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Review

Anoikis: A necessary death program for anchorage-dependent cells

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ABSTRACT

Cell to matrix adhesion is a key factor for cellular homeostasis and disruption of such interaction has adverse effects on cell survival. It leads to a specific type of apoptosis known as "*anoikis*" in most non-transformed cell types. This kind of apoptosis following loss of cell anchorage is important for development, tissue homeostasis and several diseases. Integrins sense mechanical forces arising from the matrix, thereby converting these stimuli to downstream signals modulating cell viability. Anchorage-independent growth is a crucial step during tumorigenesis and in particular during the metastatic spreading of cancer cells. The disruption of the tight control leading an "homeless" cell to death is therefore able to violate the cell defences against transformation. This review analyses the recent investigations into the molecular mechanisms governing *anoikis*, discussing the different ways in which adhesion can influence this process and addressing the relevance of this unique apoptosis mode in the development of metastatic cancers, as well as in other diseases.

Contents

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Abbreviations: DR5, death receptor 5; ECM, extracellular matrix; EGF, epidermal growth factor; EMT, epithelial to mesenchymal transition; ERK, extracellular signal-regulated kinase; FADD, Fas-associated death domain protein; FAK, focal adhesion-kinase; FGF, fibroblast growth factor; FLIP, FLICE inhibitory protein; HGF, hepatocyte growth factor; JNK, Jun-NH2-terminal kinase; IL, interleukin; ILK, integrin-linked kinase; NF-kB, nuclear factor-kB; OMM, outer mitochondrial membrane; PDGF, platelet-derived growth factor; PI3K, phosphoinositide-3-OH kinase; PKB, protein kinase B; ROS, reactive oxygen species; TNFα, tumor necrosis factor-α; TNFR, TNF-α receptor; TNFR-1, TNF-α receptor 1; TGFβ, transforming growth factor β; TRAIL, TNFR apoptosis-inducing ligand; VDAC, voltage-dependent anion channels; VEGF, vascular endothelial growth factor.

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1. The physiological meaning of *anoikis* and the control from extracellular matrix (ECM)

Anoikis, a Greek word meaning 'homelessness', is apoptosis induced by loss of cell adhesion or inappropriate cell adhesion [1]. Adhesion on the extracellular matrix (ECM) is important to determine whether a cell is in the correct location and to remove displaced cells by apoptosis. *Anoikis* following loss of cell anchorage, is of physiological relevance for development, tissue homeostasis and disease [2,3]. *Anoikis in vivo* may prevent detached cells from reattaching to new matrices and growing dysplastically. Of course this could be an important safeguard for the organism.

Integrins regulate cell viability through their interaction with the extracellular matrix and they can sense mechanical forces arising from the matrix, converting these stimuli to chemical signals capable of modulating intracellular signal transduction [4]. *Anoikis* has been described in several cell types, although it appears that cells of different tissue origin activate dissimilar pathways leading to *anoikis*. The physiological relevance of *anoikis* is confirmed by the fact that cancer cells lines, rather than normal epithelial cells, are usually not sensitive to *anoikis*, and many have developed anchorage independence, meaning they do not require adhesion to ECM to proliferate and survive [5–8]. Therefore, the ability to overcome this requirement has important implications for metastatic cancer. Indeed in neoplastic cells, alterations in cell–cell adhesion molecules, protein kinases or phosphatases, integrin-associated signalling molecules or apoptosis regulators can lead to resistance to the physiologically occurring *anoikis*, conferring by this way a constitutive pro-survival signal allowing dissemination of metastatic cancer cells [7–11].

2. The molecular program of *anoikis*

Apoptosis in response to lack of adhesion or inappropriate adhesion to the ECM has been termed *anoikis*, but, in spite of its unique definition, *anoikis* is essentially an apoptotic process.

The initiation and execution of *anoikis* is mediated by different pathways, all of which merge into the activation of caspases and downstream molecular pathways, culminating in the activation of endonucleases, DNA fragmentation and cell death. In particular, *anoikis* seems to occur following either the perturbation of mitochondria (the intrinsic pathway) or the triggering of cell surface death receptors (the extrinsic pathway) (Fig. 1, panel B) [3,12].

The proteins of the Bcl-2 family are key arbiters of both these processes [7]. The Bcl-2 family can be divided into three groups: (i) the anti-apoptotic proteins, including Bcl-2, Bcl-XL and myeloid cell leukaemia sequence 1 (Mcl-1); (ii) the multidomain pro-apoptotic proteins Bax, Bak and Bok; (iii) the pro-apoptotic BH3-only proteins, counting Bid, Bad, Bim, Bik, Bmf, Noxa, Puma and Hrk.

In the intrinsic pathway, caspase activation occurs as a consequence of mitochondrial permeabilization [13,14]. This pathway is regulated by the pro-apoptotic proteins Bax and Bak, which form oligomers in the outer mitochondrial membrane (OMM), creating channels within this membrane, thus causing its permeabilization. It has been postulated that membrane permeabilization may result not only from the intrinsic pore forming activity of the Bax proteins, but even from their interaction with mitochondrial channel proteins such as the voltage-dependent anion channels (VDACs) [15]. However, recent data obtained by Baines et al. indicate that VDACs are dispensable for Bcl-2 family member-driven cell death, since wild-type and VDAC-deficient cells exhibited equivalent cytochrome *c* release, caspase cleavage and cell death in response to the pro-death Bcl-2 family members Bax and Bid [16]. The consequence is the disruption of the OMM and the release of cytochrome *c*, leading to formation of the so-called apoptosome, composed of caspase-9, the cofactor apoptosis protease activating factor (Apaf) and cytochrome *c*, with subsequent activation of the effector caspase-3 and execution of the apoptotic process [17–19]. This intrinsic cascade is initiated by the pro-apoptotic BH3-only family of proteins, essential players during the *anoikis* programme [20]. In particular, among the members of this family, Bid and Bim are activated following detachment of cells from ECM and rapidly promote the assembly of Bax-Bak oligomers within the OMM. These members of the BH3-only protein family are termed activators [21].

