A 3D matrix platform for the rapid generation of therapeutic anti-human carcinoma monoclonal antibodies

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Efforts to develop unbiased screens for identifying novel function-blocking monoclonal antibodies (mAbs) in human carcinomatous states have been hampered by the limited ability to design in vitro models that recapitulate tumor cell behavior in vivo. Given that only invasive carcinoma cells gain permanent access to type I collagen-rich interstitial tissues, an experimental platform was established in which human breast cancer cells were embedded in 3D aldehyde cross-linked collagen matrices and used as an immunogen to generate mAb libraries. In turn, cancer-cell–reactive antibodies were screened for their ability to block carcinoma cell proliferation within collagen hydrogels that mimic the in vivo environment. As a proof of principle, a single function-blocking mAb out of 15 identified was selected for further analysis and found to be capable of halting carcinoma cell proliferation, inducing apoptosis, and exerting global changes in gene expression in vitro. The ability of this mAb to block carcinoma cell proliferation and metastatic activity was confirmed in vivo, and the target antigen was identified by mass spectroscopy as the α2 subunit of the α2β1 integrin, one of the major type I collagen-binding receptors in mammalian cells. Validating the ability of the in vitro model to predict patterns of antigen expression in the disease setting, immunohistochemical analyses of tissues from patients with breast cancer verified markedly increased expression of the α2 subunit in vivo. These results not only highlight the utility of this discovery platform for rapidly selecting and characterizing function-blocking, anticancer mAbs in an unbiased fashion, but also identify α2β1 as a potential target in human carcinomatous states.

In mammalian systems, a specialized form of extracellular matrix (ECM), termed the basement membrane, normally separates epithelial cells from the underlying type I collagen-rich interstitial matrix (1, 2). Thus, in mature animals and under physiologic conditions, the epithelium does not establish stable physical contacts with interstitial tissues (1, 2). In contrast, in neoplastic states, transformed epithelial cells (i.e., carcinomas) dissolve the intervening basement membrane barrier and establish adhesive interactions with the newly exposed type I collagen fibrillar network (1–5). As carcinoma cells begin to infiltrate the interstitial matrix, they rapidly adapt themselves to their 3D environment and initiate the proliferative phenotypes that define tumor progression at both primary and metastatic sites (2, 6, 7). Indeed, emphasizing the importance of the tumor–ECM interface, carcinoma cells do not simply use the surrounding interstitial matrix as a passive substrate, but actively promote increased type I collagen deposition within the peritumoral microenvironment as a means of further enhancing invasive activity, local growth, and cancer stem cell formation (7–12).

Despite the importance of the carcinoma cell–type I collagen interface in vivo, therapeutic interventions that directly interfere with the specific cell–ECM interactions operating within this specialized tumor milieu have yet to be identified. Traditionally, new therapeutic agents are developed by identifying a preferred candidate and then generating a specific inhibitor for a targeted effector (13). In this regard, humanized monoclonal antibodies (mAbs) have been established as important players in the therapeutic armamentarium (13, 14). Strategies that allow for the rapid identification and validation of new targets remain problematic, however (13).

Cognent arguments have been forwarded regarding the utility of phenotypic screens for the purpose of identifying new targets in an unbiased fashion (13, 15). Nevertheless, leveraging this approach requires the engineering of in vitro conditions that faithfully recapitulate carcinoma cell behavior in vivo, so that targets can be identified and their functional contribution assessed rapidly before in vivo testing. To this end, here we describe a novel screening platform wherein human carcinoma cells are cultured within aldehyde cross-linked, 3D type I collagen hydrogels similar to those found at invasive sites in vivo (16), and the cancer cell–matrix composite used to generate a library of mAbs. In turn, the mAbs are then screened for their ability to suppress carcinoma cell proliferative responses under 3D growth conditions. Validating the utility of our in vitro approach, selected mAbs are shown to inhibit carcinoma cell proliferation and metastatic activity in xenograft models in vivo. Finally, using a combination of immunopurification, mass spectroscopy, and peptide mapping, the target antigens can be identified and their expression confirmed in human cancer tissues. Taken together, these findings not only establish a platform that allows for the

