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Review

Lumen formation during mammary epithelial morphogenesis

Insights from in vitro and in vivo models

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The mammary gland undergoes a morphogenetic program during embryogenesis and puberty that leads to the development of hollow ductal system terminating in acinar units. It later expands to generate an elaborate network to deliver milk to newborn progeny. Previous studies in our laboratory using three-dimensional basement membrane cultures of mammary epithelial cells, in which acini-like structures form from single cells, have indicated that lumen formation requires clearance of the cells in the center of the acini by apoptosis. This apoptotic program in vitro requires the pro-death mediator BIM. Recently we found that BIM is also required in the mouse mammary gland for apoptosis during lumen formation, which correlates the 3D acinar model to mammary morphogenesis in vivo. Herein we put into perspective the relevance of our in vitro and in vivo findings to discuss luminal space formation and maintenance during mammary morphogenesis.

Introduction

During epithelial organogenesis, lumen formation is essential for building functional networks of epithelial tubes of different sizes and forms in organs such as the pancreas, lung, or the mammary gland. Within these various organs, different modus operandi are utilized to shape tubular structures, for example deformation of a pre-existing epithelial sheet during lung morphogenesis or cavitation in breast development.[1,2] Lumen formation is secondary to the branching morphogenesis in mammary glands, while these two processes are concomitant during lung embryonic development.[2] Within the mammary gland, epithelial cells are organized into spherical structures with a lumen, termed acini, which produce milk that is transported to the nipple through a network of ducts to feed offspring during lactation. The model for lumen formation in acini is a cavitation model,[3] in which the clearance by apoptosis of an inner cell population within newly branched epithelial chords creates a luminal space.[2] We investigated the regulation of luminal cell death during cavitation in 3D cultures of the human mammary MCF-10A cell line[4,5] which forms acinus-like, hollow structures.[6,7] In fact, the in vitro MCF-10A 3D model was critical to orient studies to better understand the regulation of cavitation process in mammary morphogenesis in vivo. In the light of our recent in vivo findings,[8] we would like to put into perspective the relevance of the MCF-10A 3D model[7,9,10] for recapitulating some aspects of mammary morphogenesis in vivo.

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Similarities between in vitro and in vivo models

As mentioned above, lumen formation during in vitro as well as in vivo mammary morphogenesis requires apoptotic clearance of the inner cells within the developing structures. In vitro, MCF-10A cells (an immortalized line of non-tumorigenic human mammary epithelial cells[4,5]) undergo a morphogenetic program leading to the development of spherical acini-like structures when cultured in Matrigel™, a gel-like mixture of basement membrane proteins (predominantly collagen, laminin and entactin).[11] In this system, a single cell proliferates to form a solid cell cluster, and after four to five days, the outer, matrix-attached cells develop an axis of apical-basal polarity and undergo growth arrest (Fig. 1A). After seven to eight days, the acini remodel in a fairly synchronous fashion to create a lumen by selective apoptotic death of cells in the center of the mass. The mature structures are composed of a single layer of epithelial cells surrounding a hollow lumen (Fig. 1A).

During mammary gland formation in vivo, the rudimentary mammary tree undergoes extensive expansion from four weeks to eight weeks after birth. This expansion results from proliferation and invasion at the leading front of the ductal outgrowths. The highly proliferative bulbous structures at the tips of these expanding outgrowths, referred to as terminal end buds (TEBs), develop in response to elevation in local and systemic reproductive hormones. The pubertal terminal end buds are composed of two main cell populations: the cap cells and the body cells.[12] The cap cells are located in the outer-layer in contact with the stroma and are considered to be progenitors of the myoepithelial cells. The majority of the body cells, organized with six to ten cell layers within the TEB, are thought to be precursors for the luminal lineage (see Fig. 1B). It was previously shown that apoptosis is detected within the body of TEBs.[13] The spatial and temporal pattern of apoptosis suggested that caspase-mediated apoptosis maintains the lumen within the expanding duct (Fig. 1B).