The anti-apoptotic Bcl-2 family of proteins are structurally related to the Bax and BH3-only families but prevents apoptosis by maintaining mitochondrial membrane integrity and avoiding pore formation and OMM disruption, although the precise relationships between these proteins are not yet completely defined [2,21–23]. The original model of Bcl-2 for the inhibition of Bax function through heterodimerization is no longer valid because of the difficulty in detecting this complex under physiological conditions. Additionally, Bcl-2 family proteins can inhibit apoptosis by sequestering the activator members of the BH3-only proteins, namely Bid and Bim, thereby preventing oligomerization of Bax and Bak [24,25]. There is another group of the BH3-only proteins, called sensitizers, which are unable to directly activate Bax and Bak oligomerization [25,26]. The sensitizer BH3-only proteins (Bad, Bik, Bmf, Noxa, Puma and Hrk) contribute to cell death by competing for the Bcl-2's BH3 binding domain, inactivating the anti-apoptotic functions of Bcl-2, thus freeing activator BH3-only proteins to induce Bax-Bak oligomer formation [27,28].

Several evidences indicate that the BH3-only proteins play a role in *anoikis* execution of different cell types. Noxa and Puma are transcriptionally regulated by p53 and have been implicated in fibroblast *anoikis* [29,30]. Bim and Bad can be controlled by the phosphoinositide-3-OH kinase (PI3K) and extracellular signal-regulated kinase (ERK) pathways, and Bid may be cleaved in the death receptor pathway [31–34]. In particular, Bim is sequestered in the dynein complex until the loss of integrin engagement induces its release and translocation to mitochondria, where it interacts with Bcl-XL neutralizing its pro-survival function [35]. In addition, both ERK and PI3K/Akt-mediated phosphorylation of Bim, elicited upon integrin engagement, leads to the proteasome-dependent degradation of Bim [36,37]. As a consequence, the loss of ECM contact, leading to inhibition PI3K/Akt and ERK signals, strongly increases Bim accumulation [35]. The BH3-only protein Bmf has been implicated in *anoikis* through its interaction with the myosin V motor complex, although further evidence is required to show whether it is activated in