Significance

To select for novel monoclonal antibodies (mAbs) with anticancer activity, an experimental platform was established wherein human breast cancer cells were embedded in 3D collagen matrices and used as an immunogen to generate mAb libraries. Fifteen mAbs capable of inhibiting carcinoma cell growth in vitro were generated. A single function-blocking mAb was selected for further analysis and then validated as a potent inhibitor of carcinoma cell behavior in vivo. The target antigen was identified as the α2 subunit of the α2β1 integrin, a major type I collagen-binding receptor whose expression was confirmed in tissues of patients with breast cancer. These findings describe a new discovery platform that allows for the rapid selection of function-blocking antibodies and identify α2β1 as a potential target in carcinomatous states.


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at the start of the 3D culture period; addition of the inhibitory cell carcinoma, ovarian carcinoma, and fibrosarcoma cell lines in similar inhibitory effects can be observed with human squamous membranes structure nor that of the interstitial matrix (1, 2), that neither recapitulates the structure of normal basement treated plastic substrata or within 3D Matrigel, an ECM extract when cultured under standard 2D conditions atop tissue culture-

Among the 15 mAbs displaying inhibitory activity in our initial screens, clone 4C3 was one of the most potent IgG1 class anti-

RNA processing, and cell division-related programs (Fig. 1 E and F). Taken together, these results identify mAb 4C3 as a po-

tent regulator of MDA-MB-231 cell function within the confines of a type I collagen-rich ECM.

mAb 4C3 Exerts Global Effects on the MDA-MB-231 Transcriptome. In an effort to identify the potential signaling networks impacted by mAb 4C3, we cultured MDA-MB-231 cells in 3D type I collagen hydrogels in the presence of either a control IgG1 or mAb 4C3 for 48 h (i.e., before the initiation of proliferative responses), and RNA was harvested for gene expression profiling. Under these conditions, mAb 4C3 exerted global effects on gene expression, with almost 1,200 unique transcripts affected (i.e., 172 up-regulated and 1,004 down-regulated transcripts, respectively, using a 2.0-

mAb 4C3 Prevents Postextravasation Carcinoma Growth In Vivo. In our in vitro model, embedded carcinoma cells are individually surrounded by a network of type I collagen fibrils, a scenario similar to that encountered when circulating tumor cells extravasate from vascular or lymphatic beds and enmesh themselves within the perivascular interstitial matrix (1–3, 6, 12). To examine the inhibitory potential of mAb 4C3 in a post-

extravasation program directly, we used a live embryonic chick xenograft model that faithfully recapitulates carcinoma cell behavior in mouse models (22, 23). As shown in Fig. 24, the chick chorioallantoic membrane vasculature is readily visualized by confocal laser microscopy. Furthermore, using second harmonic generation to image type I collagen fibrils in situ (24) revealed

Fig. 1. In vitro activity of mAb 4C3. (A) MDA-MB-231 cells were seeded in 3D collagen matrices in the absence or presence of mAb 4C3 (10 μg/mL). Cultures were evaluated by phase-contrast or confocal microscopy (red) at day 0 and day 4. (B) MDA-MB-231 cells were seeded in 3D collagen in 12-well plates (5 × 104 cells/well) with mAb 4C3 (10 μg/mL) added at day 0 or day 4 (red arrow). At indicated times, cell numbers were determined by hemocytometry. Results are expressed as mean ± SEM (n = 3). *P < 0.05. (C) MDA-

MB-231 proliferation was assessed by relative ATP levels after 48 h of treatment with indicated mAb 4C3 concentrations. Vehicle controls or control IgG1 mAb were without effect. Adhesion was assessed by allowing MDA-MB-231 cells to attach to collagen gels for 1 h, followed by staining with crystal violet. Results are expressed as mean ± SEM of three experi-

ments. (D) Relative levels of caspase 3 and caspase 7 activity were deter-

mined for MDA-MB-231 cells embedded within 3D collagen gels for 72 h in the presence of the indicated concentrations of mAb 4C3 added 24 h before the assay. Vehicle control or control IgG1 were without effect. Results are expressed as mean ± SEM of three experiments. (E) Gene Ontology terms identifying cellular processes after mAb 4C3 (10 μg/mL) treatment of 3D-

embedded MDA-MB-231 for 48 h. (F) Heat map of genes regulating cell cycle after mAb 4C3 treatment is shown.
carcinoma cells (Fig. 2A and B) and quantification of luminescent signals using luciferase-tagged MDA-MB-231 cell proliferation was markedly inhibited, where tumor colonization was readily monitored by both visual inspection and quantification. Results are expressed as mean ± SEM (n = 3). *P < 0.05.

