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A cytoskeleton-based functional genetic screen identifies Bcl-xL as an enhancer of metastasis, but not primary tumor growth

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Many mouse models of breast cancer form large primary tumors that rarely metastasize. Models with aggressive metastasis express oncoproteins that simultaneously affect growth and apoptosis pathways. To define the role of apoptotic resistance and to model a challenge faced by tumor cells during metastatic dissemination, we focused on apoptosis induced by cell shape change. Inhibiting actin polymerization with Latrunculin-A causes cell rounding and death within hours in nontumorigenic human 10A-Ras mammary epithelial cells. In contrast, MDA-MB-231 metastatic breast tumor cells resist LA-induced death, and survive for days despite cell rounding. Infecting 10A-Ras cells with a MDA-MB-231 retroviral expression library, and selecting with Latrunculin-A repeatedly identified Bcl-xL as a suppressor of cytoskeleton-dependent death. Although Bcl-xL enhances the spread of metastatic breast tumor cell lines, the distinct effects of apoptotic resistance on tumor growth in the mammary gland and during metastasis have not been compared directly. We find that Bcl-xL overexpression in mouse mammary epithelial cells does not induce primary tumor formation or enhance MEK-induced tumorigenesis within the mammary gland environment. However, it strongly enhances metastatic potential. These results with Bcl-xL provide novel evidence that isolated apoptotic resistance can increase metastatic potential, but remain overlooked by assays based on breast tumor growth.
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The principal cause of patient mortality from cancer is metastatic progression rather than tumor growth at the primary lesion. Metastasis involves dissociation of tumor cells from the organ of origin and their subsequent movement to alternate sites of colonization in the body (Chambers *et al*., 2002). Since nearly 90% of all human solid tumors arise from epithelial cells (Birchmeier *et al*., 1996), it is important to identify genetic changes that can influence the metastatic spread of epithelial tumors.

The survival of mammary epithelial cells requires continuous attachment to the extracellular matrix (Streuli and Gilmore, 1999). Detachment of epithelial cells from extracellular matrix results in apoptosis that is known as anoikis (Frisch and Francis, 1994). Beyond attachment, the maintenance of a distinct cellular morphology is necessary to suppress apoptotic cell death (Chen *et al*., 1997; Martin and Leder, 2001). Disruption of the actin cytoskeleton leads to cell rounding, and the resulting apoptosis cannot be prevented by attachment (Martin and Leder, 2001). This form of programmed cell death, following alteration of cell shape, has been termed amorphosis, which is derived from the Greek word for 'misshapen', *amorphos* (Martin and Vuori, 2004). Since detachment and significant changes in cell shape occur during metastasis, it may be necessary for tumor cells to overcome anoikis and amorphosis to disseminate. The observation that many metastatic tumor cells survive despite persistent cytoskeletal abnormalities (Ben-Ze'ev, 1997) supports this hypothesis. It is important to note that the specific type of apoptosis involved in metastatic spread remains unclear.

A differential sensitivity to amorphosis between nontumorigenic and metastatic cell lines is apparent in culture (Martin and Leder, 2001). Human MCF10A mammary epithelial cells expressing activated V12-Ras (10A-Ras) do not form tumors in nude mice and retain a typical epithelial morphology with strong cell–cell contacts (Figure 1a). Inhibiting actin polymerization with Latrunculin-A (LA) causes cell rounding by 1 h and high levels of apoptotic fragmentation by 48 h. LA seems to specifically affect the actin cytoskeleton, rather than induce general cytotoxicity, since mutant actin alleles confer resistance to LA in both human cells (Fujita *et al*., 2003) and *Saccharomyces cerevisiae* (Ayscough *et al*., 1997). The metastatic human breast tumor cell line, MDA-MB-231, appears much more mesenchymal than MCF10A cells, and rounds even more dramatically after a 1 h treatment with LA.

