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β 4 Integrin-dependent formation of polarized three-dimensional architecture confers resistance to apoptosis in normal and malignant mammary epithelium

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Summary

Tumor cells can evade chemotherapy by acquiring resistance to apoptosis. We investigated the molecular mechanism whereby malignant and nonmalignant mammary epithelial cells become insensitive to apoptosis. We show that regardless of growth status formation of polarized, three-dimensional structures driven by basement membrane confers protection to apoptosis in both nonmalignant and malignant mammary epithelial cells. By contrast, irrespective of their malignant status, nonpolarized structures are sensitive to induction of apoptosis. Resistance to apoptosis requires ligation of $\beta 4$ integrins, which regulates tissue polarity, hemidesmosome formation and NF κ B activation. Expression of $\beta 4$ integrin that lacks the hemidesmosome targeting domain interferes with tissue polarity and NF κ B activation and permits apoptosis. These results indicate that integrin-induced polarity may drive tumor cell resistance to apoptosis-inducing agents via effects on NF κ B.

Significance

Here we used a 3D model in which nonmalignant and malignant human breast epithelial cells could be repeatedly and reversibly organized into polarized tissue-like structures to study how normal tissues and some tumor cells resist apoptotic cell death by chemotherapeutic agents and immune regulators. We show that, regardless of the genetic make up, the rate of growth, or the malignant status, resistance correlates with laminin, $\alpha6\beta4$ integrins, hemidesmosome-dependent polarity and NF κ B activation. Our model sheds light on the endogenous activation of this pathway in epithelial tissues. These data indicate the critical importance of tissue architecture in cellular responses and have important implications for how tumor cells may become dormant and offer possible chemotherapeutic strategies.

Introduction

Apoptosis is essential for immune surveillance and for the efficacy of tumor therapy (Costello et al. 1999). Although considerable progress has been made towards understanding how apoptosis is executed at the cellular level (Adams and Cory, 1998; Thornberry and Lazebnik, 1998), less is known about what regulates apoptotic decisions, and how apoptotic agents could selectively target the tumor tissues.

The site of tumor cell metastasis is influenced by the composition of the extracellular matrix (ECM) and the integrins expressed by the tumor cells (Pignatelli and Stamp, 1995). Despite the fact that tumors overexpress ECM-degrading proteases, aggressive tumors often make excess basement membrane (BM) and have abundant $\beta 4$ integrins (Tagliabue et al., 1998; Rabinovitz and Mercurio, 1996). This paradox suggests that, in some cases, ECM adhesion may foster tumor progression rather than tumor inhibition (Tani et al., 1997; Pfohler, 1998). Interactions between tumor cell integrins and adhesion molecules in the ECM microenvironment may drive the selection of treatment-resistant tumors (Scupoli et al., 2000). However, the rate at which tumors can acquire resistance to treatment *in vivo* argues that mechanisms, functioning independently of genetic selection, must also operate to drive the genesis of apoptosis resistance in metastatic tumors.

The tissue ECM may modify the responsiveness of tumors to exogenous apoptotic stimuli. Adhesion to ECM rapidly and reversibly modifies the responsiveness of myeloma and lung tumor cells to chemotherapeutic drugs (Damiano et al., 1999; Sethi et al., 1999). Tumor cells selected for their drug resistance in monolayer cultures develop changes in cell adhesion and integrin expression (Nista et al., 1997; Narita et al., 1998). Tumor cells grown as three-dimensional (3D) multicellular spheroids rapidly acquire and sustain a multi-drug resistant phenotype in response to acute drug treatment (Durand and Olive, 2001; Kerbel 1994), exhibit modified adhesion (Hauptmann et al., 1995; Man and Kerbel, 1998), and secrete ECM proteins (Santini and Rainaldi, 1999). This implies that tissue organization, cell adhesion, and the ECM may synergistically generate apoptosis resistance in metastatic tumors. We showed previously that ECM-induced tissue architecture can override the proliferative and invasive malignant phenotype, but that reversion is dependent upon the 3D tissue microenvironment (Weaver et al., 1997; Wang et al., 1998). Because reversion of the malignant phenotype and polarity are associated with recalcitrance to growth factor stimulation, we have now hypothesized that the

ECM, via cooperative interactions between integrins, the cytoskeleton and tissue organization, dictates apoptosis inducibility in mammary epithelial cells (MECs).

To show this, we used the HMT-3522 MEC model of breast cancer progression (Briand et al., 1996; Weaver et al., 1996). This tumor cell series was established from a reduction mammoplasty of a woman with a nonmalignant breast lesion. Continued passage and growth factor withdrawal led to the spontaneous generation of tumorigenic cells (Briand et al., 1996). We used the nonmalignant S-1 cells at passages 50-70 ("normal"; S-1) and their tumorigenic progeny at passages 238-245 (T4-2). In the present study we asked whether BM signaling via integrins regulates apoptosis resistance in MECs, and if so how.

Results

A differentiated tissue structure is resistant to apoptosis induction

We asked whether interactions with BM modulate the sensitivity of MECs to apoptotic stimuli, and if malignant transformation altered this responsiveness. We measured the apoptotic sensitivity of S-1 and malignant T4-2 MECs to apoptotic stimuli. S-1 and T4-2 MECs, grown as monolayers on a thin coat of collagen I, exhibited comparable apoptotic responsiveness regardless of the kind of apoptotic agent used. These included ligation of receptors for tumor necrosis factor (TNF- α), Trail, Fas, or treatment with the microtubule reagent paclitaxol, the topoisomerase II inhibitor etoposide, or the actin cytoskeletal disruptor cytochalasin B (Figure 1).

Non-malignant MECs embedded in reconstituted BM (rBM) formed growth-arrested 3D, organoids (acini), whereas malignant MECs continued to proliferate to form non-polar, multi-cellular and invasive aggregates (Petersen et al., 1992; Weaver et al., 1997). The rBM conferred apoptosis-resistance to non-malignant MECs, however this mechanism was absent or no longer functioned after malignant transformation (Figure 1B).

A polarized mammary tissue structure is resistant to apoptosis induction

To determine whether there was a link between rBM-induced apoptosis resistance and formation of a differentiated 3D structure, we induced the T4-2 cells to form polarized and phenotypically reverted structures (T4-Rvt). We grew cells within the rBM, in the presence of a function-blocking β 1 integrin monoclonal antibody (mAb, Wang et al., 1998); T4-2 cells grown unperturbed within parallel rBM cultures served as apoptosis-sensitive, positive controls. Both S-1 acini and T4-Rvt structures established tissue polarity as shown by cortical actin, β -catenin at cell-cell junctions, and secreted laminin-5 and β 4 integrin at their basal surface (Figure 2A). Disruption of polarity by treating S-1 cells within the rBM with a function-blocking anti-E-cadherin mAb (Figure 2A) resulted in clusters of cells with disordered filamentous actin, dispersed β 4 integrin, and intracellular and randomly secreted laminin-5, similar to the disorganized T4-2 colonies (Figure 2A). Polarized S-1 MEC acini were completely resistant to apoptosis induction by three kinds of apoptotic agents, yet they became very sensitive when the acinar polarity was compromised (Figure 2B). Similarly, while non-polarized T4-2 cells were

sensitive to apoptotic stimuli, they became resistant to apoptosis following phenotypic reversion and tissue polarization (Figure 2B).

BM-induced tissue polarity is necessary for apoptosis resistance regardless of growth status

Because the development of tissue polarity and apoptosis resistance was also associated with a substantial reduction in cell growth (Figure 2C), we asked whether growth-arrest was sufficient for apoptosis-resistance. We compared S-1 cells grown within rBM, which form polarized, growth-arrested 3D structures, with S-1 cells within collagen I gels, which form growth-arrested, nonpolarized 3D structures (Lelièvre et al., 1998; Gudjonsson et al., 2002) and with S-1 cells that over-express EGF-R (Wang et al., 1998) but maintain basal polarity within rBM while continuing to proliferate. All three groups of MECs had adherens junctions as shown by β -catenin localized at cell-cell junctions. However, only those MECs interacting with the rBM became basally polarized, as indicated by deposition of an endogenous BM (Figure 3A), and resisted apoptosis following treatment with TNF- α , Fas mAb, or etoposide, whether growth-arrested or proliferating (Figure 3, B & C). In contrast, growth-arrested, but non-polarized S-1 cells underwent apoptosis.

To investigate whether the presence of rBM molecules is sufficient to protect S-1 cells from apoptosis induction, we grew S-1 cells as 3D non-polar spheroids within collagen I gels or as 3D polar acini in rBM. In parallel, we liberated some MEC 3D spheroids by collagenase treatment, suspended them in polyHEMA-coated dishes, and then overlaid them with serum-free medium supplemented with either BSA or rBM proteins. All of these tissue structures were growth-arrested (see Figure 3B) and showed adherens junctions as assessed by β -catenin (Figure 4A). Apoptosis could be readily induced in the S-1 non-polar spheroids in collagen I gels, but once exposed to rBM, they resembled polarized S-1 acini with basally localized β 4 and α 6 integrins and laminin-5, and they acquired apoptosis resistance (Figure 4B).

Laminin-1, the main component of the rBM, alone was sufficient for inhibiting apoptosis sensitivity (Figure 4B). These results demonstrate that tissue polarity, resulting from interaction with BM laminin-1, but not growth-arrest, is necessary for protection from apoptosis induction. Significantly, ligation of receptors by other ECM molecules such as type I collagen which leads to growth arrest but incorrect polarity was not sufficient for apoptosis resistance.