That blood vessels were uniformly invested by a dense collagenous network (Fig. 2B).

As such, fluorescent-tagged MDA-MB-231 cells were injected into the host vasculature of 11-d-old immunocompetent chicken embryos in tandem with a control IgG1 or mAb 4C3, and postextravasation growth was monitored. After a 6-d culture period in vivo, extravasated MDA-MB-231 cells initiated proliferative activity in close association with the chick vasculature (Fig. 2C). In contrast, in the presence of mAb 4C3, MDA-MB-231 cell proliferation was markedly inhibited, where tumor colony formation was readily monitored by both visual inspection and quantification of luminescent signals using luciferase-tagged carcinoma cells (Fig. 2C–F).

To rule out the possibility that 4C3 blocks proliferative responses by interfering with MDA-MB-231 extravasation itself, we injected carcinoma cells into the chick vasculature, and after a 24-h period that allows for the completion of extravasation (23), introduced mAb 4C3 intravascularly. Even under these conditions, mAb 4C3 exerted potent inhibitory effects, equivalent to those obtained when the antibody was introduced at the start of the in vivo assay (Fig. 2F).

Antimetastatic Activity of mAb 4C3 in a Mouse Xenograft Model.

Unlike humans, whose mammary tissues are dominated by type I collagen, the mouse mammary gland contains only small amounts of type I collagen that is largely confined to periductal regions, thus rendering mouse xenograft orthotopic models less useful for analyzing carcinoma cell–type I collagen matrix interactions (25). Alternatively, the organic matrix of mouse bone, like that of humans, is composed largely of type I collagen (17, 26–28). Furthermore, bone is a frequent site of breast cancer metastatic activity in human disease (17). As such, after intracardiac injection, we assessed the ability of luciferase-tagged MDA-MB-231 to generate bone metastatic lesions in nude mouse recipients in the presence of control IgG1 or mAb 4C3 by in vivo imaging as well as micro-CT analyses over a 28-d assay period.

When treated with twice-weekly dosages of 10 mg/kg of the control mAb, MDA-MB-231 cells generated large tumors in the mandible, hindlimb, and spine of inoculated mice (Fig. 3A and B). In contrast, in the mAb 4C3-treated group, carcinoma growth in the mandible and hindlimb was impaired, with significant inhibitory effects recorded in vertebral metastases where bone-erosive lesions were readily observed in micro-CT scans of the control antibody-treated group (Fig. 3A–C). Whereas ~50% of the control antibody-treated mice required euthanization due to spinal cord compression and resulting limb paralysis, fewer than 20% of the mAb 4C3-treated mice were similarly affected, consistent with the ability of mAb 4C3 to block the progression of bony metastases (Fig. 3D).

Identification of the mAb 4C3 Target Antigen and Its Expression in Human Breast Cancer Bone Metastases.

To identify the target antigen recognized by mAb 4C3, we applied whole-cell lysates of MDA-MB-231 cells to immunoblot columns constructed using the purified antibody as the capturing agent. After antigen recovery, a major bond of ~150 kDa was isolated and submitted for mass spectrometry analysis after trypsin fragmentation (Fig. 4A). Bioinformatic analysis of the generated fragments identified the target antigen as the integrin subunit, alpha 2 (α2) (29, 30).

![Antimetastatic Activity of mAb 4C3 in a Mouse Xenograft Model.](https://www.pnas.org/cgi/doi/10.1073/pnas.1410996111 Dudley et al.)
Inhibitory actions with type I collagen (Fig. 1C) triple helix. [Model adapted from ref. 29 with permission from Elsevier.]

