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Review

The regulation of cancer cell death and metabolism by extracellular matrix attachment

Cassandra L. Buchheit[1], Raju R. Rayavarapu[1], Zachary T. Schafer*

Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556, United States

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ABSTRACT

The metastasis of cancer cells to distant sites is responsible for the vast majority of cancer mortalities yet the molecular mechanisms underlying this extraordinarily complicated process have yet to be sufficiently elucidated. Recently, it has become clear that cancer cells need to inhibit anoikis, a cell death program induced by loss of attachment to the extracellular matrix (ECM), in order to successfully metastasize. These studies have motivated additional research into the relationship between ECM-detachment and cell viability, much of which reveals integral connections between ECM-detachment and cell metabolism. This review serves to thoroughly discuss the signaling pathways and metabolic changes that are induced by ECM-detachment. In addition, the molecular mechanisms by which cancer cells can alter signaling and metabolism to survive in the absence of ECM-attachment will be highlighted. Furthermore, cell death mechanisms that have been observed or implicated in cells detached from the ECM will also be examined. In aggregate, the studies discussed in this review reveal that ECM-detachment can regulate cancer cell metabolism in a variety of distinct cell types and suggest that interfering with metabolism in ECM-detached cells may be a novel and effective chemotherapeutic approach to selectively inhibit tumor progression.

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Abbreviations: ECM, extra cellular matrix; ROS, reactive oxygen species; PPP, pentose phosphate pathway; TCA, tricarboxylic acid; PDH, pyruvate dehydrogenase; PDK4, pyruvate dehydrogenase kinase 4; ANGPTL4, angiopoietin-like 4; PK, pyruvate kinase; TIGAR, TP53-induced glycolysis and apoptosis regulator; AMPK, adenosine monophosphate-activated protein kinase; mTORC1, mammalian target of rapamycin complex 1; HIF-1α, hypoxia-inducible factor 1 alpha; ATG, autophagy-related proteins; ULK-1, unc-51-like kinase 1; ER, endoplasmic reticulum; ROCK, Rho associated protein kinase; TNFα, tumor necrosis factor alpha; Rip1/3, receptor interacting protein 1/3; RHIM, rip homotypic interaction motif; BMF, Bcl-2 modifying factor; PYGL, glycogen phosphorylase; GLUL, glutamate–ammonia ligase; GLUD1, glutamate dehydrogenase 1; MLKL, mixed lineage kinase domain-like; PI(3)K, phosphatidylinositol 3 kinase; PH, pleckstrin homology; SGLT1, sodium/glucose cotransporter molecule 1; BDNF, brain derived neurotrophic factor.

* Corresponding author at: Cancer Biology, Department of Biological Sciences, University of Notre Dame, 222 Galvin Life Science Center, Notre Dame, IN 46556, United States. Tel.: +1 574 631 0875; fax: +1 574 631 7413.
E-mail address: zschafe1@nd.edu (Z.T. Schafer).

[1] These authors contributed equally to this work.

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1. Introduction

The metastasis of cancerous cells to distant and vital organs is responsible for approximately 90% of cancer mortalities [1]. Given this extraordinarily high mortality rate, there is clearly a substantial need for the development of novel therapeutic approaches that eliminate metastatic cancer cells. Traditional chemotherapeutic approaches lack the specificity and potency to successfully target metastatic disease, raising the prospect that therapies targeting unique molecular properties of metastatic cancer cells may ultimately be the most successful approach to lowering cancer mortality rates. Unfortunately, the molecular characterization of metastatic cancer cells and of the metastatic process remains poorly understood, thus precluding the development of these types of therapeutic approaches. It is widely appreciated that the successful metastasis of cancer cells is comprised eight distinct steps: (1) primary tumor formation, (2) localized invasion, (3) intravasation, (4) transport through circulation, (5) arrest in capillaries of distant organs, (6) extravasation, (7) formation of micrometastases, and (8) formation of macrometastases (Fig. 1) [2,3]. While more recent studies have begun to unmask some of the elusive mechanisms that modulate the early steps of the metastatic cascade (i.e. the facilitation of cancer cell invasion by epithelial–mesenchymal transition [4] and matrix metalloproteinases [5], signals from the tumor microenvironment [6], etc.), the understanding of the later stages of the metastatic cascade remains rudimentary. A better understanding of these processes could allow for the discovery and development of novel therapeutics.

More recently, it has become apparent that in order to successfully metastasize, cancer cells need to inhibit anoikis, a cell death program induced by detachment from the extracellular matrix (ECM) [7–10]. Anoikis induction is not limited to ECM-detached cells as even the attachment to abnormal or foreign ECM can induce cell death [11]. Thus, during metastasis, cancer cells may need to resist anoikis at *every* step of the metastatic cascade, from local invasion and survival in the circulation to implantation at a secondary site (see Fig. 1). While significant advances in our understanding of the molecular regulation of anoikis have recently been achieved, it has also become abundantly clear that ECM-detachment can induce a wide variety of cellular changes that can ultimately lead to cell death that are independent of classical anoikis induction. For example, a novel cell death mechanism known as entosis (discussed in detail below) was recently discovered in ECM-detached mammary epithelial cells [12]. In addition, ECM-detached cells undergo autophagy [13], which involves the catabolism of intracellular organelles and can serve as both a survival mechanism under conditions of nutrient starvation [14] or an alternate form of cell death if left unchecked [15]. The significance of these findings to the survival of cancer cells during the metastatic cascade remains unclear, but these data do reveal that ECM-detachment can induce a wide variety of cellular alterations independently of anoikis induction.

In addition to the above-described ECM-detachment induced changes, it has become clear that the maintenance of attachment to the ECM can actively regulate cell metabolism [16]. The discovery of these metabolic changes has important implications in the regulation of cancer cell viability and may potentially impact the ability of cancer cells to successfully metastasize. In addition, many of the signaling pathways that are involved in the metabolic reprogramming of cancer cells [17] are also actively regulated by ECM-detachment suggesting that ECM-detachment may modulate cellular metabolism in several ways. This review will discuss the recent findings implicating ECM-detachment in metabolic control and will analyze the importance of ECM-detachment-induced signaling pathways in the regulation of cell metabolism.