BM-induced tissue polarity regulates NF κ B activation to drive apoptosis resistance

NF κ B is activated early during neoplastic transformation of the mammary gland (Kim et al., 2000), and plays an important role in the pathogenesis (Sovak et al., 1997) and metastasis of human breast cancers (Nakahatri et al., 1997). Activated NF κ B is linked to repression of apoptosis during mammary involution and increased survival of mammary epithelial cells in culture (Clarkson et al., 2000). NF κ B activation also drives resistance to chemo and radiation therapy (Baldwin 2001) and modifies expression and stability of apoptosis regulators (Tanaka et al., 2000; Tergaonkar et al., 2002). Accordingly, we investigated the relationship of NF κ B p65 activation to BM-induced apoptosis resistance. Nuclear NF κ B B p65 increased within one hour of treatment of polarized S-1 acini with TNF- α , Trail or etoposide (Fig. 5A). When we used the multi-catalytic proteasome inhibitor MG 132 or expressed a mutant I κ B α , which is resistant to proteolytic degradation, nuclear translocation of p65 did not occur (data not shown) and all three of these agents induced apoptosis (Figure 5B). In contrast, ceramide, which fails to induce nuclear translocation of NF κ B, induced death in control S-1 acini (Figure 5A, B). These data show that modulation of NF κ B activity is one mechanism mediating BM-induced apoptosis resistance in MEC acini.

α 6 β 4 integrin directs apoptosis resistance in 3D mammary organoids

We next determined whether integrins mediate the effects of laminin and basal polarity on NF κ B activation and apoptosis resistance. As a direct test, we incubated non-polar, apoptosis-sensitive, S-1 spheroids isolated from collagen I gels with function-blocking mAb to α 2, α 3, α 6, β 1, or β 4 integrin or control IgG. The structures were suspended in polyHEMA-coated dishes in serum-free media supplemented with either BSA or rBM. The spheroids treated with rBM, but not BSA, that were challenged with TNF- α in the presence of mAb directed against β 1, α 2, or α 3 integrins were viable and intact, even after 96 hours of incubation (Figure 6A) and showed nuclear translocation of NF κ B (data not shown). In contrast, cells in the rBM-treated spheroids incubated with blocking mAbs to α 6 or β 4 integrins failed to establish polarity, did not activate NF κ B (data not shown) and showed significantly increased apoptosis when challenged with TNF- α for 96 hours (Figure 6A). Because S-1 MECs do not express α 6 β 1 integrin (data not

shown), these results indicate that $\alpha6\beta4$ integrins, but not $\alpha2\beta1$ or $\alpha3\beta1$ integrins, participate in regulating BM-directed apoptosis resistance in polarized acini.

To establish whether ligation of $\beta4$ integrins was sufficient to protect the non-polarized spheroids from apoptosis induction, we ligated and clustered their $\beta1$ or $\beta4$ integrins or MHC molecules with mAbs that were cross-linked to magnetic beads. The integrin-activated 3D MEC structures were suspended in polyHEMA-coated dishes in serum-free medium. mAb-bead-mediated ligation of $\beta4$, but not $\beta1$ integrins, protected the MEC spheroids from apoptosis induction (Figure 6B). Conversely when S-1 cells expressing a GFP-labeled tailless $\beta4$ integrin were embedded in rBM to form growth-arrested structures NF κ B did not translocate to the nuclei following drug treatment (data not shown) and cells did not develop polarity (Figure 6D). These structures showed disrupted hemidesmosome formation, as indicated by randomly dispersed type 1 hemidesmosome protein (HD-1) and bullous pemphigoid antigen 180 (BP180; Figure 6D). Loss of tissue polarity and NF κ B activation in these growth-arrested structures was associated with enhanced sensitivity to apoptosis induced by etoposide, Trail and Fas receptor ligation (Figure 6C). Thus the ability of $\beta4$ integrins to drive tissue polarity is essential for activation of NF κ B and apoptosis resistance.

Disrupting hemidesmosome formation perturbs BM-directed tissue polarity, inhibits NF κ B activation and permits induction of apoptosis in 3D acini.

We then investigated the role played by hemidesmosomes, a structure that functionally links the cytoskeleton to $\beta4$ integrin in induction of polarity, NF κ B activation and apoptosis resistance in MEC acini. T4-2 non-polar spheroids in rBM had sparsely dispersed hemidesmosomes (1 hemidesmosome/2 μ m plasma membrane), of which >90% were immature type II hemidesmosomes, disorganized $\beta4$ integrin, a sparse random distribution of HD-1, and predominantly cytosolic NF κ B p65 (Figure 7). In contrast, T4-2 cells in rBM, treated with a function-blocking $\beta1$ integrin mAb but not non-specific rat IgGs, reverted to polarized acini (Figure 7; see also Figure 2A). The polarized T4 reverted structures had basally organized $\beta4$ integrin and HD-1, increased hemidesmosomes (2 hemidesmosomes/3 μ m plasma membrane) of which >60% were mature type I hemidesmosomes, and nuclear localization of NF κ B p65

(Figure 7). These observations establish an association between BM-induced apoptosis resistance, $\beta 4$ integrin-directed tissue polarity and NF κ B activity in 3D structures.

Several amino acid residues in the connecting segment that resides between the four type-III fibronectin-like modules, towards the C-terminus, are critical for targeting $\beta 4$ integrin to hemidesmosomes (Figure 8A). To test the involvement of hemidesmosomes, S-1 cells were transfected with one of the following: an untagged or RFP or EGFP-tagged $\beta 4$ integrin deleted in the cytoplasmic tail connecting segment ($\Delta 1314-1486$), wild type $\beta 4$ integrin, or vector control RFP or EGFP-tagged or untagged constructs. Pooled stable populations of S-1 cells transfected with the connecting segment deleted $\beta 4$ or wild type $\beta 4$ integrins showed uniform expression of tagged RFP protein (Figure 8C) and increased total $\beta 4$ integrin expression compared to S-1 vector controls (Figure 8B). All cells in 3D rBM assembled adherens junctions judged by localization of β -catenin (Figure 8E). Expression of the $\beta 4$ integrin with the connecting segment deleted should act as a dominant negative by competing with the endogenous, wild type $\beta 4$ integrin to disturb hemidesmosome organization and perturb cytoskeletal organization. Accordingly S-1 cells transfected with mutant $\beta 4$ integrin formed structures with dispersed, faint HD-1, patchy, sparsely distributed BP180, cytosolic, non-polarized $\beta 4$ integrin, and randomly secreted laminin-5, indicating that disrupting hemidesmosome cytoskeletal structures perturbed polarity (Figure 8E). In contrast, vector control or S-1 cells infected with wild type $\beta 4$ integrin acini were polarized with intense staining for HD-1 protein, and punctate basal, BP180 (Figure 8E). Moreover, perturbing hemidesmosome organization in the 3D MEC structures with mutant EGFP-tagged $\beta 4$ integrin reduced NF κ B activation and led to a significant enhancement in apoptosis sensitivity (Figures 8D & F). These observations establish that ligation of $\alpha 6\beta 4$ integrins, formation of mature hemidesmosomes and activation of NF κ B p65 are linked to BM-directed tissue polarity, and resistance to apoptosis.

Discussion

We exploited a mammary epithelial tumor progression model, HMT-3522, and 3D assays in rBM or collagen-I gels to determine how breast epithelial cells become resistant to apoptosis. The studies described here show that tissue architecture regulates sensitivity to exogenous apoptotic stimuli and that this effect is mediated via integrin-cytoskeletal associations and

activation of NF κ B. Laminin-induced ligation of β 4 integrin directs tissue polarity and promotes resistance to apoptosis in both nonmalignant and malignant breast epithelial structures, regardless of growth status. The apoptosis resistance depends upon the 3D organization of the acini, and is functionally linked to β 4 integrin-directed hemidesmosome formation and NF κ B activation. Thus integrin-laminin interactions not only initiate signals essential for cell growth, viability and functional differentiation (Streuli et al., 1991; Boudreau et al., 1995), but also protect mammary cells that are organized into tissue-like structures from exogenous apoptotic cues. The unit structure of the tissue thus emerges as an important determinant in homeostasis.

The presence of hemidesmosomes in epithelial tissues stabilizes attachments to the underlying BM (Jones et al., 1998). α 6 β 4 integrins play a diverse role in normal epithelial physiology (Borradori and Sonnenberg, 1999) by acting as laminin receptors that interact with intermediate filaments via long cytoplasmic tails (Spinardi et al., 1995). This facilitates branching morphogenesis (Stahl et al., 1997), cell proliferation (Mainiero et al., 1997), and migration (Goldfinger et al., 1999). Here we show that ligand-activated β 4 integrin is involved directly in induction of tissue polarity in mammary epithelial acini and in modulating a program that leads to the acquisition of apoptosis resistance to most receptor-linked and many chemical stimuli. This phenotype requires β 4 integrin to mediate hemidesmosome formation and to facilitate NF κ B activation. In support of these results, β 4 integrin-null keratinocytes exhibit reduced viability in response to an exogenous mechanical stress *in vivo* (Dowling et al., 1996), and targeted disruption of the *LAMA3* gene in mice compromises keratinocyte survival (Ryan et al., 1999). Similarly, β 1 and β 4 integrins activate NF κ B to maintain survival during T cell development (Fiorini et al., 2000).

