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News and Commentary

Anoikis

AP Gilmore*,[1]

[1] Wellcome Trust Centre for Cell Matrix Research, Faculty of Life Sciences, A.3034 Michael Smith Building, The University of Manchester, Manchester M13 9PT, UK

* Corresponding author: AP Gilmore, Wellcome Trust Centre for Cell Matrix Research, Faculty of Life Sciences, A.3034 Michael Smith Building, The University of Manchester, Manchester M13 9PT, UK.
Tel: +44-161-2753892; Fax: +44-161-2751505;
E-mail: agilmore@manchester.ac.uk

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An important aspect of multicellularity is that cells only grow and differentiate when in the correct context within a tissue, and remove themselves by apoptosis when they are not. Cells sense their location through specific interactions with the extracellular matrix (ECM) as well as neighbouring cells. Apoptosis in response to inappropriate cell/ECM interactions is termed anoikis, a name that in some way implies a special case of cell death initiated by signals not used in response to other proapoptotic insults.[1] In practice, different cell types use diverse mechanisms to interpret signals from the ECM, all of which are found to regulate apoptosis in response to many other stimuli. The purpose of this review is to show that rather than anoikis being a specific stimulus of cell death, it is in fact a broad range of cellular responses to loss of adhesion that utilise diverse signalling and apoptotic pathways.

Anoikis – A Short Historical Perspective

Anoikis is apoptosis induced by lack of correct cell/ECM attachment, and many experimental systems study this by completely detaching cells from the ECM. Some thought is required regarding the significance of anoikis *in vivo*. Cells in tissues require very specific ECM attachments, and the wrong type of ECM can have the same consequences as no ECM at all. The importance of anoikis *in vivo* can readily be seen when alterations that perturb its normal control are seen to enhance tumour metastasis, a process which requires cells to survive in totally inappropriate ECM environments.[2] Anoikis, therefore, should not be considered as an experimental system *in vitro*, but the mechanism by which cells *in vivo* use ECM-derived signals to maintain tissue integrity.

Although anchorage dependence of cells has been recognised for many years, particularly regarding proliferation,[3] anoikis as we understand it was first described in the early 1990s. Almost simultaneous papers from the groups of Martin Schwartz and Steve Frisch showed that cells that were deprived of attachment to the ECM underwent classical apoptosis.[1,4] The significance of these papers was that signals from the ECM were recognised to be required to prevent cells from actively undergoing programmed cell death. These papers also demonstrated some fundamental aspects of anoikis. Firstly, ECM receptors of the integrin family are essential for cells to suppress anoikis. Thus, plating cells onto anti-integrin antibodies prevented anoikis, whereas attachment to antibodies recognising other cell surface proteins did not, suggesting that specific integrin-dependent signalling is required. Second, anoikis was inhibited by overexpression of Bcl-2, indicating a requirement for mitochondrial membrane permeabilisation (MMP) to occur. Thirdly, not all cell types are equally sensitive to anoikis. Epithelial and endothelial cells were found to be more sensitive than fibroblasts, the latter being able to survive in the absence of ECM if serum growth factors were present. Furthermore, epithelial cells can be switched between anoikis sensitive and insensitive by oncogenic transformation or treatment with scatter factor. Such processes not only induce cells to become migratory but also to become insensitive to anoikis. Transformation with H-Ras was reverted by adenovirus E1a, which restored sensitivity to anoikis. The sensitivity of cell to anoikis appears, therefore, to be associated with epithelial to mesenchymal transition, transformation and immortalisation.

Signals from the Matrix – Clarifying or Confusing?

Adhesion receptors not only provide a physical attachment to the ECM but they also create an adhesion-dependent signalling scaffold containing a number of adaptor proteins and kinases.[5,6] Thus, integrins function in an analogous way to growth factor receptors (GFRs), and indeed activate many of the same downstream pathways. Integrins also crosstalk directly with GFRs, and allow cells to respond optimally to soluble cytokines only when they are attached to the correct ECM.