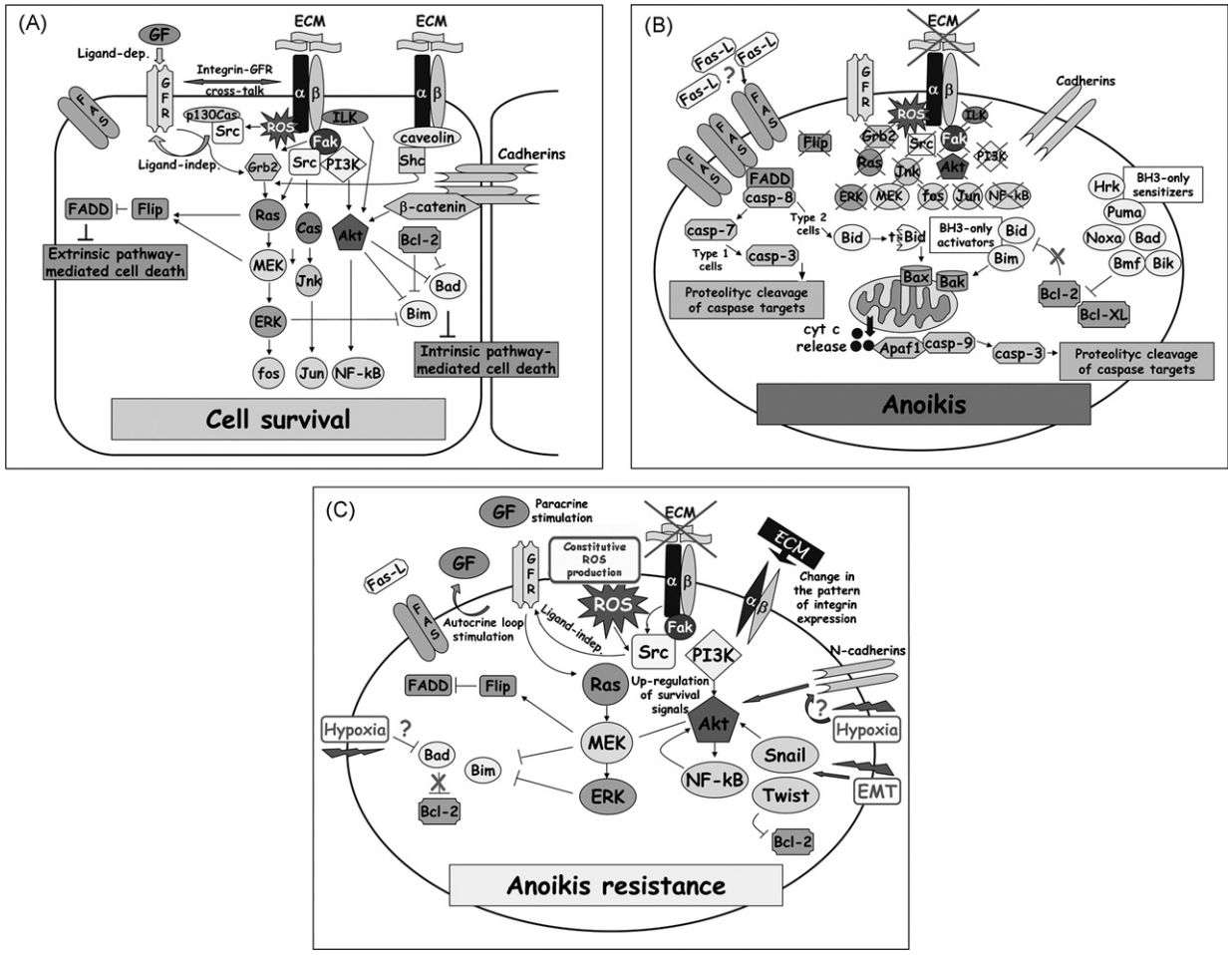

Fig. 1 – Signalling pathways activated during cell survival, *anoikis* and *anoikis* resistance. (A) Integrin engagement by ECM activate several pro-survival routes including ERK, JNK and Akt, which stimulate *fos*, *jun* and NF-kB transcription factors and, on the other hand inhibit pro-apoptotic proteins, ultimately preventing both the extrinsic and the intrinsic pathway of cell death. GFR, both in a ligand-dependent and -independent manner, collaborate with integrins in promoting cell survival mainly converging on the same signalling pathways. Cadherin-mediated cell–cell contact also promotes cell survival in a PI3K/Atk-dependent fashion, leading to down-regulation of the BH3-only pro-apoptotic proteins. (B) Lack of ECM-mediated cell contact or engagement of the wrong type of ECM fails to activate the pro-survival players, thereby removing the inhibition on the key controller of both the death receptor-mediated machinery and the mitochondria-driven *anoikis*. It has been postulated that the extrinsic pathways is further activated by an increase in the cell surface expression of Fas receptors, whose proximity results in their activation. Fas-L is also upregulated, even if it is unclear if external ligand is required for death receptors activation during *anoikis*. (C) Acquisition of *anoikis* resistance is achieved through the involvement of different strategies which converge on the stimulation of survival signals and/or on the inhibition of apoptotic pathways. Cells can circumvent *anoikis* by acquiring constitutive activity of survival pathways (*i.e.* PI3K/Akt, MEK/ERK and NF-kB) by means of an autocrine growth factor loops or by a paracrine stimulation of the neighbouring cells. Another strategy to avoid *anoikis* is to change the pattern of integrin expression, so that the correct environmental survival signals are received. ROS constitutively upregulated in cancer cells, are additional key player in conferring *anoikis* resistance by transducing pro-survival signals through a ligand-independent activation of GF. Hypoxia-mediated increase in ROS production may also concur to overcome *anoikis*, through a redox-mediated down-regulation of pro-apoptotic factors. An additional hypothesis for hypoxia-mediated cell survival is that the up-regulation of N-cadherin expression, induced by hypoxic environments, promotes resistance to apoptosis by stimulating the anti-apoptotic protein PKB/Akt and subsequently inactivating the pro-apoptotic factor Bad. EMT is another fundamental strategy to evade the *anoikis* barrier. The up-regulation of several transcription factors (Snail, Twist, NF-kB), by activating survival genes, such as the PI3K/Akt cascade, is critical for EMT success and for the overcoming of the apoptotic programme (see text for details).

a causative way following detachment of cells from the ECM [38]. In addition, the activator BH3-only proteins have been directly implicated in *anoikis*. In particular, siRNA down-regulation of Bid inhibited *anoikis* in FSK-7 mammary epithelial cells and a similar siRNA-mediated knockdown of Bim prevented apoptosis of MCF10A cells [8,39]. The sensitizer BH3-only proteins do not directly regulate *anoikis*. These proteins appear to sensitize cells for apoptosis, but are unable to induce mitochondrial permeabilization themselves. For instance, overexpression of Bad does not induce apoptosis in adherent MDCK cells, but sensitizes them to *anoikis* [40].

In addition to the involvement of the intrinsic pathway in the execution of *anoikis*, a clear evidence supports a role for the extrinsic pathway during detachment-induced apoptosis. The extrinsic pathway is initiated by the ligation of extracellular death ligands, such as Fas Ligand (FasL) or tumor necrosis factor-α (TNF-α), to their transmembrane death receptors, namely Fas and TNF-α receptor (TNFR), respectively, resulting in the assembly of a death-inducing signalling complex (DISC). The role of the DISC is to recruit adaptor proteins such as the Fas-associated death domain protein (FADD), which in turn engage and aggregate several molecules of caspase-8, thereby promoting its activation and autoprocessing. Active caspase-8 then proteolytically processes and activates caspase-3 and -7, provoking further caspase activation events that culminate in substrate proteolysis and cell death [21,41]. Cells that respond to extracellular death ligands fall into two classes, type I and type II [13]. In type I cells, activation of caspase-8 is sufficient to cleave effector caspases, leading to cell death. In type II cells caspase-8 cannot itself initiate apoptosis. Indeed, in these cells extrinsic death signals crosstalk with the intrinsic pathway, since caspase-8 cleaves the BH3-only protein Bid, which in turn, in its truncated form (t-Bid) can promote mitochondrial cytochrome *c* release and assembly of the apoptosome [42]. In both type I and type II cells, the initiating signal is caspase-8 activation. *Anoikis* requires OMM permeabilization as it can be blocked by overexpression of anti-apoptotic Bcl-2 proteins [43,44]. Nevertheless, some studies in epithelial cells have suggested that the initiating event is the activation of a death receptor, as overexpression of a dominant-negative form of FADD, which blocks caspase-8 recruitment to the DISC, inhibits *anoikis* [45,46]. However, Rytomaa et al. [46] showed that caspase activation and apoptosis occurs without requiring external ligand activation of death receptors during *anoikis*. In fact, the soluble extracellular portions of Fas, TNF-α receptor 1 (TNFR-1) and death receptor 5 (DR5), one of the TNFR apoptosis-inducing ligand (TRAIL) receptors, which can sequester the ligands, failed to inhibit *anoikis*. Furthermore, the detachment-induced activation of caspase-8 was inhibited by Bcl-2 overexpression, suggesting that caspase-8 activation occurs as a consequence of activating the intrinsic pathway. For what concern the role of Fas-receptor, Aoudjit and Vuori demonstrated that in human endothelial cells the loss of anchorage to ECM leads to a threefold increase in Fas expression, a 1.5-fold increase in Fas-Ligand (Fas-L) expression, while FLICE inhibitory protein (FLIP), an endogenous inhibitor of Fas-mediated signalling, is massively down-regulated [47]. Furthermore, it has been previously shown that changes in cell shape can induce apoptosis [48]. Indeed, cell rounding following detachment could lead to ''induced proximity'' of Fas receptors resulting in their activation [49]. Alternatively, activation of the death receptor pathway could be the result of a positive feed-back mechanism, secondary to mitochondrial damage [50]. The observation that the blockage of caspase-8 activation by Bcl-2 and Bcl-XL overexpression, as well as that cytochrome *c* release precedes caspase-8 activation during *anoikis*, support this hypothesis [51,52].