But how do tumor cells evade apoptotic therapy? Our data suggest that epithelial tumors that express β 4 integrins have the potential to acquire resistance to exogenous death stimuli if they are given the appropriate spatial and biochemical cues from the microenvironment. The T4-2 breast tumor cells express β 4 integrin and are sensitive to induction of apoptosis when they are grown in two dimensional and three dimensional cultures. However they acquire an apoptosis-resistant phenotype when they recapitulate three dimensional polarized architecture accompanied with endogenously activated NF κ B p65. These findings provide a rationale for a number of previous reports in the literature. Aggressive and metastatic breast tumor cell lines express β 4

integrins (Taylor-Papadimitriou et al., 1993; Jones et al., 1997). Furthermore, the highly metastatic, $\alpha 6\beta 4$ integrin-positive breast epithelial cell line, MDA MB-231, rapidly and reversibly acquires a multi-drug apoptosis resistant phenotype when grown in three-dimensions (Graham et al., 1994; Kerbel et al., 1996; St. Croix et al., 1998). The data are also consistent with clinical reports that individuals expressing both BM proteins and $\beta 4$ integrins in their primary tumors have the poorest prognosis among breast cancer patients (Tagliabue et al., 1998), and that breast tumors frequently show increased expression of NF κ B regulated genes such as survivin (Tanaka et al., 2000). A recent study showed that greater than 60 percent of primary breast tumor tissues express high levels of both $\beta 4$ integrin and laminin-5 (Davis et al., 2001), and more than 50 percent of dormant, metastatic cells isolated from the bone marrow of breast cancer patients express $\alpha 6$ and/or $\beta 4$ integrins (Putz et al., 1999). Furthermore, deregulated NF κ B has been implicated as an important prognostic indicator in primary and metastatic breast tumors (Nakshatri et al., 1997; Sovak et al., 1997; Kim et al., 2000), underscoring the potential relevance of our present studies to tumor pathology.

Our model suggests that integrin-directed tumor architecture may constitute a prognostic indicator of future tumor behavior and apoptosis sensitivity. The major event regulating tumor metastasis is survival of the tumor cell in the distant site (Wong et al., 2001): Accordingly, our data may explain why tumors preferentially colonize selected tissues. In addition to providing the soluble factors and blood flow dynamics deemed necessary for promoting the dissemination of metastatic tumor cells (Taylor et al., 2000), viable sites for tumor metastasis may also provide the appropriate ECM, microenvironmental and spatial information critical for tumor cell survival.

MATERIALS AND METHODS

Substrates and Antibodies

The material used were: Commercial EHS matrix (Matrigel™, Collaborative Research) for the rBM assays; Vitrogen (Vitrogen 100, Celtrix Laboratories; bovine skin collagen I), 3 mg/ml, for coating culture dishes; Cellagen Solution AC-5, 0.5% (ICN Biomedical Inc.) for the 3D collagen I assays; and poly HEMA, 6 mg/ml (Sigma Chemicals) for the cell suspension studies.

Antibodies used were: collagen IV, clone PHM-12 (Biogenex); laminin-5 $\alpha 3$ chain specific,

clone BM165 (gift of M.P. Marinkovich, Stanford; Rousselle et al., 1991M); α 6 integrin, clones J15B (gift of C. Damsky, UCSF) and GoH3 (Chemicon International); α 2 integrin, clone 10G11 and α 3 integrin, clone P15B (Chemicon International); β 1 integrin, clones AIIB2 (gift of C. Damsky, UCSF), and TS2-16 (gift of M. Hemler, Harvard); β 4 integrin, rabbit sera and clones 3E1, ASC-3 and ASC-8 (Chemicon International); BP180, rabbit sera J17 (J. Jones, Northwestern); HD-1, clone 121 (gift of K.Owaribe, Graduate School of Human Informatics, Okazaki, Japan; Okumura et al., 1999); β -catenin, clone 14 (Transduction Laboratories); FAS receptor, clones IgM CH-11 (MBL Co., Ltd.) and CD95 (Immunotech, Inc.); TNF- α 55 receptor, clone CD120a (Caltag); E cadherin clones 36 (Chemicon International) and HECD-1 (Zyomed); NF κ B p65, rabbit sera (Santa Cruz), cytokeratin 18, clone RCK106 (Transduction Laboratories); human MHC class I clone W6.32 (Sigma); and actin, FITC-conjugated Phalloidin (Molecular Probes); fluorescein- and Texas Red-conjugated, non-conjugated anti-mouse and anti-rat, and non-specific rat and mouse IgGs (Jackson Laboratories); HRP-conjugated rat and mouse secondary antibodies (Amersham Pharmacia Biotech). The multicatalytic proteasome inhibitor MG132 (5 mM stock in DMSO; Calbiochem) was used at 0.5-5.0 μ M.

Cell Culture

The HMT-3522 MECs were grown and manipulated in 2D and 3D as described (Petersen et al., 1992; Weaver et al., 1997). Phenotypic reversion of T4-2 cells, using β 1 integrin function-blocking mAb, and disruption of S-1 morphogenesis, using the E-cadherin function-blocking mAb, were exactly as described (Weaver et al., 1997; Wang et al., 1998).

Indirect Immunofluorescence Analysis and Image Acquisition

Cells were either directly fixed using 2% paraformaldehyde or in 1:1 methanol:acetone or 100% methanol or first extracted in situ using CSK buffer (50 mM HEPES, 300 mM sucrose, 100 mM KCl, 5 mM MgCl, 5 mM EDTA, 0.5% Triton X-100, containing 10 μ g/ml leupeptin, 10 μ g/ml pepstatin, 1 mM Pefabloc [Boehringer Mannheim], 10 μ g/ml E64, 25 μ g/ml aprotinin, 0.5 mM benzamide, 1 mM sodium orthovanadate and 20 mM sodium fluoride), and subsequently fixed. In some experiments cultures were embedded in sucrose, frozen in Tissue-Tek OCT compound (Miles Laboratories), and 5 μ m frozen sections were prepared for immunostaining. Cell samples

were incubated with primary mAbs followed directly by either FITC- or Texas red-conjugated secondary Abs. Nuclei were counterstained with diaminophenylindole (DAPI, Sigma), or propidium iodide (Sigma). Cells were visualized using a Bio-Rad MRC 1024 laser scanning confocal microscope attached to a Nikon Diaphot 200 microscope. All immunofluorescence images were recorded at 120X magnification.

Analysis of Cell Growth

The proliferation rate of cells was measured by assaying BrdU incorporation using a commercially available labeling and detection kit (Boehringer Mannheim). The BrdU-labeling index was determined by scoring the BrdU-positive cells, and expressing this as a percentage of total cell number, estimated by counting the number of nuclei visualized by DAPI staining (200-400 cells).

Induction and Analysis of Apoptosis

Apoptosis was initiated by direct receptor trimerization using the FAS receptor antibody IgM Ch-11 (1-2 $\mu\text{g/ml}$); by receptor ligation with CD95 (1-2 $\mu\text{g/ml}$) followed by secondary mAb-induced clustering; by treatment with recombinant, purified Trail (Apo2L, 1-4 $\mu\text{g/ml}$; BioMol Research Laboratories Inc.), recombinant TNF- α (10-100 nM; R & D Systems Inc.), the topoisomerase II inhibitor, etoposide (10-100 μM in DMSO; TopoGen Inc.), the microtubule agent paclitaxol (20-120 nM; Sigma), the actin microfilament drug cytochalasin B (1-2 $\mu\text{g/ml}$, Sigma), the membrane permeable N-acetylsphingine analogue C2-ceramide (0.5-5 μM ; BioMol), or the membrane permeable non-active N-acetylsphingine analogue C2-dihydroxyceramide (0.5-5 μM ; BioMol). Screening assays for apoptosis included: Live/Dead Assay (Molecular Probes), active caspase 3 detection and cleavage of PARP by immunoblot analysis, and increased expression of annexin V in treated cells (Pharmingen). After initial screening, routine assay for apoptosis in intact fixed cells or cryosections, used a commercially available in situ apoptosis kit (Boehringer Mannheim). The apoptotic labeling index was calculated as cells positive for FITC-labeled 3'OH DNA ends as a percentage of the total number of cells scored (200-400 cells). In some experiments cell death by apoptosis was confirmed by showing that DNA cleavage could be inhibited by prior treatment with the caspase inhibitors YVAD-CHO or DEVD-CHO (1 μM ; BioMol Research Laboratories Inc.).

BM Overlay Assay and Integrin Inhibition Studies

To assay for effects of rBM overlay on multicellular structures, cells were grown in collagen I gels to form spheroids, growth-arrested by removal of EGF for 24 hrs., collagenase-liberated, and suspended in polyHEMA-coated dishes in H14 media supplemented with either BSA or rBM proteins (2 mg/ml). To inhibit integrin function, the 3D multi-cellular structures were pre-incubated with $\alpha 2$ integrin (4-16 μg IgG/ml); $\alpha 3$ integrin (1:25-1:100 ascites/ml); $\alpha 6$ integrin (4-12 μg IgG/ml); $\beta 1$ integrin (1:25-1:100 ascites/ml); $\beta 4$ integrin (4-16 μg IgG/ml); or IgG isotype matched control mAb (4-16 μg IgG/ml).

Antibody-conjugated Beads and Integrin Activation Studies

Monoclonal anti-human $\beta 1$ -integrin (TS2-16), $\beta 4$ -integrin (3E1), human MHC class I (W6.32), or non-specific IgG control mAb was covalently attached to magnetic porous glass beads (5 μM) by hydrazide cross linking, (CPG Inc.). Briefly, IgG protein was diluted (200 $\mu\text{g}/\text{ml}$; 100 μg total IgG), dialyzed overnight in oxidation buffer (100 mM sodium acetate, 150 mM sodium chloride, pH 5.5), and then activated by periodate treatment (10 mM sodium periodate). After quenching (50 % glycerol) mAb was dialyzed into coupling buffer (100 mM sodium acetate, 150 mM sodium chloride, pH 4.5) and linked to pre-washed MPG hydrazide beads, capped (67 mM glyceraldehyde, in coupling buffer), washed and stored in buffer (PBS: 138 mM sodium chloride, 2.7 mM potassium chloride, 8.1 mM disodium phosphate, 1.2 mM potassium phosphate, 0.5 mM magnesium chloride, 0.1 % Tween 20). To monitor for apoptosis resistance induced by integrin activation in 3D spheroids, pre-washed (10 X DMEM:F12) mAb cross-linked beads (1 μg IgG/2 X 10⁵ beads/10⁵ cells) were pre-incubated with suspensions of collagenase-liberated 3D collagen I structures followed by incubation with the apoptosis inducing agent.