Specificity for particular types of ECM occurs through the range of integrins expressed on cells.[6] Humans have at least 24 different integrins, and although some are expressed on the same cells and even recognise the same ECM components, many have essential roles in specific tissues. Mammary epithelial cells adhere to a laminin-rich basement membrane via integrin $\alpha 6\beta 1$. The stroma underlying the mammary ducts and alveoli contain collagen I, which is recognised by $\alpha 2\beta 1$ on the mammary cells. However, although they express collagen-binding integrins, they do not support mammary cell survival and they eventually undergo apoptosis.[7,8] Melanocytes are similarly kept in the correct tissue compartment through integrin/ECM interactions. The underlying dermis is rich in collagen, and this fails to support melanocyte adhesion and survival, as unlike MEC they do not express suitable integrins. However, during melanoma invasion through the collagen-rich dermis, upregulation of $\alpha v\beta 3$ on the melanoma cells allows them to receive antiapoptotic signals from a normally hostile ECM environ-

ment.[9] Inhibition of $\alpha v\beta 3$ function using blocking antibodies induced melanoma cell apoptosis.

So how does adhesion via integrins keep cells alive? Integrin-mediated adhesion regulates all the same signalling pathways that control apoptosis in growth factor-mediated survival, DNA damage responses and death receptor-mediated apoptosis, although to different extents. Which pathways regulate anoikis varies depending on cell type, with different integrins activating distinct signalling cascades (Figure 1). For example, integrins can activate PI3-kinase signalling, the classical ERK pathway, as well as stress-activated MAP kinases like c-Jun N-terminal kinase (Jnk).[5] These can be activated in a number of ways specific to different integrins. Some integrins ($\alpha 1\beta 1$, $\alpha 5\beta 1$ and $\alpha v\beta 3$) recruit the src family kinases Fyn and Yes via an interaction with caveolin 1, which can then activate the classical ERK pathways by recruiting Shc, Grb2 and Sos[10] (Figure 1a). Many integrins recruit pp125FAK (focal adhesion kinase), a nonreceptor tyrosine kinase that is activated in response to adhesion[11] (Figure 1b). Pp125FAK interacts with a range of signalling and adaptor molecules, including Src, PI3-kinase, paxillin and p130CAS, and has been linked to a number of signalling pathways controlling migration, proliferation and apoptosis. Integrin-linked kinase is also recruited to adhesion sites, and has been implicated in survival signalling.[12,13] There is considerable overlap between the downstream pathways activated by these alternative integrin signalling mechanisms. I will highlight a few examples that illustrate the diversity in anoikis signalling.

Pp125FAK has been shown to be required to suppress anoikis in a number of cell types, either through expression of dominant-negative forms, microinjection of anti-FAK antibodies or use of dominant active forms to suppress cell death.[14–16] FAK can regulate PI3-kinase signalling, MAP kinase signalling, small GTPases and other tyrosine kinases such as those of the Src family, all of which can influence cell survival.[11] In MDCK cells, detachment from ECM can activate Jnk, providing a proapoptotic signal.[17] However, a different study also using MDCK cells found no correlation between Jnk and anoikis.[18] Instead, activation of PI3-kinase in adherent MDCK cells was required to suppress anoikis, similar to that seen in mammary epithelial cells.[14] Jnk can be either pro- or antiapoptotic, depending on its cellular context. A study in primary fibroblasts found that pp125FAK activation of Jnk in adherent cells was required to suppress anoikis.[19] In fibroblasts, anoikis was also found to be dependent on p53.[20] In serum-free conditions, FAK is required to suppress p53-dependent apoptosis. In the absence of FAK or adhesion, p53 is activated via phospholipase A2 and protein kinase Cγ. Anoikis was inhibited by either overexpression of Bcl-2 or by a dominant-negative form of p53, indicating that this p53-dependent mechanism still required mitochondrial permeabilisation. In the same study, growth factors provided a strong PI3-kinase-dependent survival signal and the fibroblasts were no longer sensitive to inhibition of pp125FAK.