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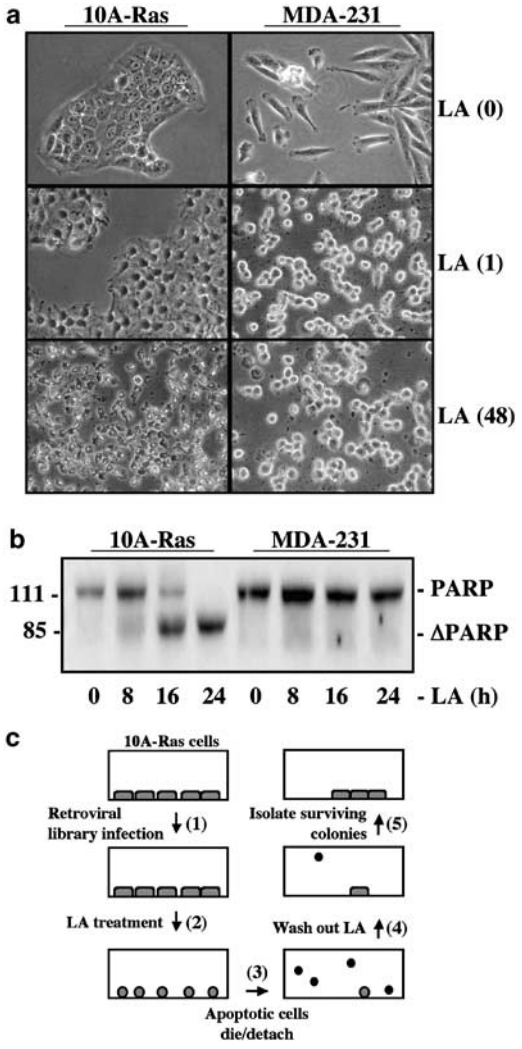

Figure 1 Differential apoptotic response to cell rounding in nontumorigenic metastatic mammary epithelial cell lines is exploited for a genetic screen. (**a**) Treatment with LA for 1 h (1 μM, Biomol) disrupts the shape of both nontumorigenic 10A-Ras and metastatic MDA-MB-231 cells, but only the 10A-Ras cells show apoptotic morphology after 48 h. (**b**) Western blot showing apoptotic cleavage of the PARP protein to an 85 kDa form (ΔPARP) in whole-cell lysates (H-250, Santa Cruz, 1 : 1000). After 16 h of LA treatment, most 10A-Ras cells have executed apoptosis, while MDA-231 cells do not show any detectable PARP cleavage, even after 24 h. (**c**) Schematic of cytoskeleton-based functional genetic screen for suppressors of amorphosis. 10A-Ras cells infected with a MDA-MB-231 retroviral expression library were treated with LA (5 μM) for 6 days and surviving colonies allowed to form for 10 days following washout of LA

However, the MDA-MB-231 cells do not fragment apoptotically after 48 h and remain attached to the plate. Measurement of caspase-dependent PARP cleavage demonstrates the differential amorphotic sensitivity (Figure 1b). After 24 h of cytoskeletal disruption, 10A-Ras cells completely cleave PARP, while cleavage remains undetectable in MDA-MB-231 cells. 10A-Ras cells do not begin to detach from the plate until after 24 h (not shown), despite showing strong cleavage of PARP by 16 h, confirming that detachment is a consequence of apoptosis under these conditions, rather than a cause. The effect of LA could be reversed by washing out LA, and MDA-MB-231 cells respread and continued to grow as a result, indicating their resistance to amorphosis (data not shown).

Given these observations, we conducted a functional genetic screen to identify genes that can confer resistance to amorphosis to allow an isolated examination of apoptotic resistance in tumor development and metastasis. Amorphotically sensitive 10A-Ras cells were infected with a retroviral expression library from the resistant MDA-MB-231 cells (Figure 1c). Treatment with Latrunculin-A induced amorphosis in the majority of cells and detachment from the plate, while surviving cells remained attached. Inhibition of actin polymerization with LA is reversible, so washing out the inhibitor after 6 days allowed surviving cells to respread and continue to grow into small colonies.