Electron Microscopy Analysis

Cells in rBM were fixed for a minimum of 30 minutes (2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2), washed 3 times in (0.1 M sodium cacodylate buffer), and post-fixed (1% OsO₄ containing 0.8% potassium ferricyanide), stained with uranyl acetate, dehydrated in ethanol, and embedded in Epon-Araldite resin (Tousimis Corp.). Thin sections of embedded

material were stained with lead nitrate and sodium citrate and viewed at 60 kV in a JEOL 100CX electron microscope (JEOL USA).

Preparation of MECs Expressing Mutant β 4 integrin and the $I\kappa B\alpha$ Mutant

S-1 non-malignant HMT-3522 cells were transfected with mutant β 4 integrin connecting segment deleted and pcDNA 3.1 plasmid DNA using LipofectAMINE (Gibco BRL) or infected with GFP-tagged tailless β 4 integrin or RFP-tagged connecting segment mutant or wild type β 4 integrin, or mutant $I\kappa B\alpha$ (P. Khavari, Stanford; Seitz et al., 2000), or untagged wildtype or mutant β 4 integrin retroviral supernatant. Vector controls were prepared by transfecting with plasmid 3.1 pcDNA, or GFP, RFP or empty retroviral vector. Resistant β 4 integrin or vector control cells were selected using G418 (plasmid) or blasticidin (retrovirus) whereas experiments conducted using the $I\kappa B\alpha$ mutant were done using non-selected pooled cell populations.

Flow Cytometry

Cells grown as 2D monolayers were isolated, and non-specific binding was blocked (60 min Dulbecco's PBS, 1% bovine serum albumin). They were then incubated with saturating concentrations of primary mAb, three times, and labeled with fluorescein isothiocyanate (FITC)-conjugated goat immunoglobulin. Stained cells were washed three times and immediately analyzed on a FACScan (Becton Dickinson).

Immunoblot Analysis

To assess total β 4 integrin levels, pooled populations of β 4 integrin and vector control transfected or infected pooled cell populations were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 1 % NP-40, 0.5% deoxycholate, 0.2% SDS containing 20 mM sodium fluoride, and 1 mM sodium orthovanadate, and a cocktail of protease inhibitors). Equal amounts of protein were separated on non-reducing SDS-PAGE gels, immunoblotted and detected with an ECL system (Amersham Pharmacia BioTech).

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Figures

Figure 1. Only non-malignant cells within mammary acini are resistant to apoptosis.

Apoptotic labeling indices calculated for S-1 and T4-2 cells treated with Trail peptide (1 $\mu\text{g/ml}$), anti-Fas mAb (IgM CH-11, 2 $\mu\text{g/ml}$), TNF- α (100 nM), etoposide (50 μM), cytochalasin B (1 μM) or paclitaxol (120 nM): Cells were treated **A**) as monolayers on a thin coat of collagen I for 24 hours; or **B**) as 3D structures in rBM for 96 hours. Results are the mean \pm SEM of 3-5 separate experiments each with duplicates or triplicates.

Figure 2. Polarized mammary structures are resistant to apoptosis induction.

S-1 acini produced within rBM were treated with E-cadherin function-blocking mAb (HECD-1; 25 $\mu\text{g/ml}$ rBM) to perturb polarity. T4-2 structures within rBM were treated with β 1 integrin inhibitory mAb (clone AIIB2; 1:50 ascites/ml rBM) to restore polarity: **A**) Confocal microscopy of nuclei (propidium iodide), F-actin (FITC-phalloidin), β -catenin (Texas Red), β 4 integrin (Texas Red) and laminin-5 (FITC) fluorescence. Note that S-1 and reverted T4-2 acini exhibit cortically organized filamentous F-actin, cell-cell junction-localized β -catenin, basally localized β 4 integrins, and basally secreted laminin-5. Contrast with the S-1 disrupted and T4-2 disorganized structures. **B**) Apoptotic labeling indices calculated for cells grown as described in (A), and treated with TNF- α (100 nM), etoposide (50 μM), or anti-FAS mAb (2 $\mu\text{g/ml}$) for 96 hours. Results are mean \pm SEM of 3-5 separate experiments each with duplicates or triplicates. **C**) BrdU labeling indices under conditions described in **B**. Results are mean \pm SEM of 3 separate experiments of 200-400 cells /experiment. All cultures were analyzed after 10 days inside the rBM. Bars: 10 μm .

Figure 3. The polarized acini resist apoptosis induction regardless of growth status

A) Confocal microscopy of β -catenin and collagen IV: Control S-1 structures in rBM, S-1 in collagen I gels, and S-1 cells over-expressing EGF-R in rBM show β -catenin (Texas Red) localized at cell-cell (adherens) junctions. However, only the structures generated in the rBM acquired basal polarity as marked by the basal deposition of collagen IV (Texas Red). All cultures were analyzed after 10 days in 3D gels. **B**) BrdU labeling indices for S-1 cells

incorporated into 3D structures as described in (A). Results are mean \pm SEM of 3 separate experiments of 200-400 cells/experiment. **C)** S-1 cells propagated as described in (A) were induced to undergo apoptosis by TNF- α (100 nM), etoposide (50 μ M) or anti-Fas mAb (2 μ g/ml) for 96 hr. Results are mean \pm SEM of 3-5 separate experiments each with duplicates or triplicates. Bars: 10 μ m.

Figure 4. BM-induced tissue polarity is necessary for apoptosis resistance

S-1 cells were either grown inside the rBM, inside collagen I, or inside collagen I followed by addition of either rBM or laminin-1. **A)** Confocal microscopy of Z sections of nuclei (propidium iodide), β -catenin (Texas Red), β 4 integrin (FITC), α 6 integrin (Texas Red) and laminin-5 (FITC) fluorescence. All structures had β -catenin localized at cell-cell junctions. Although 3D structures grown in contact with collagen I had cytosolic β 4 and α 6 integrins and dispersed laminin-5, when these cells were overlaid with rBM or laminin-1 (not shown), their β 4 and α 6 integrins became reorganized to the site of cell-rBM interactions, and they assembled an endogenous BM as shown by deposition of laminin-5 at the cell-rBM junction. **B)** Apoptotic labeling indices calculated for S-1 cells grown as described in (A). Cultures were treated with TNF- α (100 μ M) for 96 hours. Results are mean \pm SEM of 3-6 separate experiments each with duplicates or triplicates. Bars: 10 μ m.

Figure 5. BM-induced tissue polarity regulates NF κ B activation and drives apoptosis resistance in acini.

A) Confocal microscopy of cytokeratin 18 and NF κ B p65: Control S-1 acini in rBM treated for one hour with TNF- α (100 nM), Trail peptide (1 μ g/ml) or etoposide (50 μ M) show cytoplasmic (cytokeratin 18; FITC) to nuclear translocation of NF κ B p65 (Texas Red), whereas acini treated with C2-ceramide (5 μ M) do not. **B)** Cell viability calculated for control S-1 cells or S-1 cells expressing a proteolytically resistant mutant I κ B α grown in rBM to form acini and then treated as described in (A) in the presence or absence of the multi catalytic proteasome inhibitor MG 132 (5 μ M). Results are mean \pm SEM of 3-4 separate experiments of 200-600 cells scored in each.

Figure 6. $\alpha6\beta4$ integrin directs apoptosis resistance in 3D mammary organoids