2. The role of antioxidant activity in evasion of anoikis

2.1. ErbB2-mediated metabolic changes that affect cell survival

Growing evidence suggests that cancer cells rely on antioxidant activity to maintain low levels of intracellular reactive oxygen species (ROS) and to survive when detached from the ECM. A number of oncogenes have been implicated in regulating antioxidant activity within the cell in order to allow cancer cells to overcome anoikis [16,18]. More specifically, detached mammary epithelial cells were shown to have diminished ATP levels due to defective glucose transport (summarized in Fig. 2). However, ErbB2 overexpression can rescue detachment-induced deficiencies in ATP by restoring glucose uptake. This sustained ATP production during ECM-detachment is mediated by EGFR stabilization and PI(3)K activation and is dependent on metabolic flux through the pentose phosphate pathway (PPP). The PPP generates NADPH which facilitates glutathione-mediated antioxidant activity and was found to be essential for luminal filling of mammary acini. Interestingly, stimulation of antioxidant activity rescues intracellular ATP levels in ECM-detached cells via stimulation of fatty acid oxidation (FAO) and FAO is inhibited by detachment-mediated production of ROS [16]. In aggregate, these studies suggest that ECM-detached cells must regulate detachment-induced ROS levels for survival and that detachment-regulated intracellular metabolic pathways can contribute to the modulation of ROS levels.

Furthermore, ErbB2 overexpression can rescue a variety of ECM-detachment-induced metabolic defects, including decreased uptake of glucose, glutamine, and pyruvate. These decreases in nutrient uptake dramatically alter flux through glycolysis, the PPP, and the tricarboxylic acid (TCA) cycle but surprisingly, a disproportionate loss in flux through pyruvate dehydrogenase (PDH) is also observed [19]. The diminished flux via PDH is accompanied by an increase in the expression of the PDH inhibitor, PDH kinase 4 (PDK4). When examining how PDK4 was regulated by signaling from ErbB2, it was found that signaling through the ERK–MAPK pathway was able to inhibit PDK4 expression and stabilize flux via PDH. Interestingly, overexpression of PDK4 in ErbB2 expressing cells is able to negate ErbB2-mediated flux via PDH. Of note, human tumor samples are found to frequently have diminished PDK4 mRNA expression when compared to their non-tumorigenic tissue of origin [19]. Intriguingly, another study has implicated PDK4 in the evasion of cell death in ECM-detached cells through the attenuation of mitochondrial flux and subsequent decrease in ROS [20]. These findings highlight PDK4 regulation as an important regulator of metabolism in ECM-detached cells and reveal novel mechanisms by which epithelial cells can regulate their intracellular metabolism to promote survival and growth when detached from the ECM.

2.2. Nrf2 and antioxidant enzymes

In addition to ErbB2, other oncogenes are known to be important for regulating intracellular ROS levels. In order to accomplish this, previous work suggests that cancer cells utilize a tightly controlled,

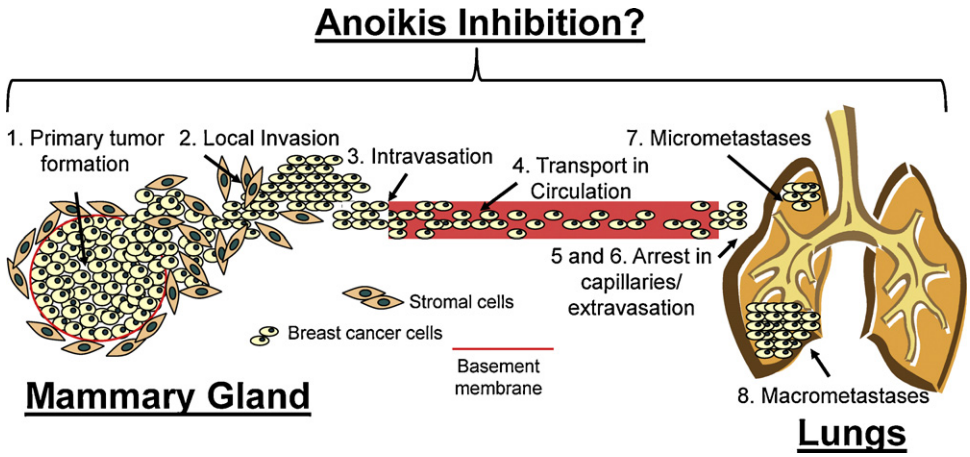

Fig. 1. Anoikis inhibition and metastasis. The eight steps of the metastasis are: (1) primary tumor formation, (2) local invasion, (3) intravasation, (4) transport in circulation, (5) arrest in the capillaries, (6) extravasation, (7) micrometastases and (8) macrometastases. Anoikis inhibition may be necessary for the completion of each of these 8 steps.

inducible antioxidant program that is regulated by the transcription factor Nrf2 [18]. Nrf2 is regulated by its repressor protein Keap1, which binds to the amino-terminal Neh2 domain of Nrf2 [21]. Keap1 knockout mice die post-natally, suggesting that tight regulation of the Nrf2 pathway is essential for viability [22]. When Nrf2 functions as a transcription factor, either by Keap1 deficiency or Nrf2 overexpression, it binds antioxidant responsive elements (AREs) in genes that are responsible for detoxification of ROS within the cell [23]. More recently, it was found that Nrf2 transcription can be induced by a number of oncogenes, including K-Ras, B-Raf, and Myc in primary murine cells [18]. Activation of Nrf2 by K-Ras can occur via the Raf–MEK–ERK–Jun pathway. This increase in Nrf2 transcription is able to sufficiently elevate the activity of the basal antioxidant program to lower ROS levels and facilitate tumorigenesis. Genetic targeting of the Nrf2 pathway in K-Ras overexpressing murine cells impairs proliferation and tumorigenicity *in vivo*. Furthermore, primary murine cells expressing oncogenic K-Ras and B-Raf and human pancreatic cancer patient samples both display oncogene-driven Nrf2 expression and enhanced ROS detoxification [18]. These data reveal an interesting mechanism that could allow ECM-detached cells to diminish ROS and facilitate their survival via oncogene driven expression of Nrf2. However, future experiments aimed at examining the importance of the Nrf2-mediated antioxidant program and other antioxidant enzymes will be critical to better understand how these data may be related to the survival of ECM-detached cells.