We repackaged integrated virus by transiently transfecting a Psi^2 helper plasmid (Figure 2a) to isolate colonies that arose from expression of the MDA-MB-231 cDNA, as opposed to either spontaneous or insertional mutation. This virus was used to ecotropically infect 10A-Ras cells that express the mCAT-1 viral receptor. A colony formation assay following challenge with LA demonstrated transfer of amorphotic resistance in five of the first 10 clones isolated from the library screen (Figure 2b). PCR amplification of the inserted cDNAs from these 10 clones also showed that while the initial clones isolated from the library often harbored multiple integrated retroviruses, this distilled to a single functional fragment after retroviral transfer of the LA-resistant phenotype (Figure 2c). These solitary amplified fragments correlated strongly with the efficiency of colony formation, also suggesting that these cDNAs were responsible for the amorphotic resistance. Sequencing of these five fragments revealed the complete coding sequence for Bcl-xL in all cases, with differences in the 5′-UTR indicating three independent isolations. This provides the first evidence that Bcl-xL expression can confer resistance to amorphosis.

Overexpression of Bcl-xL correlates with increased nodal involvement and higher tumor grade in human breast cancer patients (Olopade *et al*., 1997). In metastatic human breast tumor cell lines, overexpression of Bcl-xL can increase cell survival in the vasculature and enhance the formation of distant metastases (Rubio *et al*., 2001; Fernandez *et al*., 2002). Likewise, coexpression of Bcl-2 with E1a and activated Ras enhances survival of tumorigenic mouse embryo fibroblasts in the vasculature (Nikiforov *et al*., 1997). However, the isolated effect of apoptotic resistance on breast tumor development in the mammary gland and during metastatic spread has never been examined. It has been difficult to address this question with transgenic and knockout mouse models of apoptotic resistance for several reasons. Transgenic expression of Bcl-2 can either accelerate or repress primary tumor growth in the mammary gland (Jager *et al*., 1997; Murphy *et al*., 1999), but any role in metastasis has not been tested.