Both the intrinsic and the extrinsic pathways converge on the activation of the effector caspase-3, which initiates a downstream proteolytic cascade, a central event in programmed cell death. In particular, cleavage of signalling molecules like focal adhesion-kinase (FAK), Cas, and paxillin is important for the progression of the apoptotic cascade [7,53–55]. The cleavage of FAK by caspases shuts down its survival signal and releases the C-terminal portion of FAK. This fragment contains the focal adhesion targeting domain and inhibits FAK signalling itself, thereby enhancing the apoptotic effect of FAK cleavage [56]. Paxillin is a fundamental component of focal adhesion, which associates with integrin and recruits FAK to sites of integrin clustering. After cell detachment it is cleaved by caspase-3, generating a 42-kDa fragment [54]. p130Cas, an SH2/SH3 adaptor protein, which binds FAK and transmits integrin signals, undergoes caspase-mediated cleavage during *anoikis*, as well. This cleavage disrupts the p130Cas–paxillin interaction, p130Cas subcellular localization, and focal adhesion architecture [57]. Moreover, p130Cas cleavage produces a 31-kDa carboxy-terminal fragment which interacts with the E2A transcription factor via a helix–loop–helix motif. This interaction blocks the ability of E2A to stimulate p21Waf1/Cip1, thus contributing to the apoptotic response by blocking the cell cycle [58]. In addition, the key role of p130Cas in mediating the execution of the *anoikis* programme is further underlined by the ability of its C-terminal portion to induce *anoikis* in *anoikis*-resistant cells [59,60]. Thus, alongside with FAK, the disruption of p130Cas-controlled signalling by its cleavage and generation of inhibitory fragments could lead to the suppression of survival signals in non-adherent cells.

3. Physiological protection from *anoikis*

Protection from the execution of *anoikis* for non-transformed cells has been reported essentially in four different environmental settings: preservation of cell adhesion to ECM or cell–cell contacts, temporary destruction of focal contacts to allow cell migration and in professionally non-adherent cells like leukocytes (Table 1).

First, *anoikis* is avoided when cells are adherent on permissive ECM proteins. The role of ECM as a suppressor of apoptosis has been well established for a number of years [1,61]. However, it is misleading to think that all adhesion dependence is based on the same signal transduction events, since multiple pathways connect adhesion to the suppression of apoptosis. Many signals are affected depending on whether or not a cell commits to an apoptotic fate, and the final decision is the sum of all these inputs. Several integrins (α1β1, α2β1, α3β1, α5β1, α6β1, α6β4, and αvβ3) have profound impact on cell survival. Different factors such as cell type, composition of the surrounding

Table 1 – Critical pathways involved in physiological protection from *anoikis*

Environmental settings granting for physiological resistance to *anoikis*	Molecules and pathways involved	References
Integrin-mediated ECM contact	Activation of integrin-dependent signalling routes:	
	FAK and its downstream signalling: JNK, ERK, PI3K, PKB/Akt;	[64]
	LK and its mediators;	[65]
	caveolin-1;	[74]
	ligand-independent activation of growth factor receptor	[75–83]
Provisional detachment during cell migration of adherent cells	PI3K, Rac, Cdc42, Rho GTPases	[85–88]
Ameboid movement of hematopoietic stem cells and leukocytes	Pro-survival signals elicited by cytokines (IL-2, IL-7, IL-15, interferon-α)	[91–92]
Cadherin-mediated cell–cell contact	Activation of PI3K, and PKB/Akt	[96–97]
	Association with integrins and growth factors	[99]

matrix, tissue origin, state of differentiation or state of neoplastic transformation will determine which set of integrins are critical in transducing these survival signals [4,5,56]. Certain integrins seem to mediate survival in non-transformed cells ensuring tissue homeostasis while other integrins seem to be upregulated particularly during neoplastic transformation providing survival signals during invasion and metastatic growth [4,62,63].

Key players in integrin-mediated signal transduction leading to *anoikis* protection are FAK, integrin-linked kinase (ILK), Src tyrosine kinase, PI3K, ERK and the adaptor protein Shc (Fig. 1, panel A). Upon integrin ligation FAK is phosphorylated at tyrosine 397 and interacts with numerous signalling molecules allowing recruitment of other signalling elements to the FAK signalling complex. FAK-activated proteins include PI3K and its downstream target protein kinase B (PKB/Akt), as well as the ERK pathway and the Jun-NH_2-terminal kinase (JNK) pathway [44,64]. ILK is an integrin-interacting protein kinase which is capable of phosphorylating PKB/Akt on Ser-473, thereby stimulating its activity [65,66]. Overexpression of active FAK or ILK blocks *anoikis* in cells despite loss of cell anchorage, thereby supporting the role of these kinases in *anoikis* regulation [1,43,44,67]. In addition, the commitment of *anoikis* due to overexpression of ILK is not rescued by FAK, suggesting that the two kinases affect *anoikis* in different and parallel pathways [65].

The serine-threonine kinase PKB/Akt seems to be a vital element of cell survival signalling as integrin-, growth factor- and cell–cell anchorage-mediated signals converge to its activation. Activation of PKB/Akt leads to multiple inhibitory effects on the apoptotic machinery such as inactivation of caspase-9 [68] and phosphorylation of the pro-apoptotic protein Bad [69], activation of nuclear factor-kB (NF-kB) [70], as well as inhibition of Forkhead transcription factors [71].

Besides, ILK conveys integrin-mediated survival signals independently of FAK, as indicated by the inability of dominant-negative FAK to revert the ILK-mediated protection from *anoikis* [65,72]. Finally, integrin-mediated survival signalling can occur independently of FAK or ILK, *via* caveolin-1-mediated binding of integrins to the adaptor protein Shc, which leads to a FAK-independent activation of ERKs and consequently to protection from apoptosis and cell cycle progression through cyclin D1 accumulation [73,74].