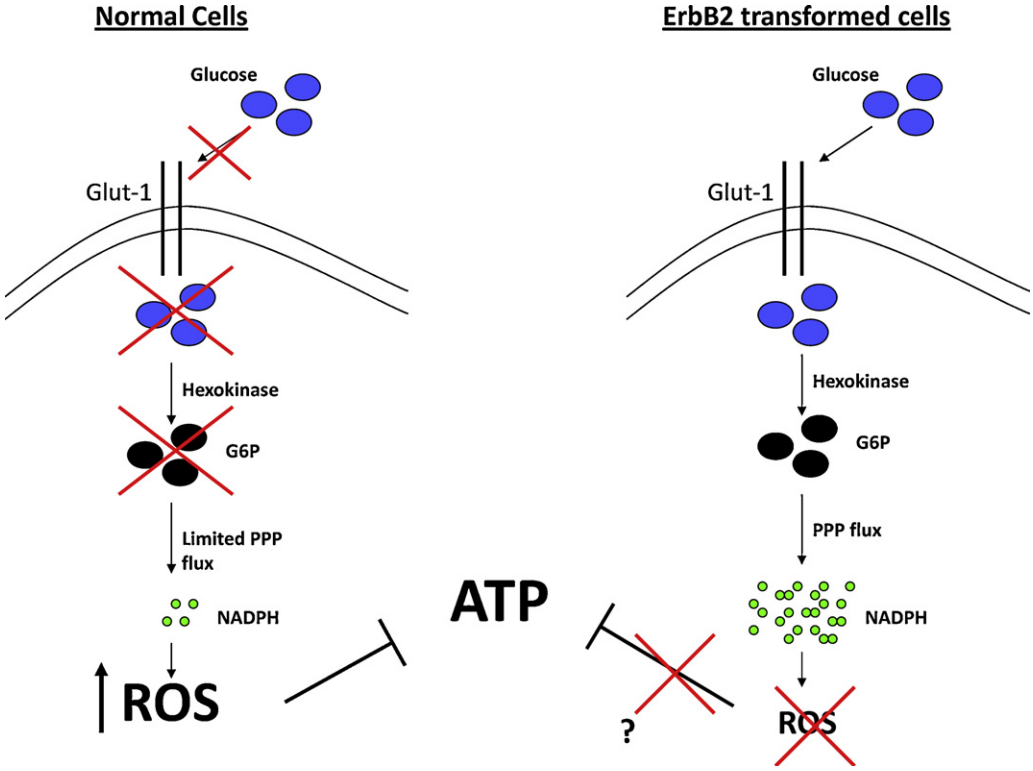

Fig. 2. The regulation of ATP levels in ECM-detached cells. In non-transformed, detached cells (normal cells), there is a defect in glucose uptake. This leads to low levels of glucose-6-phosphate (G6P) and limited flux through the PPP. The lack of PPP flux causes ROS levels to building up, which can subsequently inhibit ATP generation. When ErbB2 is overexpressed in these cells, the defect in glucose uptake is rescued. This leads to abundant G6P and enhanced flux through the PPP. ROS levels are subsequently lowered thus relieving the inhibition of ATP generation.

Although the aforementioned data suggests that cancer cells rely on antioxidant programs to mitigate ROS-mediated cell death, other studies have shown conflicting results. Recently, angiopoietin-like 4 (ANGPTL4) protein was discovered to interact with integrins to stimulate NADPH oxidase-dependent production of superoxide (O_2^-). A high ratio of O_2^-:H_2O_2 activates Src, which signals through the PI(3)K and MAPK–ERK pathways to promote the survival of ECM-detached cells and tumorigenicity [24]. This study suggests that high levels of O_2^-:H_2O_2 can be protective rather than detrimental to cancer cell survival. While on the surface, these results may seem to contradict the apparent necessity of ECM-detached tumor cells to diminish ROS, there are important differences between this work and the other studies discussed. For example, the aforementioned studies monitor total intracellular ROS levels while the ANGPTL4 study focuses on a ratio of one species of ROS to another. Additionally, mechanisms by which cells react to and regulate ROS are likely tissue-specific, and mechanisms by which cancer cells respond to ROS may be cell type dependent. Furthermore, it is possible that some of the effects of ANGPTL4 described in this study are due to regulation of cell death that is not related to ECM-detachment as anoikis assays were conducted after only 2 h of ECM-detachment.

2.3. Metabolic enzymes and ROS

While oncogenic signaling can reduce ROS levels to facilitate cell survival in ECM-detached cells, other fundamental changes in tumor cells have also been demonstrated to limit ROS production in a manner that could facilitate cell survival. The fact that tumor cells actively consume glucose and utilize glycolysis under aerobic conditions was first observed by Otto Warburg many years ago [25–27]. More recently, seminal work from Lewis Cantley's laboratory has revealed a particular isoform of pyruvate kinase (PK) that drives this phenomenon, which is now commonly known as "The Warburg Effect" [28,29]. PK is a tightly regulated metabolic enzyme instrumental in ATP production. To date, four isoenzymes of PK have been found. L-PK is expressed in tissues that undergo gluconeogenesis (i.e. the liver, kidney, and intestine) [30–32], and R-PK is expressed only in erythrocytes [33]. The other two isoenzymes, PKM1 and PKM2, are encoded by the same gene which undergoes alternative splicing to generate two products [34]. PKM1 is expressed primarily in tissues that require high energy production [35–37]. PKM2 is expressed in a number of different cell types including all proliferating cells, especially tumor cells [35]. During development, PKM2 is gradually replaced by PKM1, however, during tumorigenesis tissue specific isoenzymes are lost and PKM2 expression is regained [35,36,38,39].