The molecular mechanism underlying lumen formation in terminal end buds in vivo and MCF-10A 3D acini in vitro appears to be shared

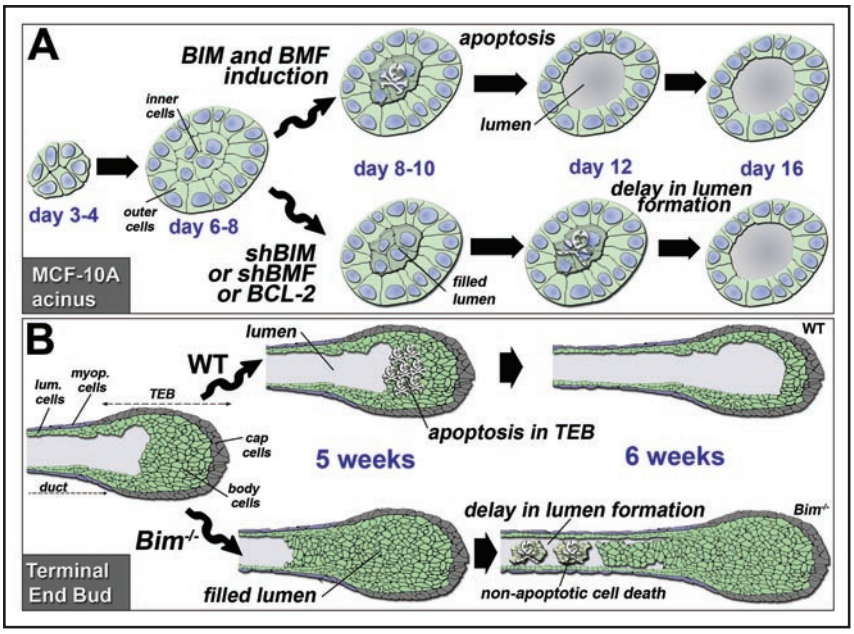

Figure 1. lumen formation mammary morphogenesis in vitro and in vivo. (A) Schematic of MCF-10A acinar morphogenesis. Apoptosis of the inner cells lacking matrix attachment correlated with the induction of the pro-apoptotic BH3-only factors BIM and BMF. This process was inhibited when BIM or BMF were downregulated by siRNA approach or in MCF-10A cells over-expressing BCL-2. Meanwhile, lumen formation was not completely inhibited but delayed. Details are provided in main text. (B) Schematic of lumen formation in terminal end bud during puberty. During puberty, apoptosis of the TEB body cells created new luminal tubes in dutcal outgrowth. Apoptosis in TEB was abolished in the *Bim*$^{-/-}$ female mouse and lumen formation in mammary ducts was delayed of several weeks. Details are provided in main text. Abbreviation: *shBIM*, *BIM* short hairpin RNA; *shBMF*, *BMF* short hairpin RNA; lum. cells, luminal cells; myop. cells, myoepithelial cells; WT, wild type; TEB, terminal end bud.

to some extent, as both require the pro-apoptotic factor BIM.[9] BIM is a BH3-only member of the pro-apoptotic BCL-2 protein family, which includes also Bmf, Bik, Bad, Bid, Puma, Noxa and Hrk. During in vitro morphogenesis, inhibition of BIM expression by transient small interfering RNA (siRNA) or stable short hairpin RNA (shRNA) significantly decreases apoptotic cell death of the central cell cluster triggering a transient luminal filling.[9] Similarly in the mammary gland in vivo, epithelial cells lacking *Bim* are deficient for apoptosis induction in the TEB and ducts at five weeks and this defect is associated with defective lumen formation[8] in TEBs (Fig. 1B).

In addition, the stimuli triggering BIM apoptotic activity may be similar in 3D acinar structures in vitro and the mouse mammary gland in vivo. In vitro studies of BIM regulation have revealed its role as a sensor that integrates growth factor signals and extracellular matrix (ECM) attachment in mammary cells.[14,15] BIM expression is upregulated upon matrix detachment as well as EGF withdrawal in MCF-10A cells,[14] while EGFR expression is lost in suspension. In MCF-10A acini, the cells in the center of the epithelial mass lack matrix attachment, and thus apoptosis may be induced by mere detachment from matrix (termed anoikis hereafter). Indeed, when MCF-10A cells are simply detached and cultured in suspension, BIM induction is required for apoptosis as inhibition of *BIM* by siRNA decreases cell death by 50%[14] (Fig. 2). Recently, the function of BIM as an anoikis sensor has been questioned.[15] In mouse mammary cells (Fsk7 cell line and primary mouse MEC), for which the lack of ECM attachment triggers a rapid apoptotic response (4 h to 8 h), BIM was induced in response to growth factor withdrawal but not integrin disengagement. The down-regulation of *Bim* expression by siRNA in mouse Fsk7 cell line had no effect on the extent of anoikis.[15] In contrast, in human MCF-10A cells BIM seems to integrate both stimuli,[14] although the apoptotic response upon anoikis is delayed in this cell line (48 h–72 h). Thus, the extent to which anoikis in MCF-10A cells is dependent on both loss of integrin signaling as well as loss of growth factor (EGF) signaling, might dictate the requirement for BIM in apoptosis as compared to other cell lines that undergo a more rapid anoikis that is not co-regulated by loss of EGFR signaling.