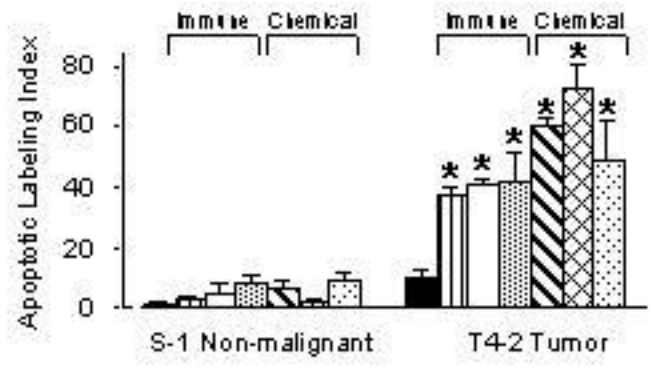
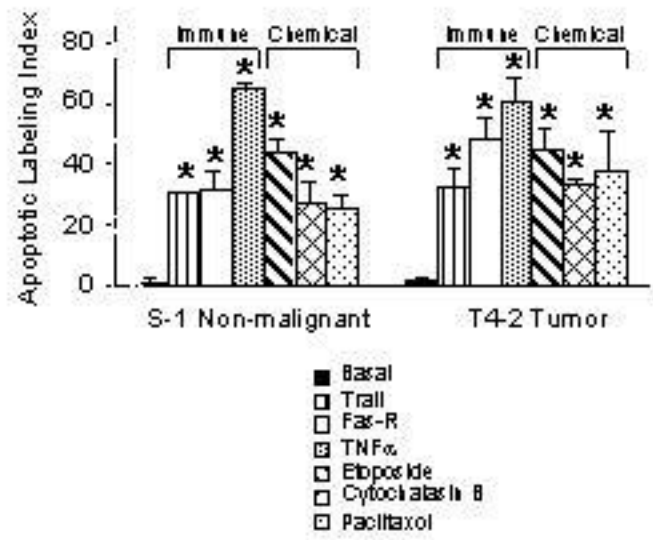
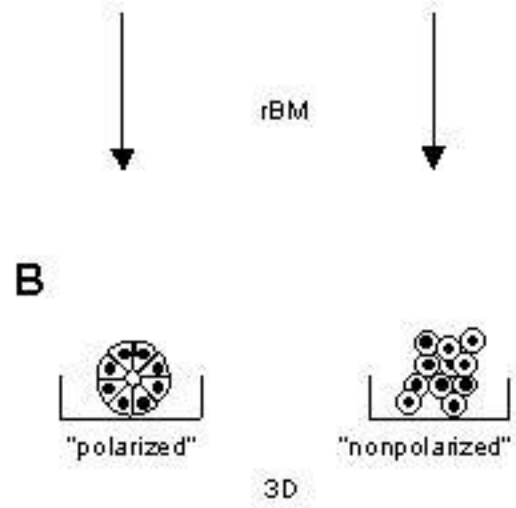
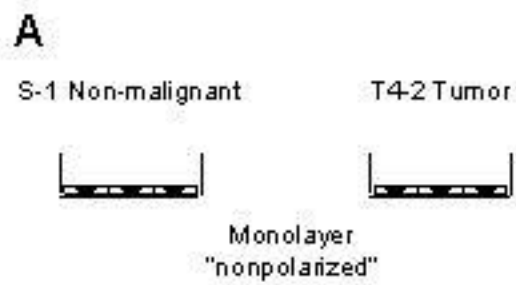
A) Apoptotic labeling indices calculated for S-1 cells grown in collagen I and pre-incubated with function-blocking mAb against $\alpha3$ integrin (clone P15B), $\beta1$ integrin (clone AIB2), $\alpha6$ integrin (clone GoH3), $\beta4$ integrin (clone ASC 3) or an isotype specific IgG control for 30 minutes, overlaid with rBM and then treated in suspension culture with TNF- α (100 μ M) for 96 hours. Results are the mean \pm SEM of 4-8 separate experiments all in triplicates. **B)** Apoptotic labeling indices calculated for S-1 cells grown in collagen I gels following ligation and clustering of $\beta1$ or $\beta4$ integrin or a MHC cell surface molecule for one hour and then treated with Trail (1 μ g/ml) for 96 hours. Results are the mean \pm SEM of 2-6 separate experiments each with duplicates. **C)** Apoptotic labeling indices calculated for S-1 vector or tailless $\beta4$ integrin expressing cells grown in rBM for 10 days and treatment with etoposide (50 μ M), Fas mAb (1 μ g/ml) or Trail (1 μ g/ml) for 96 hours. Results are the mean \pm SEM of 3 separate experiments. **D)** Immunofluorescence of HD-1, BP180, $\alpha6$ integrin, $\beta4$ integrin, collagen IV and laminin-5 (Texas Red) in S-1 control versus S-1 expressing tailless $\beta4$ integrin (GFP). Bar: 10 μ M.

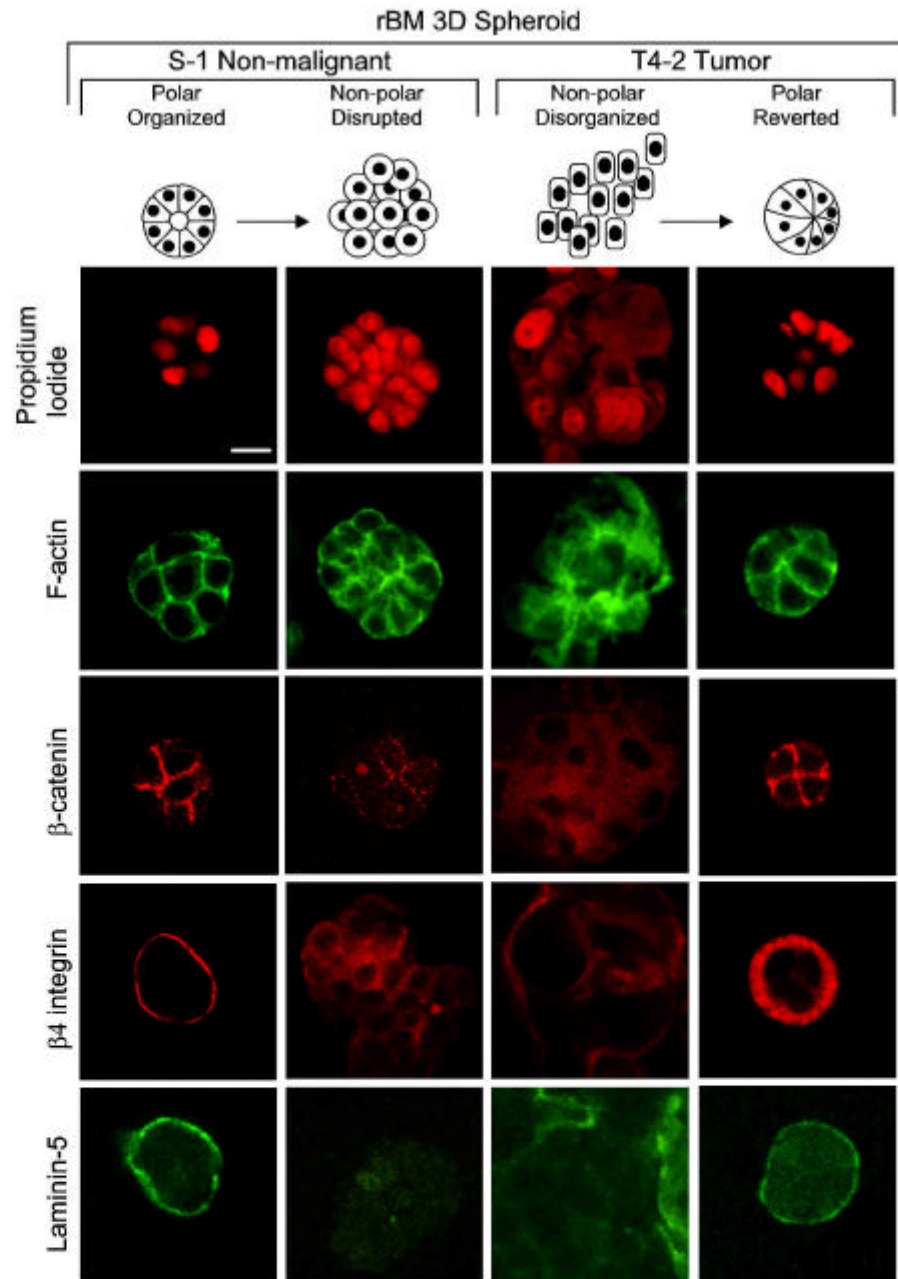
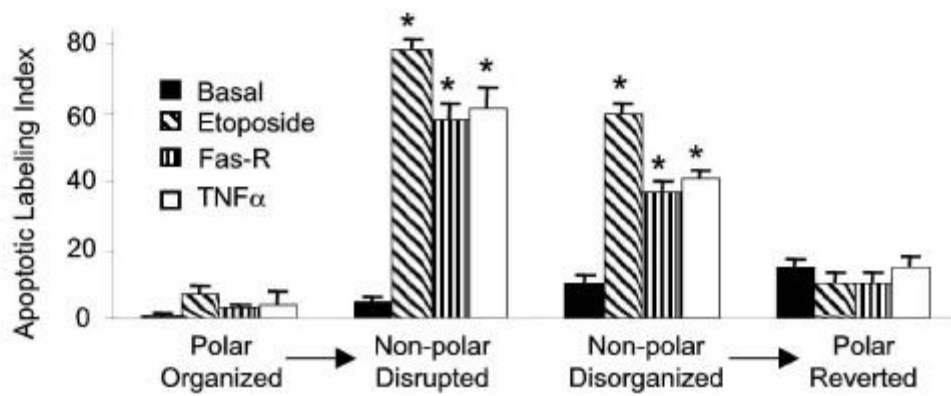
Figure 7. Apoptosis resistance in reverted tumor cells is associated with increased levels of mature hemidesmosomes and constitutive activation of NF κ B.

T4-2 cells were grown inside rBM with control mAb or were reverted with $\beta1$ integrin inhibitory mAb (AIB2), and analyzed after 12 days by transmission electron microscopy (TEM) and immunofluorescence. Both structures expressed the hemidesmosome proteins $\beta4$ integrin and HD-1 and contained hemidesmosomes; note, however, they are less abundant in the T4-2 control spheroids (1/2 μ m plasma membrane), and of these, 90% are immature type II structures. Hemidesmosomes are more abundant in the T4-revertants (Rvts), and 60% are of mature type I and are assembled at the basal tissue domain where $\beta4$ integrin and HD-1 are located. Hemidesmosome formation in the T4-Rvts is associated with constitutively localized NF κ B p65. Bars: TEM 50 nm; confocal 10 μ M.

Figure 8. Disrupting hemidesmosome formation perturbs BM-directed tissue polarity, inhibits NF κ B activation and permits induction of apoptosis in 3D acini.