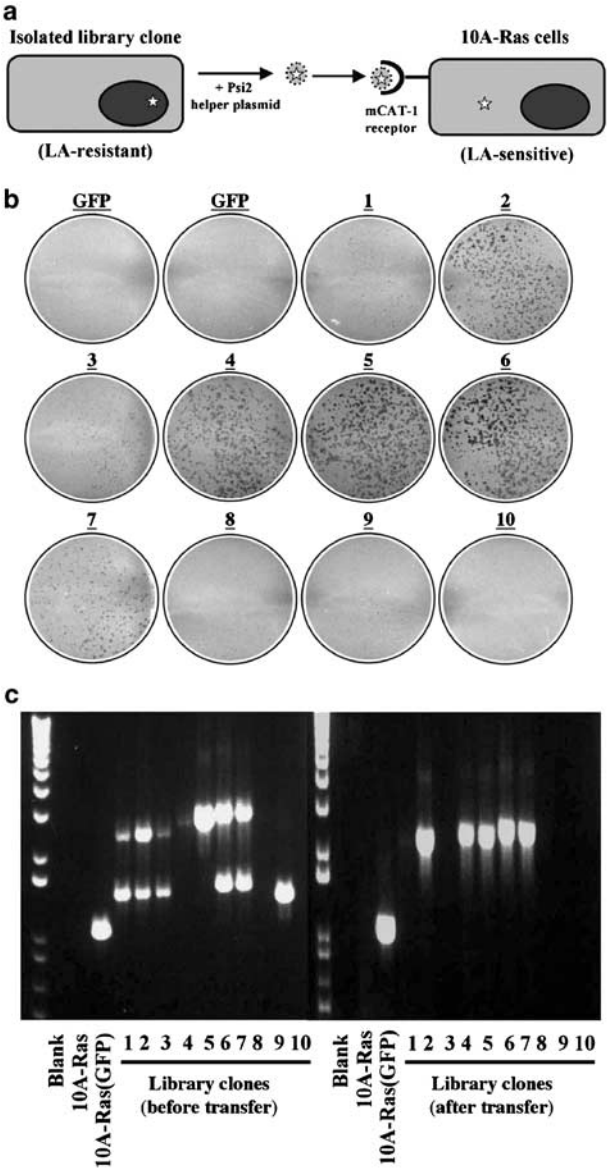

Figure 2 Linking resistance to the expressed MDA-231 cDNA. (**a**) Isolated LA-resistant library clones were transiently transfected with Psi2 helper plasmid to ecotropically repackage integrated retrovirus (star). Filter-purified virus (0.45 μM) was applied to 10A-Ras clones expressing ecotropic virus receptor (Ras-MCAT). Genomic or insertional mutations would not be transferred by this method. (**b**) Treatment with LA (4 days, 3 day washout) indicates that viral supernatants from five of the 10 library clones induced strong colony formation (crystal-violet staining) but GFP virus did not. (**c**) PCR using primers flanking the retroviral insert, shows no background amplification in 10A-Ras cells and strong amplification of integrated GFP sequence. Multiple bands seen in many of the library clones distill to a single band in resistant colonies after retroviral transfer

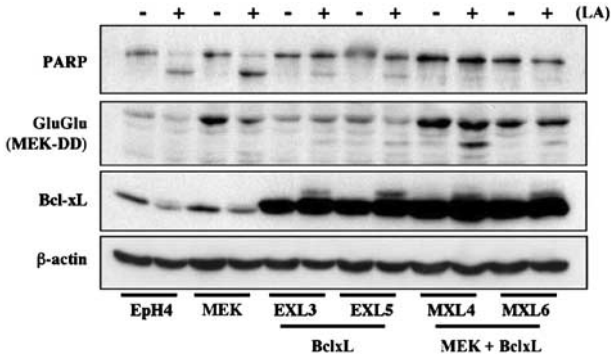

Figure 3 EpH4 cells or those expressing constitutively active MEK1-DD (βDD47), Bcl-xL (EXL3, EXL5) or a combination of MEK1-DD and Bcl-xL (MXL4, MXL6) were either left untreated or subjected to LA challenge (2.5 μM) for 24 h. PARP cleavage shows that EpH4 cells, and those expressing MEK1-DD, remain highly sensitive to amorphosis. All lines expressing Bcl-xL (EXL, MXL) resist LA-induced PARP cleavage. Expression of MEK1-DD was confirmed by Glu-Glu epitope immunoblot (Covance, MMS-115R, 1 : 2000), while Bcl-xL overexpression was confirmed using a polyclonal Bcl-xL antibody (Transduction, 610211, 1 : 1000). Equal loading was confirmed by β-actin immunoblot (lower panel, Sigma, AC-15, 1 : 3000)

Loss of the proapoptotic Bcl-2 protein, Bax, can accelerate tumor growth when combined with activated oncogenes (Yin *et al*., 1997; Shibata *et al*., 1999; Eischen *et al*., 2002). Functional redundancy between Bax and Bak, another multidomain proapoptotic Bcl-2 protein, limits the effect of Bax deficiency. This redundancy can be overcome by generating mice doubly deficient for Bax and Bak, but these animals have massive lymphocytic hyperplasia and fewer than 10% survive into adulthood (Lindsten *et al*., 2000). Similarly, mice deficient in p53 die so rapidly from lymphoma that examining the role of this type of apoptotic resistance in solid tumor development is not possible. To overcome these limitations and allow a direct examination of the role of apoptotic resistance, we have expressed Bcl-xL either alone or in combination with an activated MEK1 oncogene in nontumorigenic mouse EpH4 mammary epithelial cells (Figure 3).

As we observed with the human MCF10A mammary epithelial cells, EpH4 cells are highly sensitive to amorphosis and show strong PARP cleavage after Latrunculin-A treatment. Expression of activated MEK1-DD was not sufficient to prevent amorphosis, similar to the results with activated V12-Ras in the MCF10A background. Expression of activated forms of β-catenin and Erb2 also failed to prevent amorphosis (data not shown). Conversely, expression of Bcl-xL was able to block amorphosis almost completely on its own, or in combination with activated MEK1.