Adhesion receptors not only support a physical attachment between ECM components and the cytoskeleton but they also produce an adhesion-dependent signalling platform containing a number of adaptor proteins and kinases [4,56]. A growing body of literature indicates that integrin engagement can result in ligand-independent activation of many growth factor receptors, such as epidermal growth factor receptor (EGFR) [75], insulin receptor [76], platelet-derived growth factor receptor (PDGFR) [77], receptor for hepatocyte growth factor (HGF), namely Met [78] and vascular endothelial growth factor receptor (VEGFR) [79]. In epithelial cells either EGF binding to the EGFR or integrin-mediated ligand-independent EGFR stimulates ERK and PI3K signalling, thereby suppressing the activity of apoptotic factors. Indeed, both ERK and PI3K have been reported to negatively regulate Bim by phosphorylation, leading to its degradation. This prevents Bim from antagonizing Bcl-2 function or stimulating Bax activation. Inhibition of Bim suppresses destruction of the mitochondria and the induction of cell death. Conversely, the loss of β1 integrin engagement in detached cells repressed expression of the EGFR and increased Bim expression [80]. The adhesion survival signals suppress *anoikis* both in a Bim-dependent and -independent manner [81]. Detachment from ECM eliminates these signals, thus irreversibly committing the suspended cell to *anoikis*. In addition EGFR expression is further reduced by prolonged suspension, thereby adding this level of control to the suppression of survival signals. In keeping the re-establishment of integrin-mediated adhesion rescues the amount of EGF receptor expressed on the cell surface [82].

In the early phases of cell adhesion integrins associate with EGF receptors on the cell membrane in a supramolecular complex including the adaptor protein p130Cas and the c-Src kinase. The integrin cytoplasmic tail, c-Src kinase, and the p130Cas adaptor protein are required for a ligand-independent phosphorylation of EGF receptor in response to integrin-mediated adhesion [75,82]. The integrin-mediated ligand-independent phosphorylation of EGFR is mainly targeted to tyrosine residues 845, 1068, 1086, and 1173, but not on residue 1148, the major site of phosphorylation in response to EGF. In has been recently demonstrated that reactive oxygen species (ROS), produced through the involvement of the small GTPase Rac-1 upon integrin engagement, exert a mandatory role in transducing the pro-survival signal ensuring protection from

anoikis. In particular, ROS are responsible for the redox-mediated activation of Src, leading to the ligand-independent *trans*-phosphorylation of EGFR. The redox-dependent EGFR activation switches both ERK and PKB/Akt pathways, culminating in degradation of Bim [83].

The second physiological process in which cells need to be protected from *anoikis* is the provisional detachment which they undergo when they move towards a chemoattractant. Moving or mitotic cells are transiently protected from commitment towards *anoikis* during their transitory displacement of focal contacts. Recent key advances have challenged the view of tridimensional cell motility indicating essentially three motile phenotypes: collective, mesenchymal and amoeboid [84–86]. Mesenchymal motility is characterised by an elongated cell morphology with established cell-polarity and is dependent upon ECM proteolysis and focal contacts. Activation of several RTKs, including the c-Met tyrosine kinase, is often the initiating event for mesenchymal motility [86,87]. This leads to the PI3K-dependent activation of Rac and Cdc42 at the leading edge of the cell, which coordinate actin polymerization. The concomitant inhibition of Rho activity at the trailing edge of the cell, promotes its retraction [85]. Mechanistically, this is similar to a collective form of mesenchymal motility, with the cells at the front producing matrix metalloproteases (MMPs) and generating a 'path' for the following cells. Conversely, amoeboid migration, the most primitive form of cell migration allows cells to glide through, rather than degrade, ECM barriers through a weakening of cell–ECM attachments. Cortical actin contraction driven by Rho-ROCK signalling promotes the rapid remodelling of cell cortex characteristic of amoeboid movement. Intriguingly cell–ECM attachments are not required for amoeboid movement and focal adhesions are not organized [84,86]. Although definitive proofs are lacking, it is likely that in cells using amoeboid motility to move across ECM, the pro-survival signals elicited by integrin/focal contacts are assured in a different manner. One possibility is that during amoeboid motility, in which cells are virtually detached and round-shaped, moving cells survive due to the strong activation of the Rho family of GTPases. In keeping with this hypothesis, RhoG has been reported to regulate the suppression of *anoikis* in a PI3K-independent manner [88].

Among non-professional adhering cells, amoeboid movement is carried out by hematopoietic stem cells and leukocytes [89]. These cells use a fast crawling movement driven by weak interactions with ECM. In lymphocytes and neutrophils integrin-mediated focal adhesions are dispensable for cell migration and survival [90], thereby permitting high velocities (2–30 μm/min). T lymphocytes and other leukocytes move in a protease-independent manner across matrix barriers through adaptation of the cell shape and squeezing through narrow spaces using constriction rings [90]. These hematopoietic stem cells and leukocytes at all developmental stages require extrinsic signals for survival, although these factors are mainly growth factors and cytokines instead of ECM proteins. It is therefore conceivable that these non-adhering cells are protected from apoptosis by a continuous pro-survival signal elicited by several cytokines, including Interleukin IL-2, IL-7, IL-15 or interferon-α, which selectively abrogates induction of the pro-apoptotic BH3-only proteins [91]. In keeping with this hypothesis, in quiescent T-cells the withdrawal of survival factors leads to Bim accumulation and Bcl-XL down-regulation and final commitment to apoptosis [92].