These findings raise the question whether apoptosis of body cells during pubertal outgrowth is provoked by deprivation of matrix and growth factor signals. Our data from the mouse mammary gland clearly demonstrate that the body cells of the TEB experience a lack of matrix attachment,[8] which is likely one mechanism that triggers apoptosis associated with luminal clearing. The luminal filling observed in the absence of *Bim* suggests that inner cells require BIM to respond to loss of ECM attachment in vivo, demonstrating that BIM could be an in vivo bona fide anoikis sensor in the mammary epithelium. Nevertheless, ERK activation is decreased within the TEB as well, as evidenced by a loss of phosphorylated ERK staining in the inner-most cells,[16] suggesting that the inner cells in the TEB may also experience growth factor withdrawal, which in turn may synergize with the lack of matrix attachment to trigger BIM-mediated cell death. Thus it is possible that BIM is required for anoikis in vivo in the mammary gland because this death process, like anoikis in MCF-10A cells, is induced by a loss of both integrin and growth factor signaling.

In our in vitro model of 3D acini and also in the mammary gland in *Bim*$^{-/-}$ mice, the downregulation or loss of BIM[8] prevented apoptotic clearance of cells, however, interestingly lumen formation was merely delayed, not abrogated, in both models. A similar transient luminal filling was observed in MCF-10A cells over-expressing the anti-apoptotic factor BCL-2.[10] These data support a most interesting conclusion that apoptosis, while required for efficient luminal clearing in both models, is ultimately not required for the formation of a lumen. This is most clearly demonstrated by the fact that *Bim*$^{-/-}$ mice are able to produce milk and nurse their young with no obvious defects compared to wild-type mice. Unfortunately in vivo, the mosaic overexpression of BCL-2 in mammary gland of transgenic mice precluded evaluation of the consequences of apoptotic inhibition during pubertal outgrowth.[13]

Elucidation of the alternative mechanisms for luminal clearing would provide valuable insights into both developmental processes and also uncover potential novel tumor suppressive mechanisms, as one hallmark of breast tumors is the ability to survive and grow within the luminal space. The study of the 3D structures grown from MCF-10A cells over-expressing BCL-2 (see Fig. 1A) suggested that one alternative program of lumen formation might involve autophagy.[10,17] Although we have not yet found evidence for autophagy acting as a luminal clearing mechanism in vivo, definitive

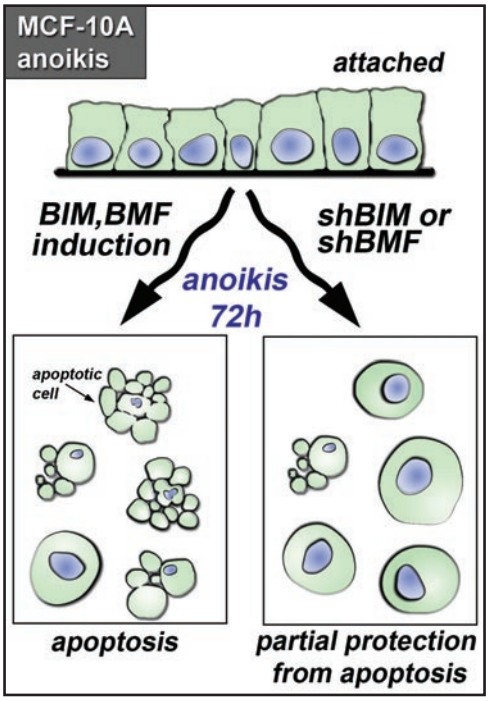

Figure 2. anoikis of MCF-10A cells upon loss of matrix attachment. Schematic of MCF-10A undergoing anoikis after at least 48h in suspension. Similarly to the 3D acinar morphogenesis, apoptosis upon loss of matrix attachment required induction of the pro-apoptotic BH3-only factors BIM and BMF. Details are provided in main text. Abbreviation: *shBIM*, *BIM* short hairpin RNA; *shBMF*, *BMF* short hairpin RNA.