Chains (green, yellow, and blue) represent a portion of the type I collagen dimer (29, 30) (Fig. 4B) cated within the subunit (Fig. 4D). Peptide mapping of the mAb 4C3-binding sites in an overlapping series of peptides (each 10-aa long) that span the α2 integrin subunit. Asterisk indicates decapeptide epitope localized within the α-I domain. (D) Schematic illustrating the putative α-I domain elements recognized by mAb 4C3 and 8F10 (labeled “C” and “F,” respectively, within the red circle). Peptide 67 (mAb 4C3 peak) lies within structural element “F,” whereas peptide 44 (mAb 8F10 peak; Fig. S4) is located within β sheet “C” of the α2 chain as described. The three colored chains (green, yellow, and blue) represent a portion of the type I collagen triple helix. [Model adapted from ref. 29 with permission from Elsevier.]

Identification of the mAb 4C3 target antigen. (A) mAb 4C3 immunocaptured a 150-kDa band from lysates of MDA-MB-231 cells as detected by SDS/PAGE/silver staining. Mass spectrometry sequencing of the band identified the protein as the α2 integrin subunit. (B) MDA-MB-231 lysates were immunoprecipitated with mAb 4C3 and immunoblotted with a second antibody directed against the α2 integrin subunit. (C) Peptide mapping of the mAb 4C3-binding sites in an overlapping series of peptides (each 10-aa long) that span the α2 integrin subunit. Asterisk indicates decapeptide epitope localized within the α-I domain. (D) Schematic illustrating the putative α-I domain elements recognized by mAb 4C3 and 8F10 (labeled “C” and “F,” respectively, within the red circle). Peptide 67 (mAb 4C3 peak) lies within structural element “F,” whereas peptide 44 (mAb 8F10 peak; Fig. S4) is located within β sheet “C” of the α2 chain as described. The three colored chains (green, yellow, and blue) represent a portion of the type I collagen triple helix. [Model adapted from ref. 29 with permission from Elsevier.]

Immunoprecipitation of MDA-MB-231 lysates with mAb 4C3, followed by immunoblotting with an independent anti-α2 antibody, further confirmed the target antigen as the α2 integrin subunit (Fig. 4B).

Consistent with the fact that α2 integrin subunit only forms heterodimeric complexes with the β1 integrin to generate the dominant mammalian type I collagen receptor, α2β1 peptide mapping of mAb 4C3 interactions with the α2 subunit identified a major epitope lying within the α-I domain of the integrin, the dominant type I collagen recognition site of the α2β1 heterodimer (29, 30) (Fig. 4C). As expected from its collagen-binding properties, mAb 4C3 inhibited MDA-MB-231 adhesive interactions with type I collagen (Fig. 1C). Interestingly, a second, inhibitory α2 integrin-reactive mAb that was identified independently in our screen (mAb 8F10) also bound to a distinct, but overlapping, epitope located within the α-I domain (Fig. S4).

Given these results, along with earlier studies demonstrating the ability of MDA-MB-231 cells to form α2β1-dependent adhesive interactions with bone matrices in vitro (26–28), we sought to determine whether our in vitro model accurately predicts α2 integrin expression patterns found in type I collagen-rich metastatic lesions recovered from human breast cancer patients. For this, bone biopsy specimens were obtained from a series of seven patients with metastatic disease and immunostained for α2 expression. Validating the results of our in vitro and xenograft models, all seven patients expressed α2 in breast cancer cells in bone metastatic sites, with both carcinoma cells and surrounding vascular endothelial cells scoring positive in blinded analyses (Fig. 5 and Fig. S5). Given that archived biopsy material was available from the original primary breast cancer site in a subset of three of these patients, and the fact that type I collagen levels are distinctly higher in human breast tissue than in mouse mammary gland (25), we assessed α2β1 staining in these samples as well. Interestingly, distinct α2 integrin expression was likewise detected in breast carcinoma cells in each of these patients (with weaker staining localized to normal myoepithelial cells), including tumor microemboli found within lymphatic vessels (Fig. S6).