In 2008, it was discovered that the M2 splice isoform of PK is *necessary* for the shift to aerobic glycolysis seen in tumor cells, and that this shift in cellular metabolism promotes tumorigenicity [28]. Knockdown of PKM2 and replacement with the M1 isoform reverses the Warburg effect and correlates with decreased tumor formation in nude mice. Interestingly, fibroblast growth factor receptor type 1 was found to negatively regulate PKM2 by direct phosphorylation. This phosphorylation event inhibits the formation of the active tetramer form of PKM2. Furthermore, phosphorylation at Y105 was commonly found in human cancers, and a mutant PKM2 that cannot be phosphorylated leads to decreased cell proliferation under hypoxic conditions, increased oxidative phosphorylation resulting in lower lactate levels, and a decreased ability to form tumors in nude mice [40]. This idea that lower enzymatic activity of PKM2 can promote tumorigenesis has been observed in multiple contexts [41,42].

Despite the observed necessity for PKM2 by tumor cells to regulate aerobic glycolysis, other groups have found PKM2 to be strictly regulated in tumor cells in an effort to monitor intracellular ROS levels. Gruning et al. find that metabolism is able to self-adapt to regulate redox processing when respiration is activated [43]. Low levels of PK activity lead to the accumulation of phophoenolpyruvate (PEP) which results in flux through the antioxidant-producing PPP. This flux is responsible for the ability of cells to utilize cellular respiration without the negative effects of increased ROS levels. Furthermore, PKM2 was recently shown to be enzymatically inhibited by acutely increasing intracellular ROS levels [44]. This PKM2 inhibition causes glucose flux to stall at the final step in glycolysis, leading to a backup of nutrients and ultimately forcing flux into the PPP. The generation of antioxidants via this mechanism allows for ROS detoxification in cancer cells. Notably, when inhibition of PKM2 is blocked in human lung cancer cells, enhanced sensitivity to oxidative stress and impaired tumor formation in mouse xenograft experiments is observed [44]. In aggregate, these data suggest that the expression of the M2 isoform of pyruvate kinase may function as another mechanism utilized by cancer cells to facilitate survival through the reduction of ECM-detachment-induced ROS.

Another metabolic enzyme, phophoglycerate dehydrogenase (PHGDH), has recently been shown to divert glycolytic flux to serine and glycine metabolism in some cancer cells [45]. PHGDH is amplified in a number of human cancers and required for cell proliferation. When PHGDH overexpressing cells are plated in a 3D cell culture model, luminal organization and polarity are substantially altered. In addition, when PHGDH is conditionally overexpressed in non-tumorigenic mammary epithelial cells (MCF-10A) and plated in 3D cell culture, PHGDH-expressing cells give rise to acinar structures that have filled lumens. Given that that the survival of cells in the luminal space is mediated by the inhibition of ECM-detachment-induced cell death, PHGDH likely plays an important role anoikis protection.

2.4. TIGAR and ROS

The utilization of the PPP to reduce ROS in cancer cells is not limited to the actions of PKM2 as other enzymes have been implicated in promoting flux through the PPP in a manner that would promote the survival of ECM-detached cancer cells. Foremost among these is TP53-induced glycolysis and apoptosis regulator (TIGAR) whose expression leads to decreased fructose-2,6-bisphosphate levels [46]. This inhibition blocks glycolysis and leads to increased antioxidant activity via the PPP. Cells expressing TIGAR mutants possess impaired anti-apoptotic activity, suggesting TIGAR could be an attractive target for chemotherapeutic intervention. In addition to this described role, TIGAR is also implicated in the inhibition of autophagy, which strongly correlates with its ability to diminish intracellular ROS levels. The implications of this autophagic inhibition on cell survival are unclear as autophagy can both promote cell survival and cell death depending on the duration for which it is active [47]. However, one could imagine that in ECM-detached cells, which have already been implicated to undergo autophagy [48], TIGAR could prevent ROS-mediated cell death while simultaneously limiting autophagy to ward off eventual autophagic cell death. Furthermore, there is a striking similarity in function between TIGAR and another p53 regulated family of proteins, Sestrins, which also decrease intracellular ROS levels [49,50]. Sestrins are proposed to be regulated in a similar fashion but the stimulation of antioxidant activity of TIGAR appears to be p53 independent and have no effects on the mammalian target of rapamycin (mTOR) pathway.

3. Utilizing the AMPK signaling pathway to evade anoikis

As described above, one way in which cells are able to escape anoikis is by minimizing the demand for cellular energy. This could

potentially be accomplished through the activation of the AMP-activated protein kinase (AMPK) energy–sensing pathway, which can regulate intracellular signaling in order to respond to a deficiency in ATP. For example, AMPK is important in the G1/S cell cycle checkpoint, and is responsible for cell cycle arrest in cells undergoing glucose deprivation. AMPK-dependent phosphorylation of p53 at serine 15 elicits a cell cycle arrest and contributes to increased cell survival even with low energy available to the cell [51]. Additionally, AMPK can phosphorylate histone H2B and directly control transcriptional and chromatin regulatory pathways to allow the cell to adapt to stressful conditions. Furthermore, ectopic expression of an H2B mutant in which the AMPK phosphorylation site is eliminated results in reduced transcription of AMPK-dependent genes and lower cell survival in stressful conditions [52].

With regards to anoikis, AMPK is activated in both transformed and non-transformed cells after detachment from the ECM [53]. However, AMPK can only suppress mTOR complex 1 (mTORC1) in transformed cells. This inhibition leads to anoikis protection through the suppression of protein synthesis. Furthermore, inhibiting signaling at either the AMPK or mTOR level leads to the induction of anoikis. Interestingly, the mTORC1 inhibition observed in ECM-detached cells relies on the expression of oncogenes such as K-Ras, which are normally responsible for pro-survival and growth signals via mTORC1 hyperactivation. Therefore, it appears that the regulation of mTORC1 by oncogenes is dependent on the microenvironment, and the inhibitory effects of stress on mTORC1 outweigh pro-growth signaling pathways in low energy states [53]. Additionally, this AMPK-mediated inhibition of mTOR has also been shown to be regulated by two p53 gene targets, Sestrins 1 and 2, which dimerize and subsequently phosphorylate TSC2. This results in GAP activation, suppression of mTOR signaling, and decreased translation, cell growth, and proliferation [50]. Together these studies display the importance of protein synthesis and AMPK signaling pathway in the survival of cells that are detached from the ECM.