A) Diagram of β 4 integrin showing the hemidesmosome targeting domain in the cytoplasmic tail of the β 4 integrin protein and the RFP tag. **B)** Western blot of RIPA lysates of S-1 cells showing that the deleted and wild type β 4 integrin transfectants expressed elevated total levels of β 4 integrin protein relative to the vector controls. **C)** Phase contrast and immunofluorescence of β 4 integrin deleted, wild type and vector control RFP-expressing S-1 cells in 2D. **D)** Immunofluorescence of NF κ B p65 (Texas Red) in S-1 cells in rBM expressing EGFP-tagged mutant β 4 integrin and EGFP vector controls before and after one hour of TNF- α treatment (100 μ M) showing reduced cytoplasmic to nuclear translocation of NF κ B p65 (Texas Red) in mutant β 4 integrin expressing S-1 cells. **E)** Immunofluorescence of HD-1 (FITC), BP180 (FITC), β -catenin (Texas Red), β 4 integrin (Texas Red,) and laminin-5 (FITC) fluorescence in S-1 cells in rBM expressing mutant and wild type β 4 integrins and vector controls. 3D cultures were analyzed after 12 days inside rBM. Bar: 10 μ m. **F)** Apoptotic labeling indices calculated after 12 days cultivation in the rBM and treatment for 96 hours with TNF- α (100 μ M), Fas receptor mAb (2 μ g/ml), or etoposide (50 μ M). Results are the mean \pm SEM of 3 separate experiments.

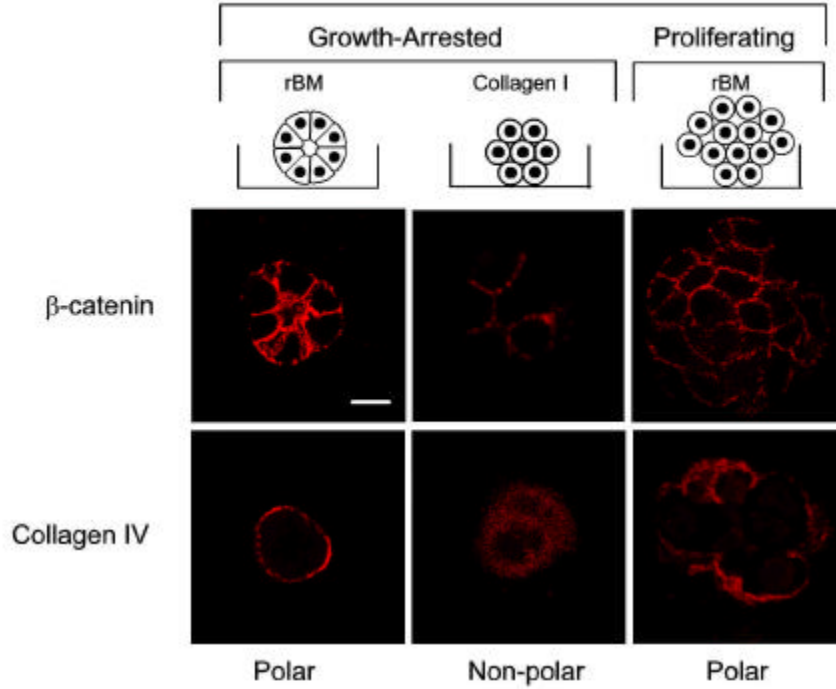


A**B****C**

BrdU Labeling Index	Polar Organized	Non-polar Disrupted	Non-polar Disorganized	Polar Reverted
	1.81 \pm 0.75	23.60 \pm 9.31	31.67 \pm 6.27	4.44 \pm 1.38

S-1 Non-malignant 3D

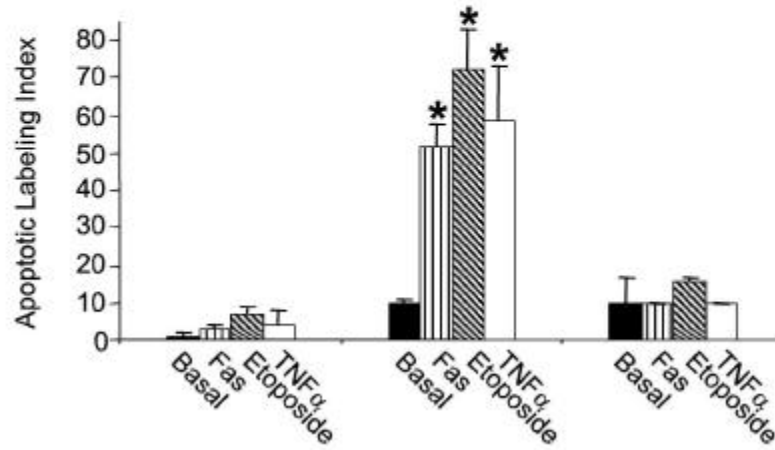
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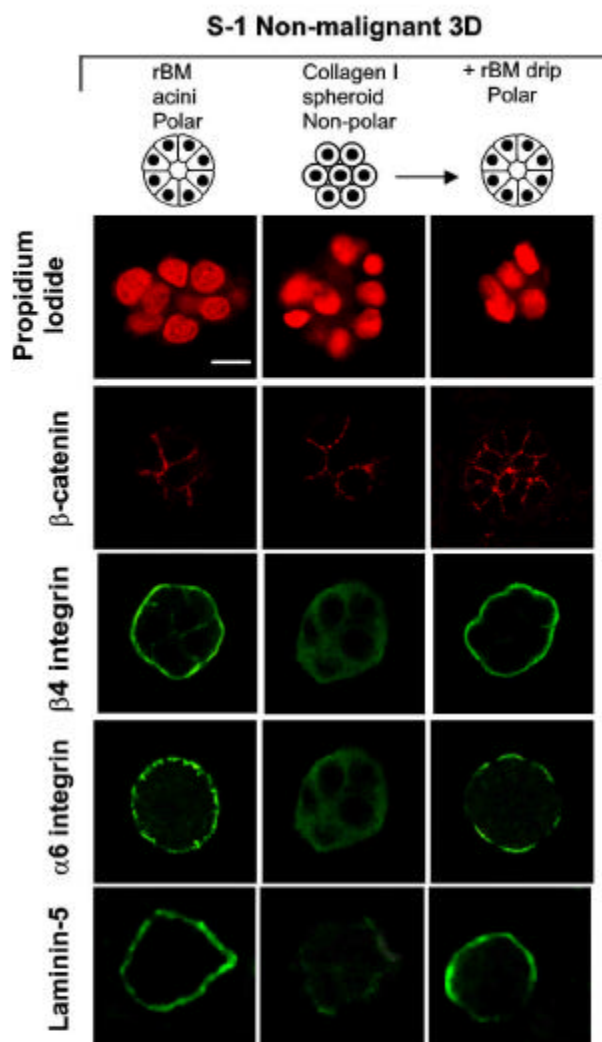
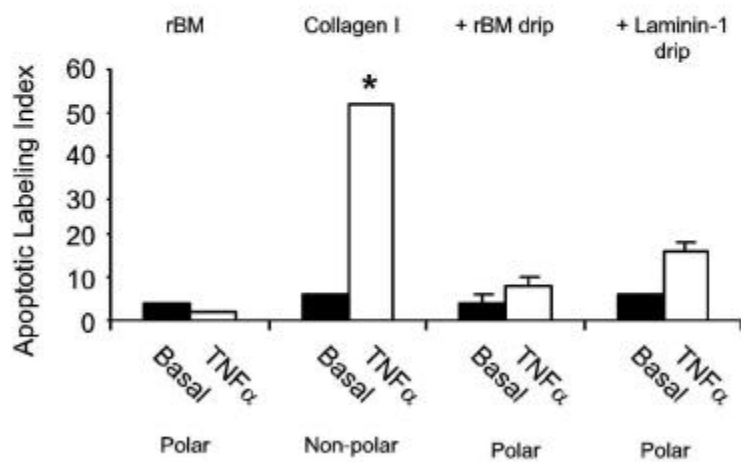


