10A detached versus attached (*left*) and in MCF-10A ErbB2 detached versus MCF-10A detached (*right*). Gray arrows represent fluxes not determined. Flux measurements were determined via metabolic flux analysis, mass isotopomer distribution data (for oxidative PPP), ^{14}C fatty acid oxidation assays, or measurements of extracellular fluxes. Color indicates fold change of flux. (*B*) Model for Erk and ECM regulation of PDH flux. Erk signaling decreases PDK4 expression, thereby preventing an inhibition of PDH flux. Increased PDK4 expression results in decreased PDH and TCA flux, leading to decreased de novo lipogenesis and proliferation. AA, Amino acid; AcCoA, acetyl coenzyme A; Akg, α-ketoglutarate; Ala, alanine; Asp, aspartate; Cit, citrate; FAO, fatty acid oxidation; F6P, fructose-6-phosphate; Fum, fumarate; Gluc, glucose; Glu, glutamate; G6P, glucose-6-phosphate; Gln, glutamine; Lac, lactate; Mal, malate; Oac, oxaloacetate; P5P, pentose-5-phosphate; PDK, PDH kinase; PEP, phosphoenolpyruvate; Pyr, pyruvate; Suc, succinate; T3P, triose phosphate.

alanine) is increased in matrix-detached cells, owing in part to the inhibition of PDH flux. Complete oxidation of glucose by the TCA cycle generates up to 36 mol of ATP per mol of glucose, whereas formation of lactate (via lactate dehydrogenase) generates only 2 mol of ATP; thus, the ECM-detached cells do not generate maximum ATP levels from glucose metabolism.

The activity of PDH is tightly regulated by PDH kinases (PDKs) 1–4, which phosphorylate and inactivate the catalytic subunit of PDH (Sugden and Holness 2006). Interestingly, mammary epithelial cells grown in suspension display a dramatic increase in PDK4 expression, which is at least partially responsible for the decrease in PDH flux (Grassian et al. 2011b). Unlike nutrient uptake, which is primarily controlled by the PI3K/Akt pathway, PDK4 expression and PDH flux are downstream from growth factor/matrix signaling through the Mek/Erk pathway. Accordingly, matrix detachment results in a dramatic increase in PDK4, and downregulation of PDK4 significantly increases PDH flux, indicating that the increased PDK4 expression inhibits PDH flux (Fig. 6B). As observed with ErbB2 rescue of PI3K signaling and glucose uptake in ECM-detached cells, overexpression of ErbB2 in the detached cells maintains Erk activity, prevents the induction of PDK4, and rescues the suppression of PDH flux (Grassian et al.

2011b). Therefore, ECM controls nutrient uptake through PI3K/Akt and intracellular nutrient utilization through Mek/Erk, thereby regulating both the amount as well as the fate of extracellular nutrient sources to best coordinate proliferative metabolism.

PDK4: SUPPRESSION OF PDH FLUX DECREASES LIPOGENESIS AND PROLIFERATION

In 1924, Otto Warburg noted that cancer cells produce large amounts of lactate regardless of oxygen availability—the "Warburg effect." Recent cancer metabolic research has, justifiably, been strongly focused on understanding how cancer cells utilize aerobic glycolysis (the Warburg effect) to meet their biosynthetic demands, respond to stress, and survive (Koppenol et al. 2011). It is important to note, however, that mitochondrial metabolism and the TCA cycle are critical for the production of biosynthetic intermediates that are required for de novo lipogenesis and amino acid and pyrimidine synthesis, and, under some conditions, mitochondria are key sites of ATP production. This raises the possibility that the Warburg effect is induced in cancer cells to meet their specific metabolic needs, rather than to specifically divert glucose-derived carbons away from the mitochondria. The increased glucose uptake in tumor cells is important for generation of glycolytic intermediates that are critical for biosynthetic pathways in proliferating cells (Tian et al. 1998; Bensaad et al. 2006; Christofk et al. 2008a,b; Vander Heiden et al. 2009, 2010; Koppenol et al. 2011; Locasale et al. 2011; Possemato et al. 2011) and for PPP intermediates necessary for generation of reducing equivalents and biosynthetic intermediates. Thus, maintaining precise control of PDH flux is critical to balance the use of glucose carbons for glycolytic and PPP metabolic pathways as well as the TCA cycle and oxidative phoshorylation. As supporting evidence of this hypothesis, we have shown that ectopic overexpression of PDK4 in the MCF-10A and MCF-10A ErbB2 cells diverts pyruvate away from the TCA cycle, decreases ATP levels in the detached ErbB2 cells, and decreases de novo lipogenesis in the attached cells (Fig. 6B) (Grassian et al. 2011b). The decrease in lipogenesis is probably partially responsible for a decrease in proliferation that is observed with PDK4 overexpression and highlights the importance of TCA metabolism for cell growth.