4. The effects of hypoxia on anoikis resistance in cancer cells

In order for cancer cells to survive as they grow further from the existing vasculature, they must adapt to hypoxic conditions. Additionally, as solid tumors form, cells located in the interior of the mass and distant from blood vessels must alter cellular energetics in order to survive. Hypoxia-inducible factor alpha (HIF-1α) has long been implicated in the adaptation of cancer cells to hypoxic conditions, and high expression levels of HIF-1α correlate with a poor prognosis ([54,55]; see accompanying review by Shay and Simon). More recently, HIF-1α has been shown to be important for anoikis resistance by regulating the expression and activity of integrins at the cell surface [56]. Loss of HIF-1α in human gastric cancer cells resulted in increased transcription of α5 integrin alone. Interestingly, the increased transcription of α5 integrin is dependent on elevated intracellular superoxide, suggesting a link between ROS levels and HIF-1α regulation of α5 integrin transcription. Ultimately, this loss of HIF-1α results in the elimination of anoikis resistance and inhibition of anchorage-independent growth. These results suggest that the increased levels of hypoxia seen in tumors may be acting to suppress α5 integrin transcription and thereby allowing cells to survive while detached from ECM [56]. In contrast with these data, another study found that S100A4 (also known as calvasculin and previously shown to be overexpressed in cancers) plays an important role in anoikis resistance via regulation of integrin expression [57]. Knockdown of S100A4 significantly increased anoikis in gastric cancer cells and decreased levels of α5 and αv integrins.

Additional studies in mammary epithelial cells have shown that HIF-1α may be acting through the EGFR–MAPK–ERK pathway to suppress Bim and BMF, two members of the pro-apoptotic BH3-only family known to be involved in anoikis induction [58,59]. Utilizing 3D cell culture approaches that mimic the glandular structure of mammary acini, hypoxia was found to disrupt acinar organization, inhibit luminal clearing, and block Bim and Bmf expression during morphogenesis of MCF-10A cells [60]. Interestingly, these effects were observed to be irreversible as they were retained even after cells were subsequently exposed to normoxic conditions [61]. These studies suggest that hypoxia cannot only regulate expression of integrins that contribute to intracellular survival pathways, but also the expression of pro-apoptotic proteins in order to survive when detached from the ECM.

5. Alternative death pathways and ECM-detachment

As previously discussed, ECM-detached epithelial cells have a number of metabolic defects (i.e. decreased ATP levels and increased ROS levels) which eventually can lead to non-apoptotic cell death [16]. In addition to anoikis, there are a multitude of alternative non-apoptotic programs that could be playing a role in this context. Although the exact role these mechanisms play in cancer and other disease states has yet to be definitively determined, it is clear that these non-canonical cell death mechanisms are tightly regulated, and this regulation may be important for ECM-detached tumor cells to survive (see Fig. 3 for a summary).

5.1. Detachment-induced autophagy

Autophagy is the mechanism by which cells induce the lysosomal processing of their own machinery in order to respond to nutrient stress [62,63]. It is believed that autophagy may provide a temporary means to avoid cell death as a result of starvation due to ECM-detachment. During autophagy, cells form a double membraned vesicle (known as the autophagosome) within the cytoplasm that acts to package cellular proteins and organelles. The vesicles are then transported to the lysosome and catabolized. The degraded products are transported out of the lysosome such that the cell can utilize the degraded products in order to create new proteins.

Cells under duress due to ECM-detachment will undergo autophagy [64]. As discussed previously, this loss of integrin engagement and its associated signaling can result in the induction of anoikis signaling. However, it was found that the loss of β1 integrin is sufficient to promote the induction of autophagy [13]. Furthermore, in the presence of oncogenes or other tumor promoting insults, cells can activate autophagy in order to prolong cellular survival until angiogenesis or other nutrient rescue mechanisms are activated.

Autophagy is regulated within the cell by a family of proteins known as autophagy-related proteins (ATG) [48]. During ECM-detachment, the loss of ATG proteins results in a substantial increase in apoptosis [13,65]. This induction of autophagy has been strongly correlated with the activation of proteins that sense cellular metabolic stress. For example, it has been shown that AMPK can strongly regulate the induction of autophagy [47]. AMPK is activated by cellular levels of NAD+ and AMP [45], which indicate that the cell is undergoing substantial metabolic stress. This induces the activation of unc-51-like kinase 1 (ULK-1), a mammalian homologue of yeast ATG1 [66], which in turn, is able to mobilize the autophagy machinery. mTOR has been found to inactivate ULK-1 in order to prevent the induction of autophagy.