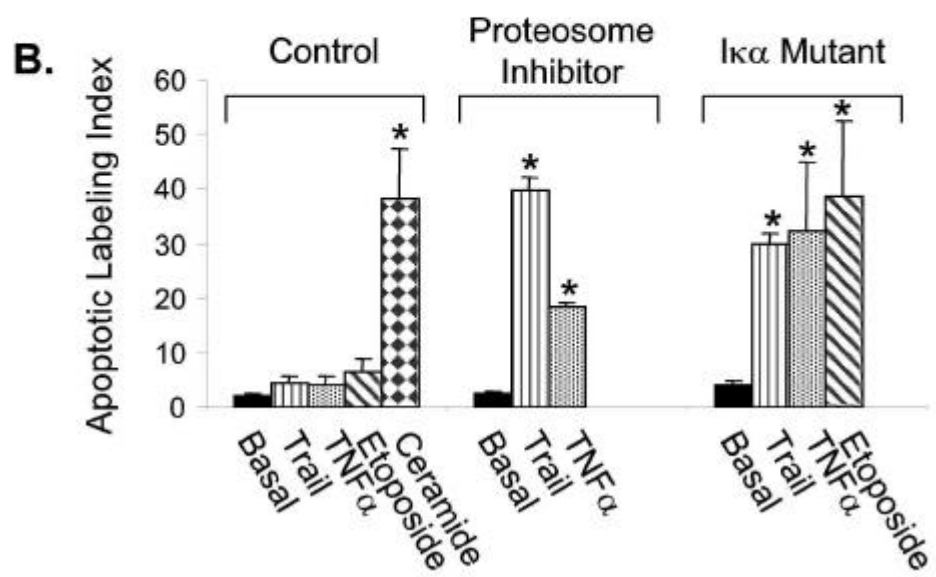
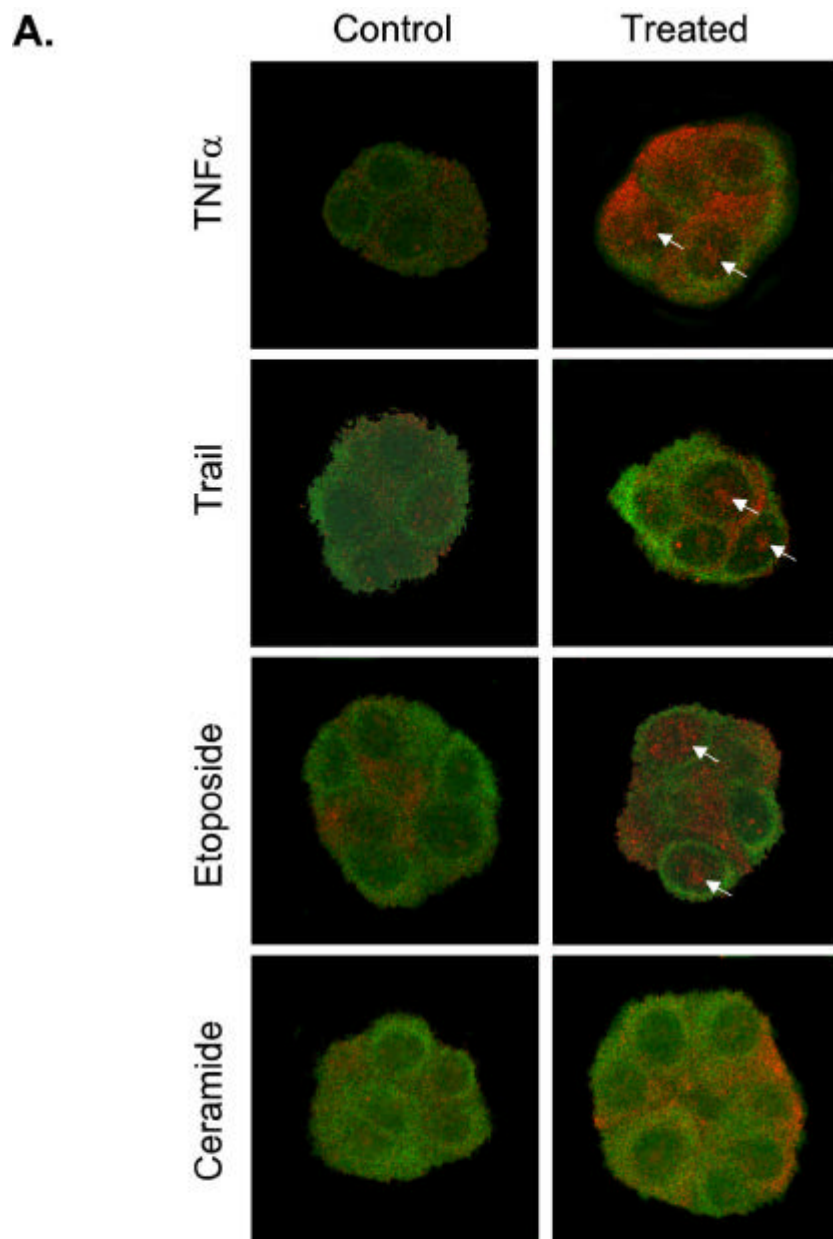
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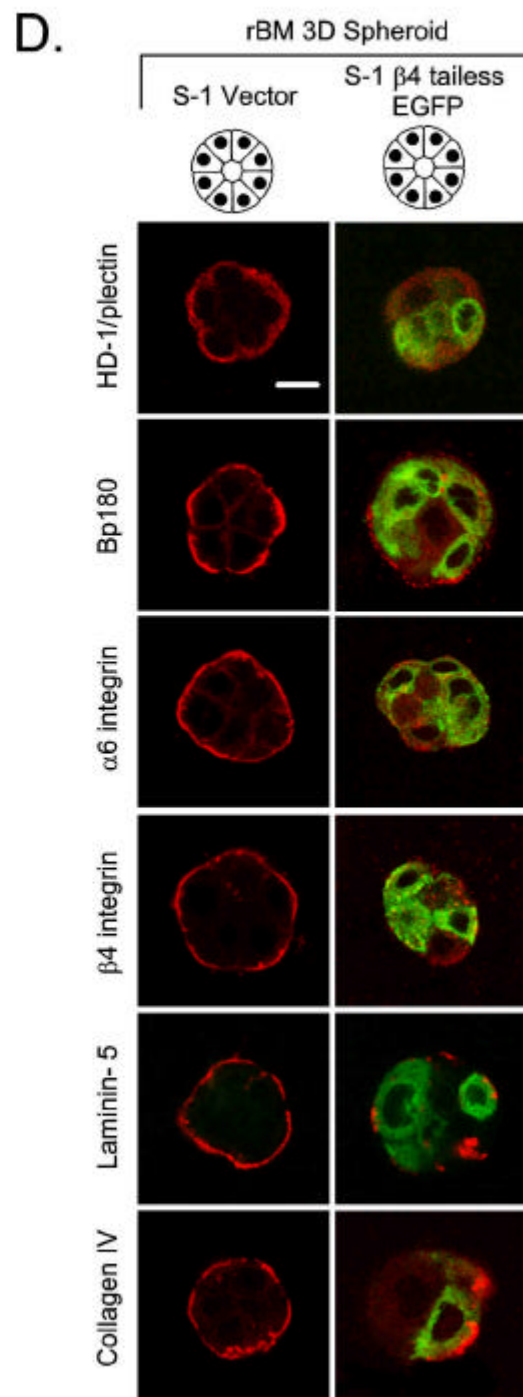
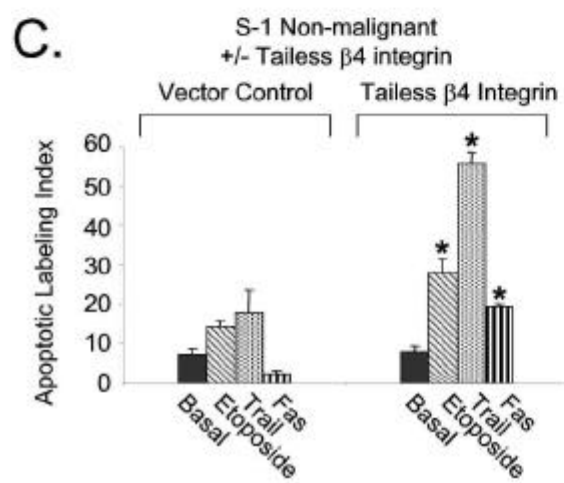
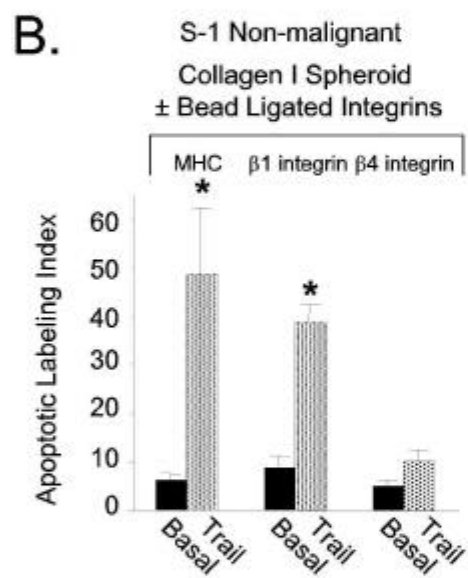
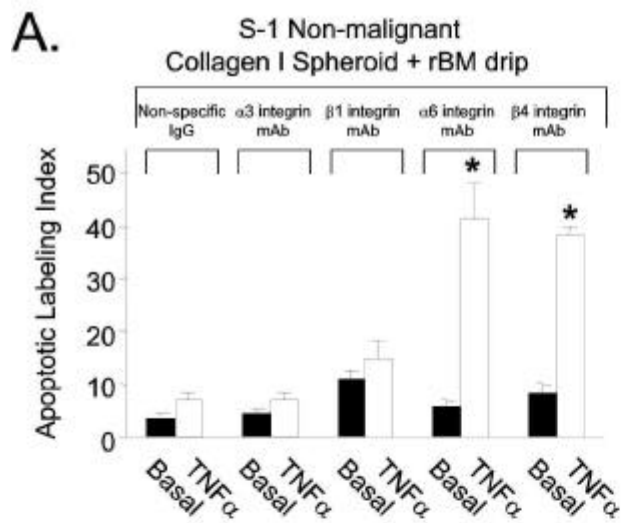
BrdU Labeling Index	2.38±0.25	5.72±2.78	32.33±2.75
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C