PDK4 may function as a growth suppressor through its ability to decrease TCA cycle flux and lipogenesis. We have observed that elevated PDK4 associates with decreased proliferation in several other systems. For example, during the morphogenesis of MCF-10A 3D structures, expression of PDK4 correlates with growth arrest and with an increase in expression of the cell cycle inhibitor, p57 (Fig. 7A). Additionally, PDK4 levels are decreased in a wide variety of tumor samples relative to normal tissue controls (Fig. 7B) (Grassian et al. 2011b), suggesting that PDK4, by inhibiting PDH flux, de novo lipogenesis, and cell proliferation, may function as a tumor suppressor in some situations and must be reduced to promote rapid proliferation. Recent data also shows that pyruvate entry into the TCA cycle via pyruvate carboxylase is required for survival and proliferation under conditions of low glutamine (Cheng et al. 2011), further highlighting the importance of mitochondrial metabolism in cancer.

Our data suggest that, in addition to aerobic glycolysis, maintenance of an optimal level of PDH flux may also be required for proliferation. Accordingly, there may be a selection against PDK4 expression during tumorigenesis to maintain the proper level of PDH flux to optimally balance the use of glucose carbons. Thus, whereas the levels of secreted lactate are elevated in cancer cells, this may reflect the increase in glucose uptake as well as the need for cofactor (NAD^+) regeneration rather than inhibition of PDH flux. Several studies have shown that PDH flux is maintained in tumor cells (Forbes et al. 2006; DeBerardinis et al. 2007; DeBerardinis 2008) and glucose-derived carbons that enter the TCA cycle via PDH flux are the primary source of acetyl-CoA for lipogenesis in tumor cells (Hatzivassiliou et al. 2005). However, there are circumstances under which PDH flux may be detrimental to cell growth and viability. For example, cells in solid tumors often need to survive in hypoxic environments, and under these conditions, cells would not have access to sufficient oxygen to operate the electron transport chain that is necessary for cofactor regeneration and TCA metabolism. Therefore, fine-tuning of PDH flux is required in varying cellular conditions, and control of PDK4 expression through extracellular cues via the Mek/Erk pathway allows remodeling of intracellular metabolism to optimally match cellular needs. Although unrestricted PDH flux may limit cell viability, a complete absence of PDH flux could also compromise cell growth.

CONCLUSION

Despite extensive research and numerous clinical trials, cancer remains the second leading cause of death in the U.S. Many new treatments for cancer are aimed at identifying and targeting the specific genetic aberrations unique to subsets of cancer patients. Whereas this strategy holds great hope, properties that are common to multiple tumor types are also attractive therapeutic targets that may be useful as either single agents or in combination with targeted therapies. Because survival in the absence of appropriate matrix environments is probably important for most types of epithelial tumors, including the most lethal metastatic tumors, understanding how and what proteins and pathways are required for tumor cell survival may provide clues on how to better eliminate them pharmacologically.