tests of this hypothesis will require breeding the *Bim*$^{-/-}$ mice with mouse lines defective for autophagy (e.g., *Atg5*$^{-/-}$[18]). Within the *Bim* defective filled ducts in vivo, the inner cells lacking matrix attachment appeared to undergo an alternative, non-apoptotic death at 6 weeks (see Fig. 1B). The inner cells undergoing the non-apoptotic cell death in the *Bim*$^{-/-}$ mammary ducts did not any display the morphological features characteristic of dying cells in late apoptosis. Rather, these dying cells displayed necrotic features such as compromised cytoarchitecture, karyolysis and DNA fragmentation; and lacked apoptotic features such as cellular blebbing, caspase activation and nuclear condensation. Unlike apoptosis, non-apoptotic cell death processes are poorly characterized (even in vitro). Given the absence of defined and distinct markers and the lack of reporter activity, it is a difficult to dissect this process. We have shown that reactive oxygen species (ROS)-induced stress might contribute to this process. Indeed, detachment from matrix, growth factor withdrawal, and other events that compromise cellular metabolic activity can lead to elevated levels of reactive oxygen species (ROS) which can contribute to necrotic cell death, independent of apoptosis.[19]

In conclusion, our in vivo data validated previous in vitro studies that identified BIM as a critical player in the induction of apoptosis associated with lumen formation of 3D MCF-10A acinus-like structures. At this point, one could argue like the philosopher Pangloss[20] that "all is for the best in this best of all possible worlds". However, as we will discuss below, the MCF-10A 3D model of mammary acini does not mimic all the aspects of the molecular regulation of lumen formation recently revealed in vivo.

Discrepancies between in vitro and in vivo models

Even though the 3D MCF-10A model can be useful to define cellular pathways that control lumen formation in vitro,[10] this culture system does not precisely replicate the in vivo microenvironment of the breast. As we mentioned before, these structures are formed by a monolayer of mammary cells organized in an acinus-like structure (see Fig. 1) within a solubulized preparation of basement membrane proteins extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma[10,11] Another consideration is that MCF-10A cells do not represent a true luminal population, as they express markers of both luminal and basal/myoepithelial cell lineages,[21] with predominance of basal/myoepithelial markers. In vivo, the breast acini and ducts are organized in an epithelial bilayer comprised of luminal cells surrounded by a myoepithelial cell layer in a basement membrane of somewhat distinct in composition from the EHS secreted matrix. However, the double-layered breast acinus can be recapitulated in vitro with co-culture of primary myoepithelial and luminal cells.[22] While use of primary heterotypic cultures offers some advantages, these cells are highly heterogeneous and it is difficult to use them to in a experimental approaches that involve genetic manipulation of genes in order to examine effects of loss or gain of function due to this significant heterogeneity. There are several other mouse and human cell lines that have been used for in vitro 3D cultures.[23-27] Thus, while there are limitations of immortalized cell lines, they offer attractive models for mechanistic studies.

In vitro and in vivo studies showed that apoptosis during lumen formation is a BIM-mediated process in both the mouse terminal end bud[8] and human MCF-10A 3D system.[9] However, we have evidence that the regulation of BIM pro-apoptotic activity might diverge between these in vitro and in vivo mammary systems. Three major forms of BIM protein, small (BIM-S), large (BIM-L), and extra-large (BIM-EL), can be generated by alternative mRNA splicing. BIM-EL is the major isoform expressed in the MCF-10A cells[9] and in the mouse mammary gland (Mailleux AA, Brugge JS, unpublished data). This isoform can be specifically degraded by the proteasome after ERK1/2 mediated phosphorylation on serine 69 (human) or serine 65 (mouse).[28,29] During MCF-10A 3D morphogenesis, BIM was not detectable during early stages and its expression was highly upregulated concomitantly with apoptosis and lumen formation. BIM was detected in both the outer and inner cells of acini and its expression was maintained even in mature hollowed MCF-10A acini at later days.[9] BIM was also phosphorylated on serine 69 in mature hollow acini, suggesting that ERK mediated proteasomal degradation of BIM may protect these cells from apoptosis. In contrast, our in vivo study shows that BIM is constitutively expressed in the mouse mammary epithelium from the first stage of mammary embryonic development.[8] This difference does not seem to be specific to mouse mammary gland, as BIM is also ubiquitously expressed by the adult human mammary epithelium, in the alveoli and the ducts as well (Mailleux AA, Brugge JS, unpublished data). In mouse mammary gland protein extract, we failed to detect a shifted band corresponding to the phosphorylated BIM-EL form seen in MCF-10A cells by western blotting; suggesting that BIM-EL is predominantly unphosphorylated in mammary gland during pubertal development (Mailleux AA, Brugge JS, personnal communication). Unlike the MCF-10A system, BIM apoptotic activity may not be regulated by