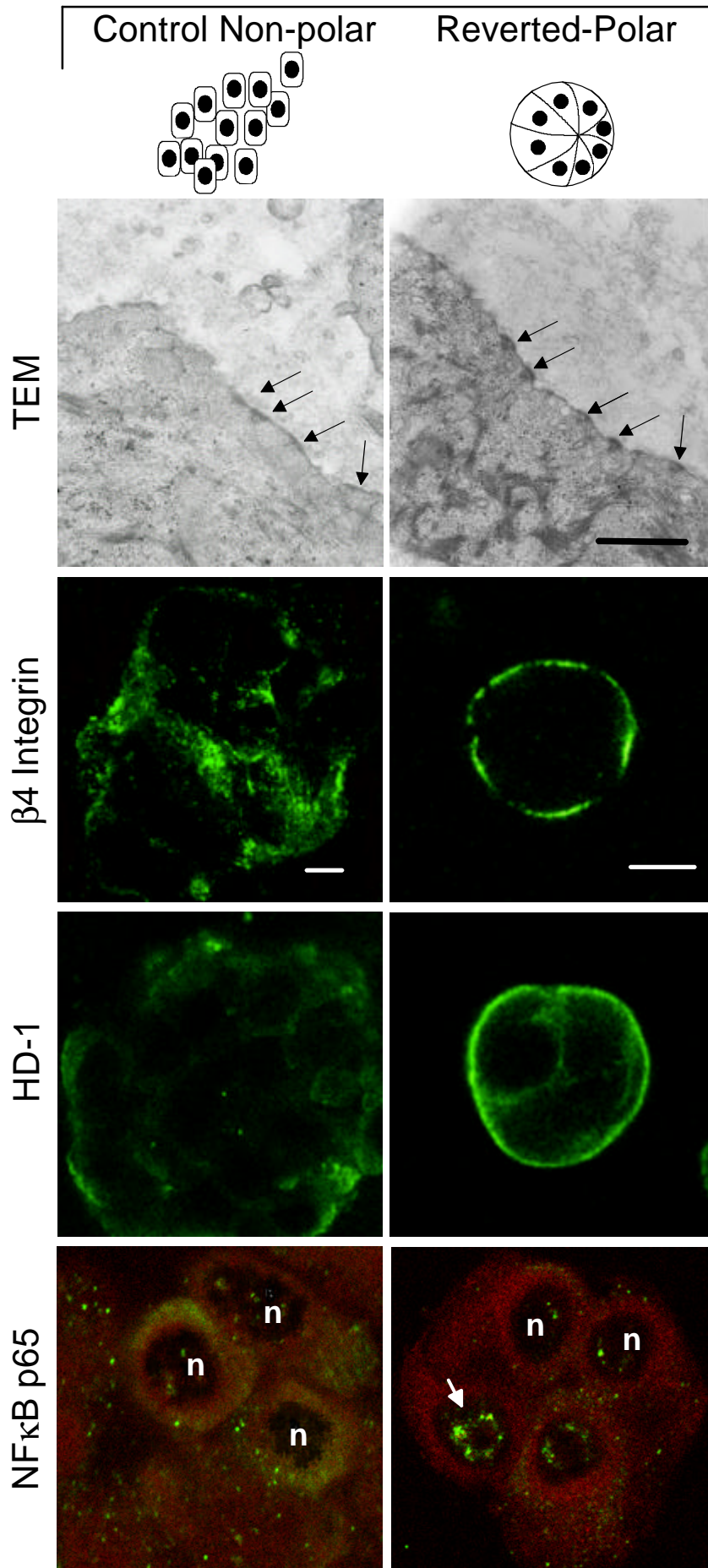


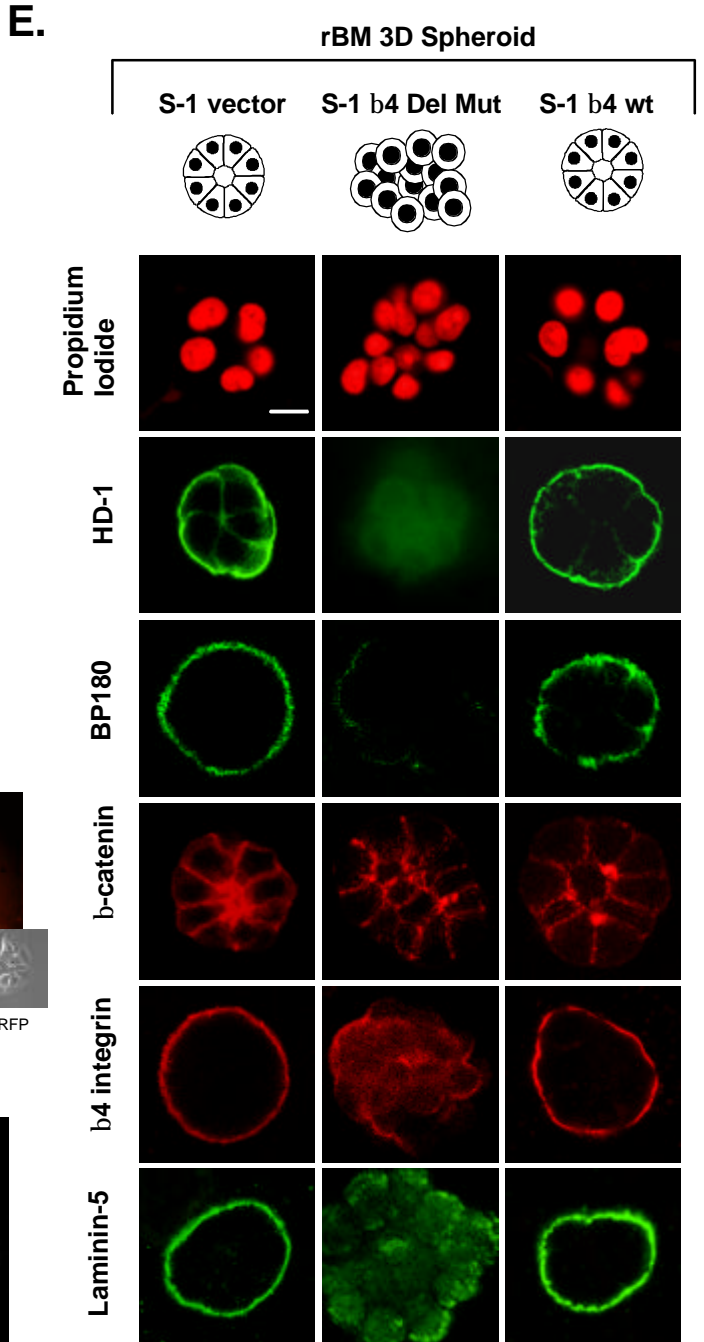
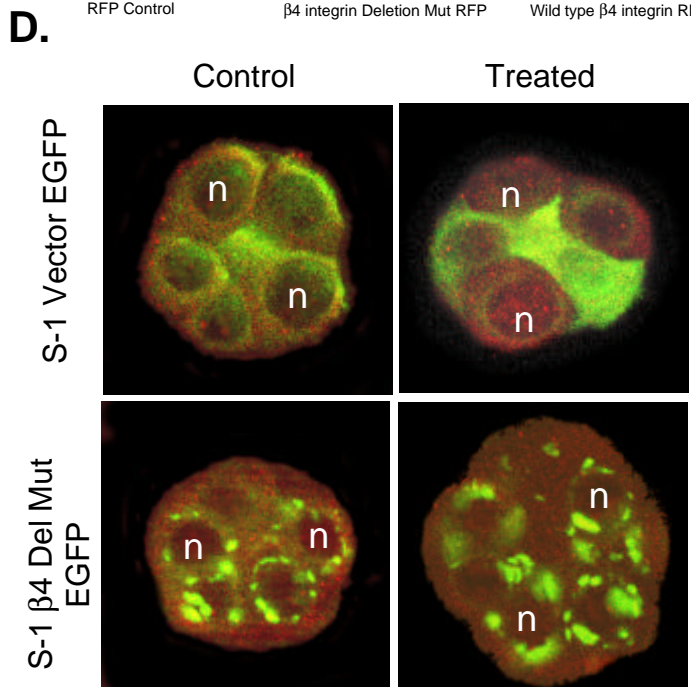
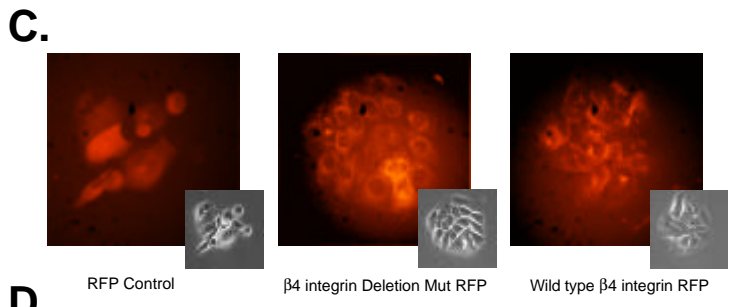
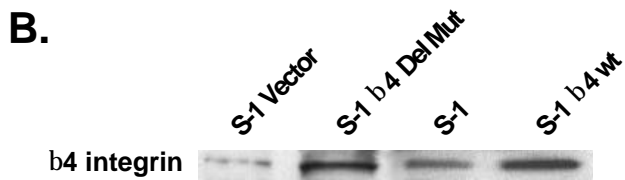
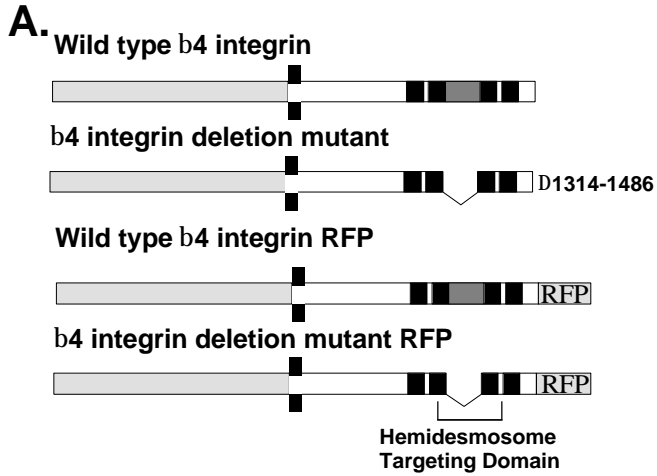
A**B**





T4-2 cells within rBM





F. Apoptotic labeling index in 3D structures formed by S-1 cells transfected with $\beta 4$ integrin wt, deletion mutant, and vector control.

	Basal	Etoposide (50 μ M)	Fas Receptor Ligation (2 μ g/ml mAb)	TNF α (100 nM)
S-1 Vector	6.74 \pm 1.63	5.91 \pm 0.99	4.25 \pm 0.77	6.74 \pm 1.51
S-1 $\beta 4$ Del Mut	8.95 \pm 1.52	32.81 \pm 5.37	28.76 \pm 5.00	34.03 \pm 1.62
S-1 $\beta 4$ wt	5.48 \pm 1.35	12.25 \pm 3.13	4.31 \pm 0.82	11.93 \pm 0.50

Mean \pm SEM of 200-400 cells per experiment (three separate experiments).