The findings discussed in this chapter have important implications for our understanding of anchorage independence and tumorigenesis. In early stages of tumorigenesis,

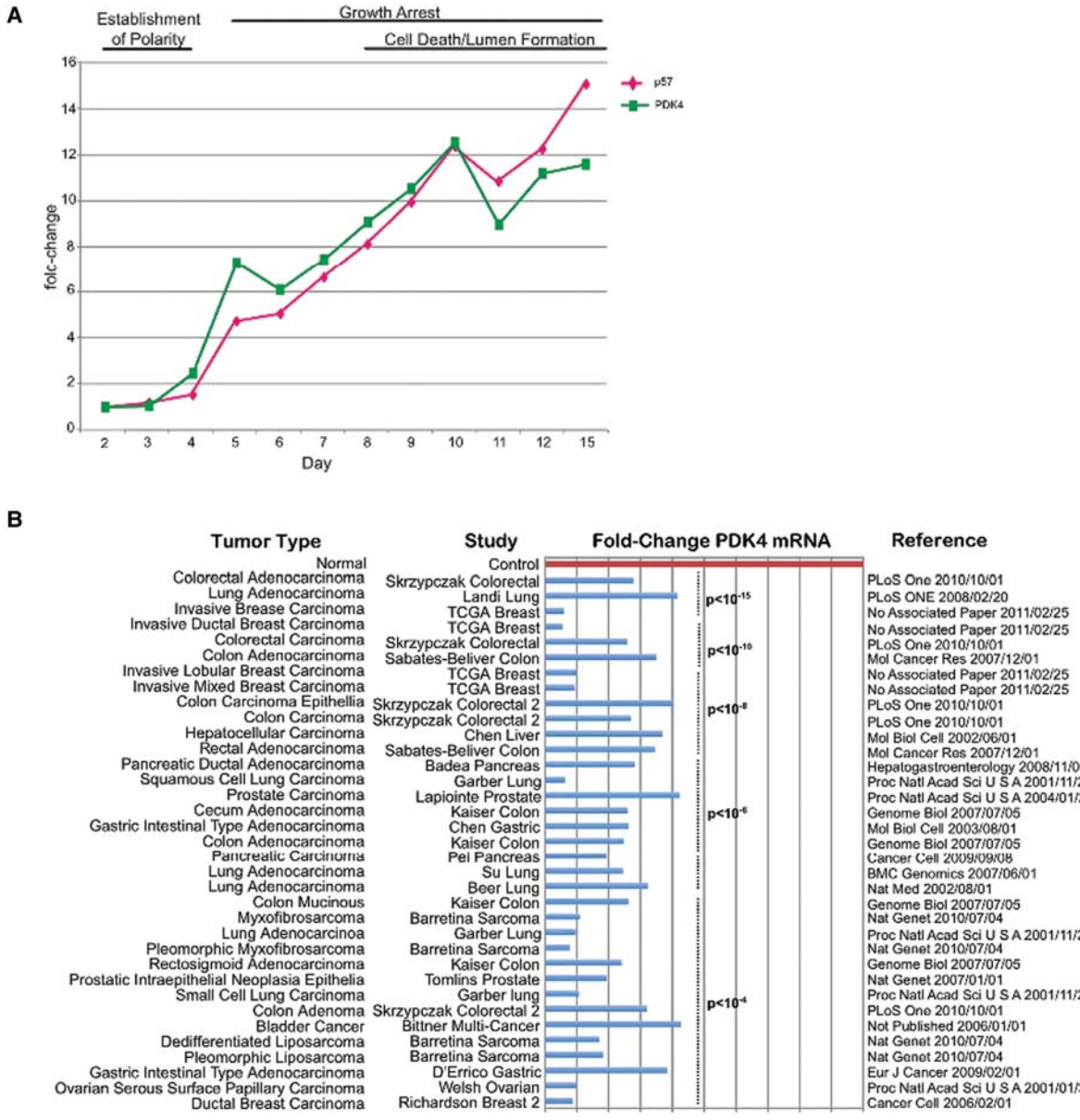

Figure 7. PDK4 expression increases during growth arrest of MCF-10A acini and is decreased in many tumor types. (*A*) Correlating with the decreased proliferation rate, expression of the cell cycle inhibitor p57 and PDK4 increase in expression throughout MCF-10A acinar morphogenesis. (Adapted, with permission, from Grassian et al. 2011b.) (*B*) PDK4 mRNA levels are decreased in a wide variety of tumors. Data are represented as the fold change in PDK4 mRNA in tumor versus their tissue of origin. (Data from oncomine.)