phosphorylation and proteasomal degradation in normal mammary cells, in vivo. The study of mouse models carrying a mutation of the ERK targeted Serine 65 in BIM would help to clarify this point.

Recent findings in our laboratory showed that BIM pro-apoptotic function overlapped with BMF, another BCL-2 BH3-only factor, during acinar morphogenesis in vitro. Downregulation of BMF conferred similar protection from apoptosis induced by loss of matrix adhesion during anoikis and acinar morphogenesis as well.[30] However, this overlap between BIM and BMF functions in MCF-10A cells did not seem to be conserved in vivo as Bmf expression was not detected by in situ hybridization in TEBs at week 5 during puberty. While the regulation of lumen formation in vitro requires cooperation between several BH3-only factors, namely BIM and BMF,[9,30] apoptosis of the body cells in TEB during puberty may only necessitate BIM.[8] However, the generation of a more sensitive reagent for the detection of BMF in mammary tissue as well as the study of the mammary gland phenotype of the *Bmf*$^{-/-}$ mice will really rule out a potential overlap between BIM and BMF during puberty in vivo.

Are in vitro models utilizing mammary cells relevant to study the regulation of apoptosis during mammary morphogenesis in vivo?

As mentioned above, several other cell lines have been utilized in 3D reconstituted basement membrane cultures. These other mammary cell models recapitulate acinar morphogenesis with a hollow lumen in 3D culture but the regulation of apoptosis of the inner cells lacking matrix attachment is poorly understood. Unfortunately, human primary mammary epithelial cell lines such as hMEC cells, which could potentially represent a physiological model for studying mammary morphogenesis in vitro, are not suitable for such study. The hMEC cells[31] grown in 3D do not form a lumen by cavitation like the MCF-10A cells, but rather the inner cells of hMEC acini undergo squamous differentiation.[8] On the other hand, the mouse mammary gland does not completely mimic the human breast environment. The human breast is organized in a hollow ductal system terminating in acinar units, while the virgin mouse mammary gland displays a ductal network, which will develop alveolar units only during pregnancy. In addition, the composition of mammary stroma is different between the human breast and mouse mammary glands: the fibroblastic component is predominant in the human breast. In contrast, the mouse mammary fat pad is mainly constituted of adipocyte with few fibroblasts. The humanized orthotopic xenograft model in which both the stromal and epithelial components of the reconstructed mammary gland are of human origin might be a very interesting avenue to circumvent this discrepancy between mouse and human mammary glands.[32]

We would like also to emphasize that the MCF-10A 3D culture system[9,14] was critical to orient the in vivo study of the role of BIM in lumen formation during mammary gland morphogenesis.[8] In addition, the MCF-10A 3D culture has been utilized to model the early steps of breast tumorigenesis such as the repopulation of the luminal space.[6,9,10,33-35] These in vitro studies showed that oncogenes promoting only cell proliferation such as HPVE7[10] or Cyclin D1[10] failed to repopulate the lumen as the proliferating cells lost in the luminal space were cleared by apoptosis. Luminal filling was observed only with oncogenes such as ERBB2,[6] CSF-R1[33] or v-SRC[9] which are able in addition to inhibit this apoptosis driven clearing process. In ERBB2 and v-SRC expressing MCF-10A acinar structures, activation of ERK MAPK pathway negatively regulated BIM expression and therefore a luminal filling was observed during morphogenesis.[9] Similarly to the study of the role of BIM in lumen formation, the next step will be to assay these in vitro predictions[9] during mammary tumor initiation and progression in vivo—that is, whether BIM is suppressed in human breast tumor and if so, whether Erk phosphorylation is involved in this suppression.

Is anoikis a developmental process in vivo?