Last, cells are protected from *anoikis* commitment through the establishment/maintenance of cell–cell contacts. These contacts are mediated by cadherins, a family of membrane proteins allowing homotypic or heterotypic calcium-dependent cell–cell anchorage. Several studies have shown that cadherins are yet further elements in the complex network of survival signalling. Indeed, disruption of cadherin function leads to increased apoptosis in intestinal epithelial cells [93]. In addition, several epithelial [94] or mesenchymal [95] cells are able to overcome *anoikis* induced by cell detachment from ECM when cell–cell-contact is preserved. In addition, blockage of E-cadherin binding induces *anoikis* [96,97], while over-expression of β-catenin, a common downstream regulator of cadherin signalling, gives rise to *anoikis* resistance in epithelial cells [98]. Cadherin signalling mainly promotes survival in a PI3K/Akt-dependent fashion [96]. In addition to the direct regulation of Akt signalling, cadherins may also affect cell survival through indirect association with integrins and their signalling. Indeed, some integrins can be localized at cell–cell contacts. For example, the α2β1 and α3β1 integrins can be found at cell–cell contacts and mediate survival signals despite loss of cell–matrix anchorage. Fascinatingly, these integrins are functionally associated with the EGFR [99], although proofs of a functional role of EGFR *trans*-activation as a pro-survival signal are lacking.

4. Pathophysiological protection from *anoikis*

Clarifying the molecular mechanism that regulates the execution of the *anoikis* program is of great importance for the onset and progression of several diseases.

Cells within a tissue require very specific ECM attachments, and the wrong type of surrounding ECM can have the same consequences as no ECM at all. Integrin engagement triggers the activation of cellular survival pathways, which allows the cell to determine whether it is occupying the correct environmental niche. Introducing a normal cell into the wrong microenvironment leads to the incorrect integrin engagement, with the consequence that the correct survival signals are not received and the cell is committed towards *anoikis* [100]. As a functional phenomenon, *anoikis* can suppress expansion of oncogenically transformed cells by preventing proliferation at migrating locations, whereas migrating cancer cells that overcome *anoikis* can survive outside of their own microenvironmental niche and grow in totally inappropriate ECM environments [101,102]. Resistance to *anoikis* is thus emerging as an hallmark of metastatic cancer cells, especially because anchorage-independent growth of tumor cells is a distinct property of different types of human malignancies [100,103]. The underlying mechanisms rendering tumor cells resistant to *anoikis* are not fully understood, but it has been postulated that it may comprise the stimulation of survival signals and inhibition of apoptotic pathways (Fig. 1 panel C and Table 2).

One strategy which allows the early-stage tumor cells to circumvent *anoikis*, is to acquire constitutive activity in the pathways responsible for cell survival, such as those trans-

Table 2 – Mechanisms and pathways conferring *anoikis* resistance to cancer cells

Mechanisms responsible for *anoikis* resistance of tumor cells	Specific activated pathways	References
Constitutive activation of pro-survival pathways (*e.g.* PI3K, MEK/ERK, and NF-kB)	Melanoma cells: acquisition of autocrine loops for HGF, IL8, PDGF-AA or bFGF to grant proliferation, survival and migration	[104–105]
	Ovarian cancer cells: up-regulation of TrkB and subsequent activation of the PI3K/Akt pathway	[106–107]
Change of the pattern of integrin expression	Melanoma cells: *de novo* expression of αVβ3 integrin allows for survival within the *dermis*	[108–113]
Constitutive oxidative environment	Prostate cancer cells: constitutive oxidation/activation of Src, granting for activation of EGFR-dependent pro-survival signals	[Manuscript in preparation]
EMT	Activation of mesenchymal genes, *e.g.* Snail, Twist, HGF/Met and NF-kB, is involved in the acquisition of *anoikis* resistance during the EMT process	[120–132]
Hypothized mechanisms		
Hypoxia	Hypoxia-mediated N-cadherin expression could promote *anoikis* resistance by inducing PKB/Akt activation and Bad inhibition	[137]
	Hypoxia-induced ROS increase could be responsible for down-regulation of pro-apoptotic molecules	

duced by PI3K, MEK/ERK and NF-kB. There are several ways in which this can happen, either through the constitutive activation of the pathways themselves, or the loss of the pathway inhibitors. In some cases, tumor cells acquire autocrine growth factor loops which activate these survival pathways. In melanoma, one of the earliest mechanism is the acquisition of an autocrine basic fibroblast growth factor (bFGF) loop [104]. Other growth factors, such as HGF, IL8 and PDGF-AA also act in an autocrine manner to aid proliferation, survival and migration of melanoma cells [105]. In addition, it has been recently described a specific pro-survival function of TrkB, a neurotrophic tyrosine kinase receptor, overexpressed in human tumors which possess a high aggressive nature and metastatic ability. In particular, TrkB, whose expression is increased in *anoikis*-resistant cells, acts as a potent suppressor of *anoikis* by activating the PI3K/Akt pathway [106,107].

Another strategy used by cancer cells to avoid *anoikis* is to change the pattern of integrin expression, so that the incorrect environmental survival signals coming from a wrong ECM may be correctly received. Only transformed melanocytes can survive in the altered environment of the *dermis*, this survival being due in part to the *de novo* expression of the correct integrins. Melanoma cells express αVβ3 integrin, which binds to fibronectin, vitronectin, collagen and laminin, and integrin αVβ1, which selectively binds fibronectin [108]. Expression of the β3 integrin subunit cannot be detected in melanocytes or early melanoma, but it correlates with progression to later stage of malignancy [109–111]. αVβ3 is important for adhesion of melanoma cells to dermal collagen and the suppression of *anoikis* [112], most likely by altering the Bcl2/Bax ratio [113].

Recent evidence obtained in our lab, suggests that ROS also play a fundamental role in conferring *anoikis* resistance to cancer cells. It is now largely accepted that cancer cells exhibit a higher amount of intracellular ROS with respect to their untransformed counterpart. By the comparison between untransformed prostate epithelial cells and metastatic prostate carcinoma cells, we observed that in cancer cells, the oxidative intracellular milieu strongly correlates with their resistance to *anoikis* induction. In particular, the high amount of intracellular ROS accounts for the constitutive oxidation and activation of the Src kinase in tumor cells, granting for a constitutive Src-dependent and ligand-independent phosphorylation of EGFR, thereby activating the pro-survival pathways [83]. Antioxidant treatment of prostatic cancer cells completely abolishes the ligand-independent activation of EGFR and the resistance to *anoikis*, thus restoring the apoptogenic stimuli. Conversely, exogenous addition of physiological doses of H_2O_2 in untransformed epithelial cells allows cells to escape from *anoikis*, underlining the crucial role of ROS in ensuring *anoikis* resistance (manuscript in preparation).