Injection of these cells into the mammary fat pad showed that while activated MEK1 formed robust tumors in this environment, cells expressing Bcl-xL alone did not form detectable tumors (Figure 4a). Coexpression of Bcl-xL with MEK did not enhance tumor formation and actually repressed tumor growth significantly in one of the two cell lines (MXL4). Bcl-xL expression can repress cell cycle progression (O'Reilly *et al*., 1996; Greider *et al*., 2002) as can Bcl-2 (O'Reilly *et al*., 1996), and this may explain the reduction in primary tumor growth. To assess the metastatic potential of these different cell lines, we performed tail-vein injections and examined the efficiency of experimental lung metastasis (Figure 4b). As in the

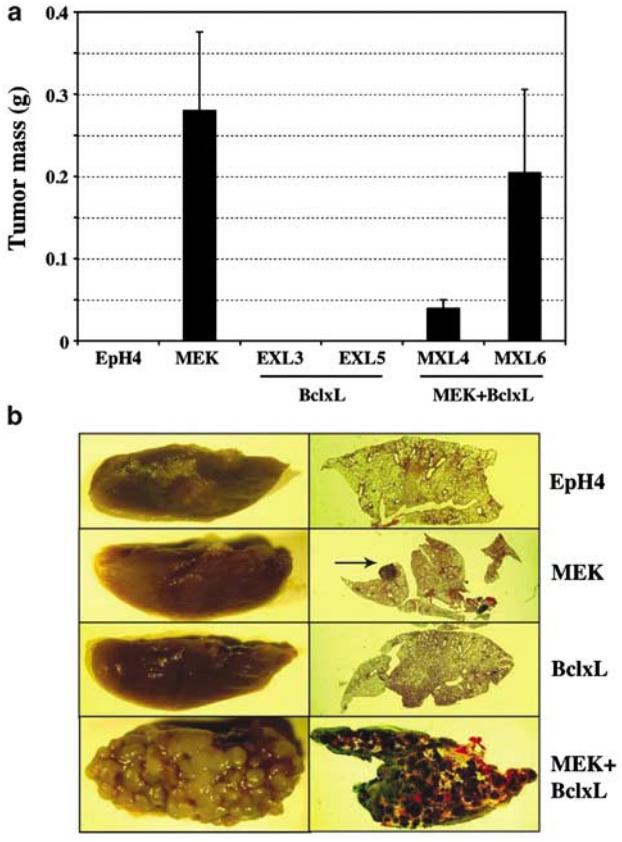

Figure 4 Bcl-xL expression does not induce tumor formation but enhances metastatic potential. (**a**) Following subcutaneous injection of 5×10^5 cells into the mammary fat pad of nude mice, MEK-expressing cells form robust tumors by 3 weeks as gauged by tumor mass. EpH4 cells or those overexpressing Bcl-xL (EXL3, EXL5) do not form detectable tumors. Two different lines coexpressing MEK1-DD and Bcl-xL (MXL4, MXL6) also form tumors, but of reduced size. Bars represent average tumor mass + s.d. (**b**) Nude mice were injected intravenously via the tail vein with 5×10^5 cells. Representative lungs from mice killed at 6 weeks are shown. EpH4 cells or those expressing Bcl-xL do not form macroscopic metastatic lesions, upon gross examination. Histopathological staining of central lung sections showed that cells expressing constitutively active MEK1-DD form few metastatic lesions (black arrow) with an average of 0.16 ± 0.4 nodules per lung section (one of six mice). Cells expressing a combination of MEK-DD and Bcl-xL show a high level of metastatic burden in the lungs (average of 28 ± 15 nodules per lung section, seven of seven mice), that often resulted in the death of the mice

mammary gland environment, neither the EpH4 parental cells nor those expressing Bcl-xL alone formed metastatic lesions. Despite forming robust tumors in the mammary gland, cells expressing MEK1 alone formed few metastatic lung lesions with a low frequency (one of six mice, average of 0.16 ± 0.4 nodules per lung section). Mice injected with cells expressing both MEK1 and Bcl-xL showed a very high tumor burden in the lungs (seven of seven mice, average of 28 ± 15 nodules per lung section). Resistance to amorphosis, conferred by Bcl-xL, was not a selective advantage during growth in an environment with abundant extracellular matrix, like the mammary gland. However, during transit through the vasculature during metastasis, resistance to amorphosis predicted a greater ability to form metastatic lesions.