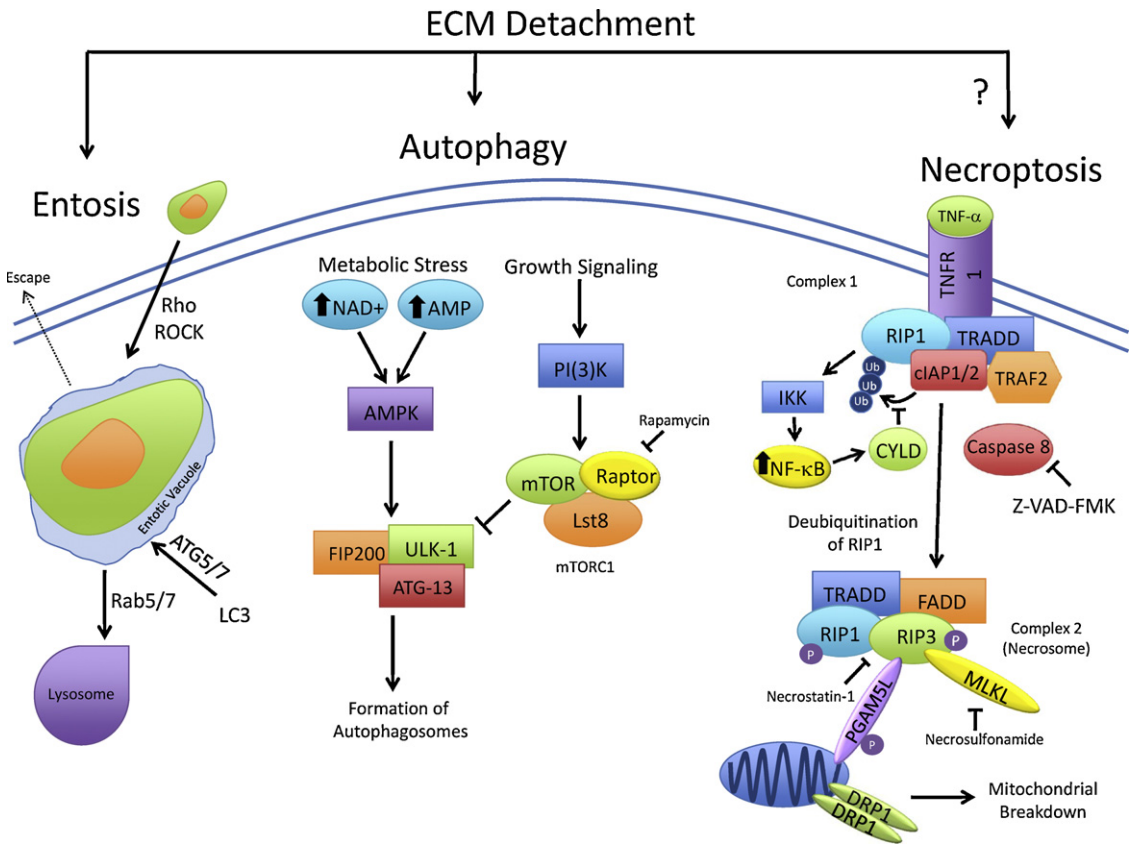

Fig. 3. Alternative mechanisms implicated in the death of ECM-detached cells. Entotic cells are internalized by host cells through the activation of Rho and ROCK which results in the contraction of host cytoskeleton. These cells are wrapped in a single membraned vacuole that can then be targeted for non-autophagic degradation utilizing components of the autophagy machinery (ATG5/7). Entotic vacuoles are then fused with the lysosome and degraded. Some entosed cells, however, are able to exit the host cell and return to normal cell behavior. Autophagy is a cell death/survival mechanism that results in self-cannibalism due to cellular stress. Autophagy can be triggered due to increased levels of NAD^+ and AMP which activate the AMPK pathway. AMPK can activate ULK-1 and the autophagy complex which results in the formation of autophagosomes. Growth signaling is able to activate PI(3)K and the mTORC1 complex. mTORC1 can suppress formation of the ATG complex. Exposing cells to exogenous TNF-α and a pan-caspase inhibitor (z-VAD-FMK) induces a programmed form of necrosis, termed necroptosis. In the presence of TNF-α, complex 1 (consisting of Rip1, TRADD, cIAP1/2, and TRAF) forms with the intracellular domain of TNFR-1. cIAP1/2 functions to polyubiquitinate Rip1 and target it for proteasome-mediated degradation. Downstream of complex 1, the IKK pathway is activated, which leads to translocation of NF-κB to the nucleus to act as a transcription factor. NF-κB acts to upregulate CYLD, which inhibits cIAP1/2 activity. By blocking cIAP1/2 activity, Rip1 protein levels are stabilized. Thus, when caspase-8 is inactivated, either endogenously or by z-VAD-FMK treatment, complex 2 (the necrosome) forms. Phosphorylation of Rip1 and Rip3 are essential for complex 2 formation. Recent studies have demonstrated that MLKL and PGAM5L both interact with Rip3. PGAM5L is a mitochondria-associated protein phosphatase that recruits Drp1 to the mitochondria, which facilitates mitochondrial degradation and necroptotic cell death.

In addition to the activation of autophagy by metabolic stress, it has been found that autophagy can be induced by enhanced oxidative stress produced by a number of metabolic pathways in cancer cells. Given the evidence that ECM-detachment induces oxidative stress, this provides another clear link between ECM-detachment and the promotion of autophagy. This oxidative stress-mediated induction of autophagy has been found to be controlled by the activity of PERK, an endoplasmic reticulum (ER) protein [67]. Normally, PERK is localized in the ER and is utilized by the cell to attenuate translation due to the presence of misfolded protein. PERK has also been previously implicated in the hypoxia response [68]. However, in the presence of enhanced oxidative stress, PERK can act in a fashion to induce autophagy. Initially, PERK induces reversible growth arrest and an antioxidant response. However, once the tumor mass reaches a critical threshold, PERK phosphorylates and activates eIF2α, thus inducing transcription and translation of the ATG proteins required for autophagy [67]. PERK also induces the upregulation of proteins that facilitate survival when ROS levels are high. These proteins include Glty1 and cysteine/glutamate transport proteins which are key components of the ROS neutralizing GSH (glutathione) [67]. Thus, the activation of PERK due to stress results in both the induction of nutrient rescuing autophagy as well as the neutralization of ROS.

5.2. Entosis

Another highly regulated and non-canonical cell death mechanism that is induced upon ECM-detachment is entosis. Entosis is defined as a mechanism by which a live cell invades another neighboring cell, leading to a state in which the invading cell is kept alive by the host cell [12]. The phenomenon was initially observed in a variety of distinct cancers where it was classified as a "cell-in-cell" cytological phenotype [12,69] and initial studies postulated that entosis might occur due to nutrient starvation. However, the discovery that cancer cells grown in serum rich media would also undergo entosis moved these studies in a different direction. It is now believed that entosis occurs due to compaction forces experienced by cells and may largely be dependent on integrin signaling and loss of ECM-attachment. For example, the reintroduction of Matrigel (a source of synthetic ECM) was found to rescue the entotic phenotype. Cells engulfed during entosis are not phagocytosed and have been observed to be fully functional, metabolically active cells. These entosed cells can maintain mitochondrial activity and, in many cases, can undergo cell divisions and form multicellular bodies while still inside the host cell. Furthermore, these internalized cells can be released in order to reenter the cell cycle when stress stimuli are lost or can be killed off when the host cell is exposed

to prolonged stress. Entotic activity is mediated by the activity of Rho family of GTPases and ROCK (Rho associated protein kinase) protein, which acts on the cellular cytoskeleton [12].