During tumor progression, two important changes affect the dynamics of tissue plasticity: the epithelial to mesenchymal transition (EMT) and the formation of a reactive stroma, reflecting intense structural rearrangement of the ECM. Both these phenomena participate to confer ability of cell to overcome *anoikis*.

EMT is an essential process that occurs during both physiologic and pathologic conditions [114,115]. Such transition, necessary for proper embryonic development, also provide a convenient system for epithelium-derived tumors to become highly invasive and rapidly metastasize [114,116], following a mechanism resembling a reactivation of the embryonic program of EMT. In both embryonic and tumorigenic EMT, migrating cells change their relationship with the ECM and become able to overcome *anoikis* and to successfully grow and survive in inappropriate location, ultimately colonizing distant organs. The activation of several mesenchymal genes in epithelial cells is critical for EMT success and for the overcoming of the apoptotic programme [117]. Molecules such as Snail, Twist, HGF/Met and NF-kB play a role in the acquisition of *anoikis* resistance during the EMT process.

Snail, a zinc-finger transcriptional factor, represses the transcription of E-cadherin, a critical step towards the malignant development in several epithelial-derived cancer [118], and activates the transcription of genes associated with mesenchymal differentiation, such as vimentin and fibronectin [119]. In addition, Snail confers apoptosis resistance by activating survival genes, such as the PI3K/Akt cascade, and by inhibiting caspase-3 activation [120,121].

Twist, a helix–loop–helix transcriptional factor, is considerably upregulated in several malignancies and its induction is linked to poor prognosis. Twist, which has an acknowledged function as a regulator of EMT by inducing the expression of mesenchymal markers such as fibronectin and N-cadherin [122], has been reported to play a key role as an anti-apoptotic factor. In particular, Twist signalling can modulate the apoptotic machinery by increasing the Bcl-2/Bax ratio that may provide a molecular basis for the ability of Twist to confer therapeutic resistance to taxol and vincristine in bladder, ovarian, prostate, and nasopharyngeal tumors [123]. Loss of twist expression renders the cancer cells to become more sensitive to *anoikis* induction and TNF-α-induced apoptosis [124,125].

HGF/Met, a widely recognized player of EMT in several cancers, has been correlated to the ability of tumor cell to escape *anoikis*. Indeed, upon EMT the *de novo* expressed Met receptor, induces *anoikis* resistance in uterine endometrial cancer cells by up-regulation of cyclooxygenase-2 expression, as well as in head and neck squamous cell carcinoma cells by activation of ERK and Akt signalling independent of NF-kB [126,127].

NF-kB, constitutively activated in human breast, prostate, colorectal, and ovarian tumors [128], is closely associated with tumorigenesis not only by maintaining these cells in the mesenchymal state [129], but also conferring apoptosis resistance to tumor cells [130]. Indeed, several lines of evidence point to the NF-kB signalling as a contributor to *anoikis* resistance within the tumor microenvironment, suppressing apoptosis via activation of the PI3K/Akt signalling cascade [70,131,132].

Another key player that promotes *anoikis* resistance, thus ensuring tumor cell spreading during the metastatic process, is hypoxia [133]. The intratumoral hypoxia is an indicator of poor patient prognosis and is associated with a malignant phenotype characterized by uncontrolled tumor growth, increased tumor cell invasiveness and metastatic potential, angiogenesis, and the development of resistance to radiotherapy and chemotherapy [134–136]. The mechanism by which hypoxia is able to induce escaping from the *anoikis* program has not been yet fully clarified. Several lines of evidence suggest that hypoxia contributes to loss of E-cadherin and expression of N-cadherin in solid tumors, inducing what is commonly indicated as the cadherin switch. One hypothesis for hypoxia-induced cell survival is that N-cadherin expression induced by hypoxia could be responsible for conferring *anoikis* resistance. Indeed, Li et al. demonstrated that in melanoma cells, N-cadherin-mediated adhesion promoted inappropriate survival and resistance to apoptosis by activating the anti-apoptotic protein PKB/Akt and subsequently stabilizing β-catenin and mild inactivating the pro-apoptotic factor Bad [137]. Moreover, it has been extensively demonstrated that hypoxia (1–3% O_2) induces a significant increase in the amount of intracellular ROS, mainly involving deregulation of the respiratory chain of mitochondria as a source [138]. As above reported, ROS play a fundamental role in transducing a pro-survival signal elicited by integrin engagement, through the redox regulation of the Src kinase and the ERK- and PKB/Akt-mediated polyubiquitination and degradation of the pro-apoptotic protein Bim [83]. Therefore, a further hypothesis for hypoxia-induced protection from *anoikis* is that the pro-oxidant environment induced by hypoxia could be responsible for conferring *anoikis* resistance, through a ROS-mediated down-regulation of pro-apoptotic factors.

Tumor cells do not exist as isolated entities but are instead embedded in a matrix of structural extracellular proteins, surrounded by other cells, such as endothelial cells, fibroblasts, inflammatory and immune cells [5,105,139]. These multiple proteins and cell types, the ''so called'' reactive stroma, are in continuous dynamic interactions with tumor cells. The stromal cells generate myriad of adhesive and chemical signals which converge to determine the metabolic, growth and survival behavior of the tumor cell. However, this communication is bi-directional, and tumor cells can re-model the stroma to suit its changing needs [139]. For instance, melanoma-derived growth factors, such as PDGF, transforming growth factor-beta (TGF-β) and bFGF, act in a paracrine manner to stimulate the growth and activation of the surrounding tumor-infiltrating fibroblasts [140] and to induce fibroblasts to produce ECM proteins, such as laminin, collagen and fibronectin [141,142]. There is also evidence that PDGF stimulation releases insulin-like growth factor (IGF-I) from stromal fibroblasts, which then promotes the survival of early-stage melanoma cells, through activation of pathways that suppress *anoikis* [143,144]. In particular, IGF-I is a potent activator of the Akt survival pathway and is able to block induction of apoptosis by TGF-β [145], thus conferring resistance to *anoikis* in a variety of cancer cells [146,147].