Adhesion to the correct ECM alone is not sufficient to provide a survival signal. Cell spreading and shape can profoundly influence phenotype, and the role of the cytoskeleton in these aspects of adhesion signalling is critical. The degree of spreading of endothelial cells on micropatterned substrates caused a switch between proliferation, differentiation and apoptosis, independent of the type of ECM and integrin used for attachment.[21,22] Similarly, mammary epithelial cells require a specific three-dimensional (3-D) arrangement to suppress apoptosis.[23] This regulation of apoptosis through the arrangement of cells within a 3-D architecture may contribute to mammary gland morphogenesis.[24] Cell shape is controlled by the cytoskeleton and its connections with integrins at cell/ECM and cell/cell junctions. Changes in these mechanical forces can alter cellular signalling pathways associated with cell adhesion, thus influencing survival.[25,26] The ways in which cells sense mechanical forces associated with spreading and tissue architecture, and how these affect signalling, are reviewed in detail elsewhere.[27–29]

A further complication arises when we consider crosstalk between integrins and GFRs. Many GFRs are influenced by adhesion to ECM, allowing, for example, anchorage-dependent control of proliferation.[30] Pp125FAK signalling can directly influence the ability of GFR to control ERK activation and G1–M transition. It is not surprising, therefore, that this crosstalk can influence anoikis (Figure 1c). In primary oligodendrocytes, integrins crosstalk with GFR, allowing target-dependent survival in conditions of limiting growth factors.[31] At physiological levels of neuregulin, attachment of newly formed oligodendrocytes to laminin on axons, via $\alpha 6\beta 1$, was required to fully activate survival signals. The laminin/$\alpha 6\beta 1$ interaction allowed neuregulin to activate a strong ERK dependent survival signal. Epithelial cells also show regulation of anoikis through integrin GFR crosstalk. Epithelial cells are dependent on both growth factors and adhesion for survival, and the absence of either results in apoptosis. However, they are not necessarily working through distinct signals. Primary mammary epithelial cells depend upon adhesion to laminin along with the insulin-like growth factor 1 (IGF-1).[32] Activation of the IGF-1 receptor suppresses apoptosis through a PI3-kinase-dependent pathway.[33] Attachment to laminin appears to be a requirement for this IGF receptor signalling. Primary MEC grown on collagen do not efficiently activate PI3-kinase in response to IGF-1 and undergo apoptosis. The human MEC line MCF10A shows requirement for epidermal growth factor (EGF) receptor (EGFR) signalling, although in this case it appears to function through the classical ERK pathway.[34] In the absence of adhesion, MCF10A cells rapidly lost cell surface expression of EGFR, leading to upregulation of the Bcl-2 protein Bim. A breast tumour cell line did not show this detachment-induced downregulation of EGFR, and overexpression of EGFR in MCF10A cells inhibited anoikis. Interestingly, different MEC lines appear to show differences in how integrins link with GFRs to control anoikis.[35] Thus, the boundary between integrins and growth factors in apoptosis regulation appears blurred.

Links to the Cell Death Machinery – Intrinsic *versus* Extrinsic Control of Anoikis

The above discussion has highlighted how suppression of anoikis is extremely variable between different cells. Conse-