Recent work by Overholtzer's group has further elucidated the mechanism by which entotic cells are processed by the host cell. After the internalization of a cell, entotic vacuoles are targeted for degradation by fusing with the lysosome. Interestingly, this phenomenon utilizes components of the autophagy machinery yet remains distinct from canonical autophagy [70]. Thus, given that entosis is linked to metabolic stress, cell death, and autophagy, additional studies will be necessary to more fully understand the link between this non-canonical death process and the ultimate survival of ECM-detached cells.

5.3. Necroptosis

Necrosis has for years been thought to be a passive, unregulated form of cell death as opposed to the orderly and highly regulated apoptotic cell death. However, recent evidence suggests that necrosis may also be a highly regulated process that can be activated in a variety of distinct physiological situations (including ECM-detachment). This "programmed necrosis" has been termed necroptosis and was initially discovered to be induced by tumor necrosis factor alpha (TNFα) [71]. This initial study was followed by a genome-wide siRNA screen to look for genes involved in necroptosis, and 432 genes were initially identified of which subsets were enriched in the immune and nervous systems [72]. In addition, receptor interacting protein 1 (Rip1) was found to be involved in the necroptosis signaling pathway after it was identified as the target of the necroptosis inhibitor, necrostatin-1 [73]. Rip1 kinase can be found in the membrane-bound complex 1 of death receptor signaling in the presence of TNFα [74]. Rip1 is typically polyubiquitinated in this complex and the end result is activation of the IKK pathway and translocation of NF-κB to the nucleus to act as a transcription factor [75]. However, when Rip1 is deubiquitinated by enzymes like CYLD, Rip3 was found to associate with Rip1 via a Rip homotypic interaction motif (RHIM) leading to the phosphorylation of Rip1 and the formation of cytosolic complex 2. Complex 2 appears to be critical for necroptosis when caspase-8 activation is inhibited [76]. Both the RHIM and kinase domains of Rip3 and Rip1 were found to be essential for necroptosis to occur after treatment with TNFα and a pan-caspase inhibitor z-VAD-FMK [77–79]. Even though the mechanisms are not well defined, it appears that Rip1 is critical for Rip3 phosphorylation, although not directly. Once both proteins are phosphorylated, they interact to form a complex known as the necrosome [78,79]. In the absence of Rip3, Rip1-mediated apoptosis can still occur, but the presence of Rip3 facilitates a molecular switch to necroptosis [77]. This kinase cascade is a crucial factor that separates necroptosis, which is carried out by a phosphorylation cascade, from apoptosis, which relies on caspase cleavage events. Of note, Bcl-2 modifying factor (BMF), a pro-apoptotic Bcl-2 protein family member, was found to be required for necroptosis, suggesting a role for Bcl-2 protein family members in both apoptosis and necroptosis [72].

Interestingly, necroptosis can be linked to ECM-detachment and metabolism through its regulation of ROS. For example, Rip3 was found to regulate downstream ROS production during necroptosis as Rip3 knockout MEFs exhibited lower ROS levels and necrotic cell death [79]. In addition, another study found that Rip3 could activate a number of metabolic enzymes to regulate TNF-induced ROS production [77]. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) revealed seven metabolic enzymes in the Rip3 complex in cells treated with TNFα and z-VAD-FMK. Three of these proteins, including glycogen phosphorylase (PYGL), glutamate–ammonia ligase (GLUL), and glutamate dehydrogenase 1 (GLUD1) were confirmed as binding partners for Rip3 by coimmunoprecipitation. PYGL plays a role in utilizing glycogen stores for energy production, and Rip3 can enhance PYGL activity in vitro. The precise molecular mechanism by which this occurs is still unclear, but a role for PYGL in necroptosis was confirmed. GLUL is an enzyme found in the cytosol that is responsible for the condensation of glutamate and ammonia to form glutamine. GLUD1 is an enzyme localized to the mitochondrial membrane and converts glutamate to α-ketoglutarate. Both enzymes are essential for the use of glutamine and glutamate as substrates for ATP production via oxidative phosphorylation. GLUL and GLUD1 were found to be required for TNFα and z-VAD-FMK induced cell death, establishing a role for these metabolic enzymes in necroptosis. Additionally, knockdown of each enzyme resulted in reduced levels of ROS accumulation and cell death [77]. In aggregate, these studies reveal an intimate link between metabolic enzymes, the regulation of oxidative stress, and necroptosis and suggest that necroptosis may (like apoptosis) need to be inhibited during tumor progression.

More recent studies from Xiaodong Wang's group have unveiled key components in the signaling cascade that mediates necroptosis. Mixed lineage kinase domain-like (MLKL) protein was identified as the substrate of necrosulfonamide, an inhibitor of necroptosis that acts downstream of Rip3. MLKL is phosphorylated by Rip3 at threonine 357 and serine 358, and these phosphorylation events are imperative for necroptosis induction [80]. Although MLKL has no kinase activity itself, it appears to function as a vital adaptor protein for downstream signaling, as MLKL knockdown attenuated necroptosis [81]. Additionally, PGAM5, a mitochondrial protein phosphatase, is involved in the Rip1 and Rip3 containing protein complex during necroptosis. Two splice variants of PGAM5 were identified, PGAM5L (long form) and PGAM5S (short form), and when either was knocked down, necroptosis and ROS levels diminished. Interestingly, when Rip3 or MLKL are inhibited, only necroptosis is blocked without any effect on ROS levels. This suggests that PGAM5 may be acting at a convergence point of various upstream necrosis pathways, including Rip1/Rip3-mediated necroptosis. Furthermore, the phosphorylation of the two splice variants was found to be differentially regulated by necrosulfonamide, suggesting unique roles for each variant in the necroptosis cascade. Necrosulfonamide treatment can inhibit the phosphorylation of PGAM5S (but not PGAM5L) and following PGAM5S phosphorylation during necroptosis, Drp1 (a mitochondrial fission factor) is activated via dephosphorylation at serine 637. This stimulation of the GTPase activity of Drp1 subsequently leads to mitochondrial fragmentation. Interestingly, PGAM5L is a substrate for Keap1, an inhibitor of Nrf2, which is important in the intracellular ROS detoxification program which is discussed above [18,82]. These studies reveal a molecular mechanism that further links ROS with the execution of the necroptotic program.