Table 3 – Disease states associated with a pathophysiological induction of *anoikis*

Diseases related to an over-induction of *anoikis*	Regulatory or signalling pathways involved in disease	References
Renal glomerular disease (postinflammatory scarring of the kidney)	Changement in ECM components of diseased glomeruli promote *anoikis* of renal mesangial cells	[148,149]
Cardiovascular degenerative pathologies	Induction of *anoikis* caused by the cleavage of adhesive proteins	[150–152]
Vascular dysfunction in diabetes	ECM glycation in hyperglycemia impairs endothelial cell survival	[153]
Ethanol-induced central nervous system dysfunctions	Astrocytes *anoikis* induced by a Rho-dependent actin cytoskeleton disorganization	[157–159]

5. Pathophysiological induction of *anoikis*

While the bulk of literature addresses the role of *anoikis* resistance during pathogenesis of cancer, other diseases seem to be the result of aberrant induction of *anoikis* (Table 3).

Irreversible postinflammatory scarring is an important and untreatable complication of tissue inflammation and represents a failure of this process, as it is associated with loss of functional resident cells. The progressive loss of normal resident cells is induced by changes in ECM composition, a phenomenon characterizing the postinflammatory scarring. In glomerular inflammation there is evidence that *anoikis* of mesangial and other resident cells may mediate the progression to irreversible glomerulosclerosis. This is associated with marked changes in the ECM of the glomerular mesangium. Indeed, different ECM components may differ in their capacity to support mesangial cell survival by suppression of apoptosis [148]. It has been demonstrated that collagen IV and laminin, which are normally limited to the interstitium components of normal mesangial ECM, protect rat mesangial cells from apoptosis induced by serum starvation and DNA damage, by a β1 integrin-mediated mechanism. In contrast, collagen I and fibronectin, which are overexpressed in diseased glomeruli, failed to promote rat mesangial cell survival. Hence, glomerular mesangial cell survival is dependent on interactions with ECM whose composition changes in renal glomerular disease and promote apoptosis of renal mesangial cells, providing insights into potential mechanisms by which resident cell loss may occur during acute inflammation and postinflammatory scarring of the kidney and other organs [149].

Moreover, enhancement of *anoikis*, not compensated by cell healing or overcompensated by a dysfunctional healing process is probably responsible for cardiovascular degenerative pathologies, such as cardiac myocyte detachment in heart failure, plaque rupture in atherosclerosis and smooth muscle cell disappearance in aneurysms and varicose veins [150,151]. Proteases able to degrade adhesive glycoproteins, such as fibronectin, induce *anoikis* of vascular endothelial cells. Active proteases can either be secreted directly by inflammatory cells, as elastase and cathepsin G by polymorphonuclear leukocytes, chymase and tryptase by mast cells, and granzymes by lymphocytes, or generated from circulating zymogens by activation in close contact with the cells. This is the case for the pericellular conversion of plasminogen to plasmin, which degrades fibronectin and induces *anoikis* of smooth muscle cells. The absence of cell adhesion and growth resulting from cleavage of adhesive proteins also represents a major obstacle to cellular healing, including the absence of cell recolonization of proteolytically injured tissue and the low efficacy of cell transplantation [152].

Interestingly, Dobler et al. have recently reported that chronic vascular disease in diabetes is associated with disruption of ECM interactions with adherent endothelial cells, compromising cell survival and impairing vasculature structure [153]. The authors show that methylglyoxal, whose formation is improved in hyperglycemia, provokes strong modification in integrin-binding sites of vascular basement membrane type IV collagen, causing endothelial cell detachment, *anoikis*, and inhibition of angiogenesis. Thus, increased formation of methylglyoxal and ECM glycation in hyperglycemia impairs endothelial cell survival and angiogenesis and likely contributes to similar vascular dysfunction in diabetes [153].

A clear evidence exists regarding the developing brain sensitivity to damage from environmental toxic compounds [154], and experimental findings also suggest that glial cells are susceptible to neurotoxic agents [155]. Ethanol is one of the most important teratogens that profoundly affects the developing fetal brain, and its consumption during gestation can produce mental retardation and neurobehavioral disorders, as well as fetal alcohol syndrome [156]. Indeed, it is widely reported that ethanol induces marked central nervous system dysfunctions. In particular, ethanol profoundly disorganizes the actin cytoskeleton in astrocytes, leads to the disappearance of stress fibres and to a decrease in the content of focal adhesions, thus adopting a 'rounded' morphology and mimicking the *anoikis* process [157]. These changes mimic some of the features observed during the initial steps of an *anoikis* process [158]. Interestingly, Minambres et al. have recently confirmed that astrocytes exposed to ethanol undergo morphological changes associated with *anoikis*, including the peripheral reorganization of both focal adhesions and actin–myosin system, cell contraction, membrane blebbing and chromatin condensation. Although in partial disagreement with current literature indicating a pro-survival role of Rho [3,12], the authors proposed that ethanol induces a Rho kinase activation and myosin light chain phosphorylation that is mediated by RhoA but independent by caspase-3. Hence, these findings suggest that ethanol-exposed astrocytes undergo *anoikis* and that RhoA participates in this process [159].

6. Concluding remarks

Anoikis regulation and dysregulation have recently and promptly emerged as leading topics in literature. This is particularly true for the several and unfortunately different manners that transformed cells use to elude the natural suicidal response that an ''homeless'' cell possesses. Unfortunately, it is misleading to think that in different cells the adhesion dependence is based on the same signal transduction pathways. Conversely, depending on whether or not a cell undergoes an apoptotic fate, several signals are affected in different tissues, and the final decision is the sum of all these inputs. Despite significant advances, it is clear that additional work remains to be done to establish mechanistic linkages between cell adhesion and *anoikis* execution. One of the actual open question and real challenge of *anoikis* research is to connect cytoskeletal organization driven by ECM with molecular signalling leading cells to execute apoptosis. Once defined, the molecular mediators of *anoikis* commitment may represent a major target for specific therapeutic manipulation of anchorage-independent cells.

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