Our in vitro and in vivo studies highlighted the potential role of anoikis during morphogenesis. Although the molecular mechanisms of anoikis are well-described in forced suspension cell cultures, the contexts in which these specific mechanisms, or even matrix detachment in general, act to initiate cell death in vivo remain poorly defined. In the mammary gland, we have defined a BIM-dependent death program that mediates luminal clearing during pubertal development; however, matrix detachment might also be an important trigger of apoptosis during involution of the mammary gland after pregnancy. Interestingly, we did not observe any major involution defect in *Bim*$^{-/-}$ mammary glands after a forced weaning, suggesting that this program is distinct from luminal clearing during development. We were able to detect expression of BMF in apoptotic cells within the lumen during involution, suggesting that another aspect of the anoikis program described in MCF10A cells, the BMF requirement, might also be relevant for anoikis in vivo but in a different context than BIM.

While the formation of luminal spaces in epithelial structures in some organs, such as the lung and pancreas, does not involve cell death, there are other developmental death programs resembling anoikis that appear to be important for cavitation in vivo. For some of these developmental anoikis-like processes there are also in vitro models that mimic the cavitation process, like the MCF-10A model for the mammary gland, which will facilitate elucidation of the molecular mechanisms involved in cell death.

Another organ that exhibits cell death during lumen formation in epithelial acini is the salivary gland. Apoptotic cells are found within the forming luminal spaces of acini in the submandibular gland in mice.[46] It has been hypothesized that the TNFα death receptor apoptotic pathway plays a role in luminal apoptosis based on expression patterns of TNF and its receptor TNFR1.[46] An in vitro model system was developed where human salivary cells form spherical acini and ducts when grown on 3D gels of collagen and Matrigel.[47,48] Notably the cells within these acini formed tight junctions, which are required for secretion of salivary fluid in vivo. This in vitro system will allow for a more detailed analysis of the processes that form a lumen in salivary acini.

Within developing blood vessels, one process that can lead to the formation of a luminal space involves the formation and fusion of intracellular vesicles dependent on RAC and CDC42.[36,37] However, there is also evidence for an apoptotic program associated with lumen formation. Endothelial cell apoptosis can be detected during the development of specific vessels, and most importantly, endothelial cell-specific overexpression of BCL-2 in transgenic mice inhibited proper vessel organization and lumen formation in small blood vessels in the skin, placenta, and other organs where apoptosis was observed in wild-type animals.[38] While it is not known whether

apoptosis is required specifically for lumen formation, or rather plays a role in vessel remodeling or regression, these data correlate with previous observations in 3D collagen cultures of human umbilical vein endothelial cells (HUVEC), where apoptotic cell engulfment was observed during lumen formation and apoptotic cell debris was detected within luminal spaces.[39] It is interesting to note that the formation of large lumens in these 3D cultures is enhanced by blocking integrin engagement, suggesting that lumen formation might occur by an anoikis-like process.[39,40] A requirement for TGFβ signaling in endothelial apoptosis in glomerular capillaries in vivo has been demonstrated by the inhibition of lumen formation and TUNEL positivity induced by a TGFβ blocking antibody.[41,42] It is likely that further characterization of the apoptotic processes involved in capillary lumen formation in vivo will be facilitated by discoveries in cell death regulation in the in vitro model.

During intestinal development in mice, epithelial cells that are multilayered at E13.5 remodel by apoptosis to form a single-layered epithelium attached to basement membrane by E16.5.[43-45] Selective apoptosis of epithelial cells facing the lumen, and not directly attached to the ECM, appears to be responsible for this remodeling.[45] This cell death process is preserved in organ cultures of intestinal segments. Interestingly, the survival of epithelial cells in the single-celled layer, which is attached to basement membrane, is dependent on signaling from EGFR, as these cells undergo cell death when incubated with the EGFR inhibitor AG1478.[45] Thus, the apoptotic program that remodels the intestine might be regulated by both integrin and growth factor signaling as in the mammary gland. In conclusion, these in vitro and in vivo studies highlighted the potential role of anoikis during morphogenesis as a luminal clearing mechanism.

In summary, clearance of a cell population lacking matrix attachment by apoptosis (e.g. anoikis) is an important process during epithelial morphogenesis as revealed by in vitro and in vivo studies. These data raise interesting questions about anoikis as tumour suppressive mechanism in mammary gland. Anoikis may contribute synergistically with growth factor withdrawal and hypoxia to limit tumor progression, as repopulation of the luminal space with cancer cells is a hallmark of early breast tumors.

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