hyperproliferation forces cells to proliferate outside of their normal ECM environment. Data from our laboratory suggest that acquisition of resistance to apoptosis will not allow for long-term survival without appropriate ECM attachment owing to the compromised metabolic activity; thus, epithelial tumor cells must acquire resistance to apoptosis as well as maintain energy levels to survive without matrix attachment (Fig. 8). These findings identify multiple mechanisms by which tumor cells could maintain sufficient metabolic activity both in ECM-detached conditions and throughout tumorigenesis. First, oncogenes that promote constitutive growth factor signaling could sustain nutrient uptake and thus provide carbon sources for ATP production. Second, an increase in antioxidant capacity would allow for FAO to provide ATP in the absence of sufficient quantities of other oxidizable carbon sources. Third, alterations to intracellular carbon metabolism, such as continued PDH flux, could contribute to production of ATP and biosynthetic precursors. As such, ECM attachment provides multiple prosurvival

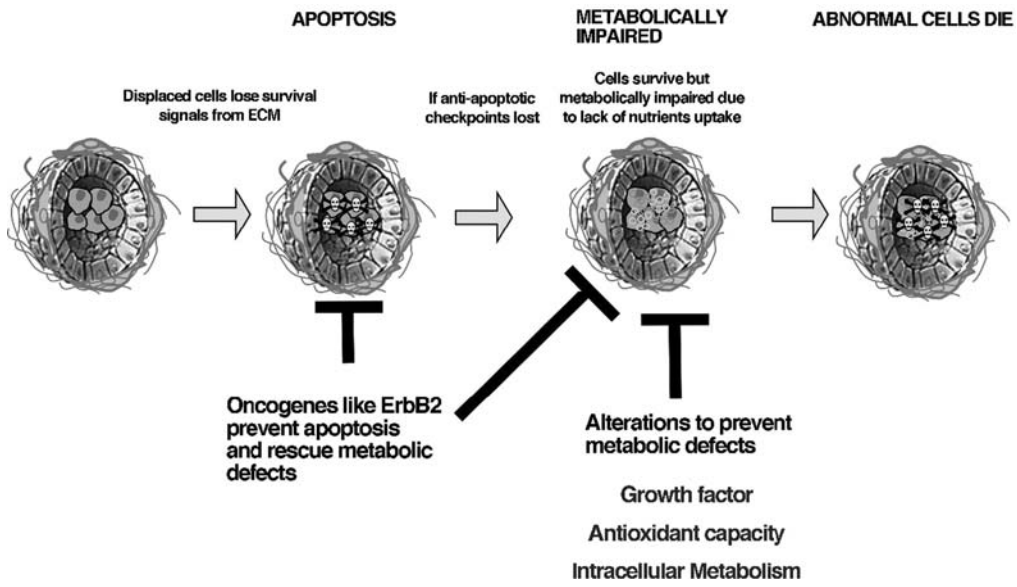

Figure 8. Implications for tumorigenesis and anchorage independence. Normal epithelial cells undergo apoptosis if deprived of matrix contact; however, cells that are resistant to apoptosis are still cleared owing in part to metabolic defects. This indicates that, in order for epithelial tumor cells to acquire anchorage independence, they must develop both resistance to apoptosis as well as maintenance of ATP levels after matrix detachment. The results discussed in this chapter outline three potential mechanisms for prevention of the metabolic defect: ECM-independent growth factor signaling, increased antioxidant capacity, and alterations in cellular metabolism (e.g., increased pyruvate dehydrogenase flux).

signals, including critical controls of cellular metabolic function.

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