With the recent understanding of programmed necrosis, it seems reasonable that necroptosis may be (partially) responsible for the death of ECM-detached cells. If this is the case, it would suggest that tumor cells must overcome both anoikis and necroptosis in order to metastasize. Further understanding the signaling cascade responsible for necroptosis may help identify chemotherapeutics that would target the cancer cells' ability to evade both anoikis and necroptosis. This multimodal approach may prove to be more efficient for long-term cancer-free survival.

6. PI(3)K regulation of survival and metabolism during ECM-detachment

PI(3)K is a potent mitogenic kinase that has been implicated in the oncogenic behavior of a variety of distinct cancer cells [83–85]. This kinase activity is responsible for converting PI to triply phosphorylated PI (PIP_3) which can then act as a site for the recruitment

of pleckstrin homology (PH) domain containing proteins like Akt. Activation of Akt is implicated in a variety of distinct survival mechanisms including those that are intimately linked to metabolism. It is widely appreciated that PI(3)K plays a role in cancer survival through the modulation of glucose uptake [94]. As discussed above, cancer cells have for many years been known to consume profuse amounts of glucose (the Warburg effect) [25–27]. PI(3)K signaling is intimately involved in this enhanced uptake of glucose through the increase in trafficking of glucose transporters to the plasma membrane [86]. This regulation of glucose transporters by PI(3)K signaling is not limited to canonical glucose transporters (i.e. GLUT1, GLUT4) as recent studies reveal that the stabilization of the sodium/glucose cotransporter molecule 1 (SGLT1) can drive glucose uptake downstream of EGFR, independent of EGF ligand [87]. Thus, given the already established links between ECM-detachment, PI(3)K signaling, and glucose metabolism, it seems clear that PI(3)K signaling is critical for the survival of ECM-detached cancer cells.

One specific and important link between PI(3)K signaling and ECM-detachment is the Trk family of proteins. Trks are a family of receptor tyrosine kinases that are activated by the ligand BDNF (brain derived neurotrophic factor), and are overexpressed in a variety of cancers [88,89]. The Trk family consists of three members (TrkA, TrkB and TrkC), and TrkB becomes oncogenic due to hyperactive PI(3)K signaling. Interestingly, elegant studies have linked this signaling to the inhibition of anoikis and the survival of ECM-detached cells [90,91]. In addition, other studies have linked the activity of Trk family members to both metastasis and anoikis avoidance [89]. For example, the activation of TrkA results in a more aggressive phenotype and a higher metastatic capacity. The constitutive activation of TrkA results in anoikis resistance and activation of the PI(3)K pathway which can be abolished by treatment with K252a, a TrkA inhibitor [92]. This suggests that the activation of Trk family members results in increased PI(3)K signaling which helps ECM-detached cells survive while undergoing metastasis. Additionally, the overexpression of TrkC also results in increased metastatic capacity and anoikis avoidance through the induction of the PI(3)K pathway in breast cancer [93].

7. Conclusions, future directions, and perspectives

In conclusion, the plethora of evidence discussed here that links ECM-detachment to alterations in cancer cell metabolism suggests that ECM-detached cancer cells may be uniquely vulnerable to novel chemotherapeutic agents aimed at altering metabolic activity. For example, the design of novel therapeutics that selectively target the antioxidant activity of ECM-detached cancer cells may be effective in inducing cell death in potentially metastatic cells. This could be accomplished through interfering with PPP flux, modulating NRF2 activity, stimulating pyruvate kinase activity, or inhibiting the activity of PGHDH. In addition, given the deficiency in ATP production and subsequent AMPK activation in ECM-detached cells, the selective inhibition of AMPK signaling may serve as an effective method to induce ECM-detachment mediated cell death in cancer cells. A similar strategy to induce cell death in cancer cells by interfering with ECM-detachment-induced HIF-1α signaling may also be productive. That being said, a better understanding of the molecular mechanisms underlying the relationship between these metabolic changes and cell death as well as the completion of studies in additional cell types is necessary prior to investing resources in the development of these types of therapeutic strategies.

In addition, the myriad cellular and metabolic changes associated with the various types of cell death caused by ECM-detachment present further avenues for chemotherapeutic intervention. For instance, the design of therapeutic approaches that tilt the balance of fate for entotic cells toward cell death could be highly effective at selectively eliminating cancer cells. As entosis seems to be relatively specific to ECM-detached cells and cancer cells lose attachment to ECM during the metastatic cascade, the induction of entotic cell death in cancer cells could have a dramatic effect on the inhibition of metastasis. However, knowledge of this novel type of cell death is still preliminary such that these types of approaches are impractical at this time. The same is true for the modulation of autophagy and necroptosis in cancer cells. Given that cancer cells will need to prevent the induction of multiple types of cell death during tumor progression, the induction of either autophagy or necroptosis in ECM-detached cancer cells seems like an attractive strategy. It is important to note however, that autophagy induction can also promote ECM-detached cell *survival* and an understanding of necroptotic mechanisms is not yet sophisticated enough to allow for its selective induction in certain cell types. Additionally, an inflammatory response will accompany the induction of necroptosis, which could have deleterious effects on patients. Thus, these types of potential therapeutic approaches are unlikely to come to fruition until future mechanistic studies are completed.

In summary, the studies discussed in this review reveal an intimate link between ECM-detachment, cancer cell survival, and alterations in cellular metabolism. This link has only begun to be appreciated over the past few years and there is no doubt that the next few years will also bring an abundance of exciting studies examining the topic. Thus, while the identification of a therapeutic window to take advantage of this unique characteristic in cancer cells remains unfeasible at this time, the pace of scientific discovery suggests that therapeutic elimination of ECM-detached cancer cells by metabolic modulation may be an impending and achievable goal.

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