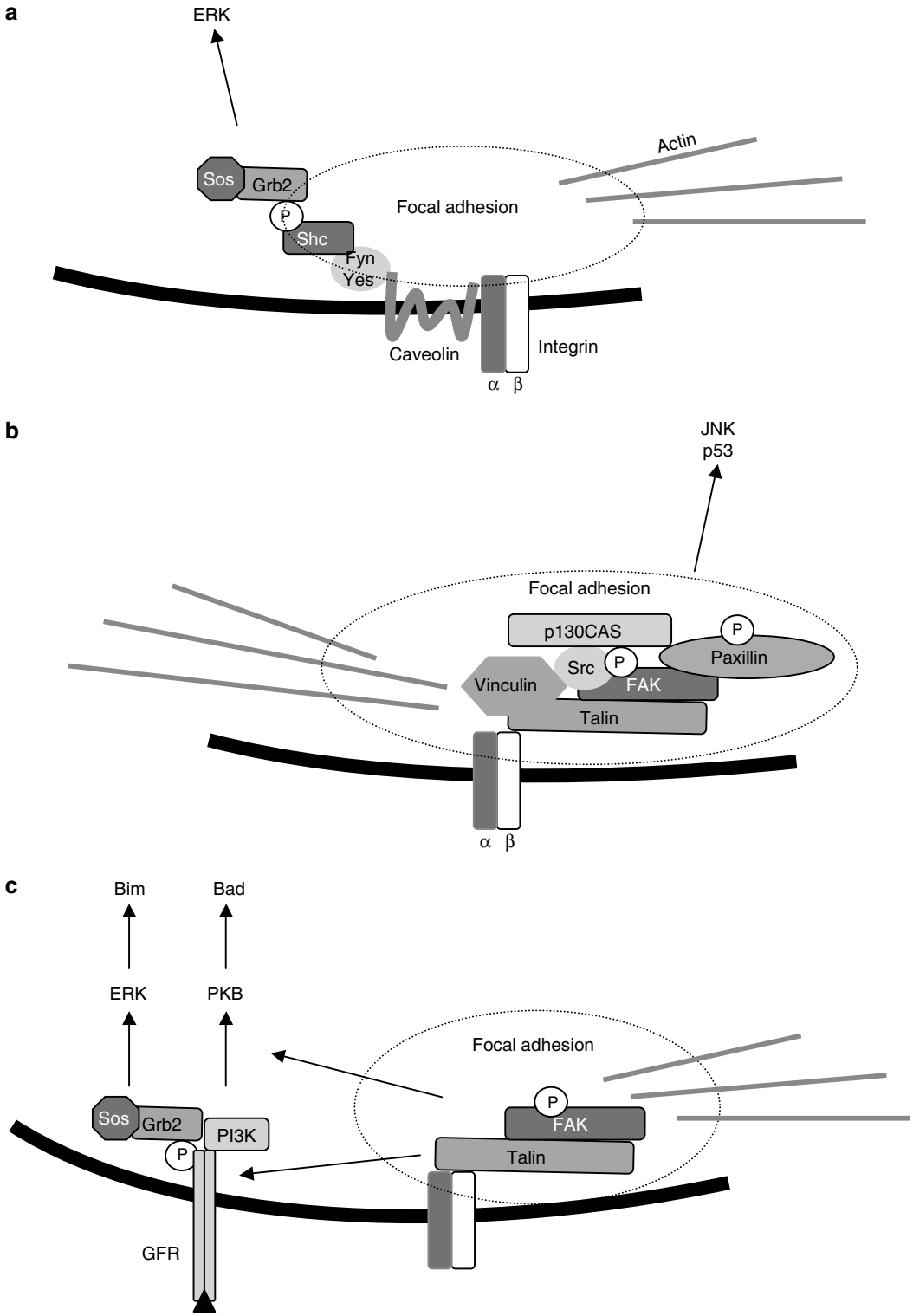

Figure 1 Schematic illustration of just some of the molecular connections from adhesion complexes (focal adhesions) that are implicated in survival signalling. The assembly of adhesion complexes is dependent not only on interactions between integrins and the underlying ECM but also on connections with the cytoskeleton. Activation of integrin signalling is, therefore, more complex than a simple receptor/ligand interaction. Refer to the main text and references therein for details. (**a**) The α-subunits of certain integrins can recruit the Src family kinases Fyn and Yes through interactions with caveolin, which then activate the classical ERK pathway. (**b**) Most integrin β-subunits recruit FAK through interactions with proteins such as talin. Talin, vinculin and other focal adhesion proteins act both as linkers between integrins and the actin cytoskeleton, and as a scaffold for the recruitment of signalling molecules like FAK and paxillin. (**c**) Adhesion and GFR signalling are intimately linked, and regulation can occur at a number of levels. Adhesion to ECM can control the expression of GF receptors, whether or not they can be activated, or if they can interact with downstream effectors such as the classical ERK and PI3-kinase pathways

quently, how they activate the cell death machinery downstream of these signals can also be expected to be diverse. Individual BH3-only proteins, such as Bmf, have been proposed to control anoikis, as has activation of death receptors. Reality is likely to be more complex and cell type specific.