These observations provide a possible explanation as to why so many transgenic breast cancer models in mice form robust tumors that metastasize inefficiently and only after long latency (Webster and Muller, 1994). The transgenic models that metastasize most efficiently are based on viral oncoproteins, such as SV40 large-T antigen (Shi *et al*., 2003) or polyoma middle-T antigen (Guy *et al*., 1992), each of which can simultaneously affect both the cell cycle and apoptosis. If there exist two classes of oncogenes, those that stimulate growth at the primary site and those that allow survival during metastatic dissemination, then a combination of the two would be required for efficient metastasis. More importantly, these two classes of oncogenes may not overlap completely, and may actually be in competition. Our results would suggest that Bcl-xL is either neutral or slightly repressive on primary tumor growth, despite strongly enhancing survival and the formation of metastatic lesions in the lungs. MEK, on the other hand, seems to increase tumor growth without providing a significant survival advantage. Expression of activated MEK2 can prevent apoptosis upon detachment, but not as efficiently as overexpression of Bcl-xL (Reginato *et al*., 2003). Although our level of expression of activated MEK1 is relatively low, it is sufficient to induce strong tumor growth in the mammary gland but cannot provide significant resistance to amorphosis or survival in the vasculature. Along these lines, it is important to distinguish distant spread of tumor cells from successful formation of metastatic lesions. Our earlier work showing that MEK1-expressing cells could be recultured from distant tissues suggested that MEK1 could promote metastasis (Pinkas and Leder, 2002). However, these animals never developed significant metastatic lesions in those distant tissues after continued study (data not shown), so the shed tumor cells either remained incapable of outgrowth or died by apoptosis. A similar phenotype is observed in several transgenic breast tumor models (Webster and Muller, 1994).

Recent results support such a divided and possibly counteracting model for genes affecting primary tumor growth and metastatic spread. In breast cancer patients, comparative genome analysis suggests that primary tumors and metastatic cells evolve independently via distinct mechanisms, and that metastatic cells may disseminate long before they acquire growth-activating mutations (Schmidt-Kittler *et al*., 2003). Our results would suggest that an apoptotic block, like that conferred with Bcl-xL, may manifest itself in such a way. Apoptotically resistant cells that escape into the vasculature may disseminate but fail to grow at the distant site. Under these circumstances, distinct growth-enhancing mutations could be sustained in the primary tumor and metastatic lesion. Situations in which apoptotic resistance is a prerequisite for primary tumor growth certainly exist, as with myc-induced tumors (Jager *et al*., 1997; Pelengaris *et al*., 2002). In such cases, expression of Bcl-2 can accelerate primary tumor growth. In other models, a counteracting role is more

apparent, and Bcl-2 expression is repressive to primary tumor growth until mutation segregates the antimitotic and prosurvival functions of Bcl-2 (Furth *et al*., 1999). The recent observation that activated TGF-βRI signaling can promote lung metastasis while repressing primary tumor growth induced by an activated Neu oncogene (Siegel *et al*., 2003), is another example of a counteracting role, as we observe for Bcl-xL and MEK1.

It is therefore important to realize that genes capable of enhancing metastasis may actually remain undetectable by assays based on primary tumor growth. Our functional genetic screen for suppressors of amorphosis successfully identified Bcl-xL as a gene that can enhance metastatic potential independent of effects on primary tumor growth. The observation that resistance to amorphosis predicts enhanced metastatic spread without inducing tumor growth, supports a model in which the independent evolution of primary and metastatic tumors is possible.

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