Anoikis has been proposed to be regulated via both the intrinsic and extrinsic pathways. In the intrinsic pathway, caspase activation occurs as a consequence of mitochondrial permeabilisation.[36,37] This is regulated by the Bcl-2 family of proteins, which control the formation of pores in the outer mitochondrial membrane (OMM), releasing proapoptotic factors such as cytochrome *c*, which activates caspase.[9] The extrinsic pathway is initiated by the ligation of death receptors on the cell surface, such as TNFR or Fas, resulting in the assembly of a death-inducing signalling complex (DISC). At its simplest level, the role of the DISC is to recruit and activate caspase 8, via an adaptor molecule, FADD.[38] Cells that respond to extracellular death ligands fall into two classes, type 1 and type 2.[36] In type 1 cells, activation of caspase 8 is sufficient to cleave effector caspases and result in cell death. In type 2 cells, which make up the majority, caspase 8 cannot itself initiate apoptosis, and instead cleaves the BH3-only protein Bid, which then initiates the intrinsic apoptosis pathway. In both type 1 and type 2 cells, the initiating signal is caspase 8 activation. The question regarding anoikis is which pathway is required. The answer may not be clearcut.

Anoikis requires OMM permeabilisation as is it can be blocked by overexpression of antiapoptotic Bcl-2 proteins.[17,20] However, 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.[39,40] However, extracellular inhibitors of death receptors (soluble extracellular portions of the receptors, which can sequester the ligand) 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. How death receptors are activated during anoikis is not clear, as in the above studies, sequestering extracellular death ligands did not inhibit cell death. In endothelial cells, adhesion to ECM appeared to suppress activation of Fas by inducing expression of c-Flip, a noncatalytic antagonist of caspase 8, as well as suppressing expression of Fas itself.[41] Expression of c-Flip was regulated by adhesion-dependent Erk activation, although pp125FAK did not appear to be involved. Another study has recently suggested that FAK can directly bind to RIP, a serine/threonine kinase that interacts with death receptors.[42] RIP has been shown to have a role in determining if TNF-R stimulates an apoptotic response or not, although exactly how this occurs is unclear. It is also not clear if FAK regulates RIP in an adhesion-dependent way. It will be important to know if RIP is localised to cell/ECM adhesion sites and if detachment results in its relocalisation.

Other studies have indicated that anoikis proceeds through the intrinsic pathway. The proapoptotic proteins Bax and Bak are activated following detachment of cells from ECM. In mammary epithelial cells, Bax translocates from the cytosol to the OMM within 30 min of loss of ECM attachment.[14,43] This is accompanied by the exposure of N-terminal epitopes indicative of it switching from the inactive to proapoptotic conformation. These cryptic epitopes can be detected in primary MEC attached to collagen, an ECM on which they undergo apoptosis. Primary MEC deficient for Bax were resistant to anoikis. Bak also shows exposure of cryptic N-terminal epitopes during anoikis of MEC (our unpublished data). Adhesion can also regulate the antiapoptotic members, and fibroblast adhesion via $\alpha 5\beta 1$ integrin increases Bcl-2 expression.[44]

The question of how signalling cascades regulated by adhesion activate Bax and Bak is less clear. Current models for apoptosis regulation indicate that the BH3-only subfamily of the Bcl-2 proteins are likely to be involved.[45] These act as sentinels that respond to diverse apoptotic signals and then regulate the function of the multidomain Bcl-2 proteins like Bcl-X_L and Bax. Interestingly, a number of the BH3-only proteins appear to be controlled by signalling pathways that have been implicated in anoikis in one or more cell types. Noxa and Puma are transcriptionally regulated by p53, which has been implicated in fibroblast anoikis, although their expression has not been tested in those studies.[46,47] Bim and Bad can be controlled by the PI3-kinase and ERK pathways, and Bid may be cleaved in the death receptor pathway.[48–51] Recently, noncleaved Bid has been shown to translocate to mitochondria in MEC detached from ECM, cells in which activation of the death receptor pathways does not occur during anoikis.[52] Another 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 it is activated in a causative way following detachment from the ECM.[53] Some BH3 proteins have been directly implicated in anoikis. siRNA downregulation of Bid inhibited anoikis in FSK-7 mammary epithelial cells, and in another study, similar knockdown of Bim prevented apoptosis of MCF10A cells.[34,52]

Other BH3-only proteins have not been shown to directly regulate of anoikis, although they appear to influence sensitivity. Overexpression of Bad did not induce apoptosis in adherent MDCK cells, but did sensitise them to anoikis.[54] Furthermore, primary MEC deficient for Bad did not undergo apoptosis when cultured on collagen, unlike their wild-type counterparts.[55] Therefore, although Bad does not appear to cause anoikis directly, it does appear to affect cell sensitivity. Recent results on different BH3-only proteins and BH3 domain peptides indicate that some of these molecules work to directly activate Bax and Bak (specifically Bim and Bid), whereas others inactivate the antiapoptotic functions of Bcl-2 and Bcl-X_L.[56,57] Those that inactivate Bcl-2 and Bcl-X_L appear to sensitise, or prime, cells for apoptosis in response to Bid or Bim, but are unable to induce mitochondrial permeabilisation themselves. Conversely, Bid and Bim can induce the proapoptotic proteins Bax and Bak to induce OMM permeabilisation. It follows then that either Bid or Bim should be activated in response to anoikis, and as discussed above, this appears to be the case in two different MEC lines. How Bim is upregulated is well understood, but our understanding of Bid activation during intrinsic apoptosis signalling is poor and requires much further work, largely due to it long being thought

to be exclusively involved in death receptor signalling. Clearly, multiple adhesion-dependent signalling pathways can influence diverse members of the Bcl-2 family, ultimately controlling OMM permeabilisation.

Conclusions

Anoikis is an essential mechanism for maintaining the correct position of cells within tissues. Induction of anoikis occurs when cells lose attachment to ECM, or adhere to an inappropriate type of ECM, the latter being the more relevant *in vivo*. However, this superficial similarity in how anoikis is initiated masks the many diverse ways in which cells signal via adhesion receptors to regulate apoptosis. How one cell responds to incorrect adhesion will be quite different from another type of cell, involving a distinct set of signalling enzymes and apoptosis regulatory proteins. Also, given the complexity of integrin/growth factor crosstalk, it also becomes difficult to determine the boundary between adhesion and cytokine dependent survival. As such, it is perhaps misleading to think that anoikis is regulated by, for example, specific BH3-only proteins. Instead, loss of adhesion can control signalling pathways that can activate most of, if not all, the BH3-only proteins, depending upon which cell type you examine. Choice of cell type for study has a further important implication. Cell lines have been selected for their ability to grow over many passages in culture. Such lines therefore show a marked loss in sensitivity to anoikis, as every passage is detached from ECM before being replated. Many studies on anoikis have therefore been based on culture models that are not truly anchorage dependent for survival. Mammary cell lines, such as FSK-7 and MCF10A, do not require adhesion to laminin to suppress apoptosis, unlike primary MEC that absolutely require laminin. Changes in cell lines most likely dampen the exquisite sensitivity of primary cells to anoikis. To truly understand the significance of anoikis, a greater understanding of how it operates *in vivo* is required.

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