Discussion

Recent interest has focused on designing unbiased phenotypic screens wherein the identification of function-blocking effects precede efforts to dissect the underlying molecular mechanisms that give rise to the desired outcomes (13, 15, 31, 32). With increasing evidence that cell behavior in 3D culture systems more faithfully recapitulates in vivo function, greater emphasis has been placed on developing improved in vitro models for screening purposes, including the use of basement membrane-like gels, pepsin extracts of dermal collagen, and synthetic hydrogels (1, 2, 6, 33–35). The degree to which any of these constructs recapitulate the structure or function of the native ECM deposited in vivo remains controversial, however (1, 2, 16, 34). In carcinomatous states, neoplastic cells at both primary and metastatic sites are known to interface a network of covalently cross-linked type I collagen fibrils whose physical properties modulate tumor phenotypes (2, 3, 5–12, 16). Consequently, we elected to use type I collagen hydrogels that are naturally cross-linked by lysyl oxidase-derived aldimine bonds (16) to promote carcinoma cells to express a more in vivo-like display of surface antigens that could serve both as an immunogen for mAb production and as a physical platform for functional screening.

With this experimental approach, ~5% of the generated cell-reactive mAbs displayed inhibitory effects. mAb 4C3 was selected for additional analysis based on its inhibitory activity in our in vitro screen, and further characterized as a proof-of-principle prototype to determine (i) whether function-blocking activity detected initially in vitro can be extended into in vivo settings, (ii) whether the mAb-reactive antigen can be identified, and (iii) whether target antigens discovered using human carcinoma cell-type I collagen composites can faithfully predict in vivo patterns of expression in patient samples. As described above, mAb 4C3 successfully inhibited the perivascular proliferation of extravasated MDA-MB-231 cells within the type I collagen-rich interstitial matrix of the chicken embryo, a model xenograft system in which cancer cell behaviors, including invasion, proliferation, and metastasis, recapitulate those observed in mouse xenograft models (22, 23).

We were initially surprised to find that mAb 4C3 did not affect primary tumor growth after orthotopic injection of MDA-MB-231 cells into the mouse mammary gland (Fig. 5). However,
assessment of the local ECM environment at the site of carcino-
ma inoculation confirmed earlier studies describing the paucity
of type I collagen in the mouse mammary fat pad (25). As an
alternative, we chose mouse skeletal tissues as a surrogate tissue
for assessing mAb 4C3-mediated effects on MDA-MB-231 pro-
life, given its rich type I collagen content and the pre-
dilection of human breast carcinomas to metastasize to this organ
system (17, 26–28). Although the effects of mAb 4C3 on carci-
noma growth within the mandible and hindlimb supported mAb-
mediated inhibitory effects, the high variability of this in vivo
model did not allow these trends to reach statistical significance
(Fig. 3). More importantly, however, MDA-MB-231 proliferation in
the vertebral column was almost completely inhibited, with
significant effects on the development of paralysis-associated
morbidty. Further studies are needed to assess the ability of mAb
4C3 to affect tumor growth within preestablished metastases, ei-
ther as a single agent therapeutic or in combination with other
interventions. Nevertheless, these findings provided sufficient im-
petus to warrant identification of the mAb 4C3 target antigen.

After immunoaffinity purification and mass spectroscopy, the
mAb 4C3 target antigen was identified as the α2 integrin subunit,
whose only known partner, the β1 integrin chain, forms a hetero-
dimeric complex that serves as a major type I collagen-binding
receptor (29, 30). Peptide mapping characterized the mAb 4C3
epitope within the C1 domain of the α2 integrin, a metal ion-
dependent adhesion site that is responsible for ligand recognition
and binding (29, 30). Although these results complement a number
of reports documenting important roles for α2β1 in med-
iating cancer cell–type I collagen interactions in vitro, ranging
from proliferation and invasion to epithelial–mesenchymal tran-
sition and cancer stem cell formation (36–48), the function of
the α2 integrin in neoplastic states in the in vivo setting is less clear.
Recently, Ramirez et al. (49) concluded that α2β1 serves as a
metastasis suppressor in mouse models as well as human can-
cers. Using α2 integrin-null mice that were bred into a mouse
mammary tumor virus-Neu transgenic line, they demonstrated
that despite the complete absence of α2β1, tumor initiation was
only marginally affected, whereas lung metastatic activity was
actually enhanced (49). In this mouse model, however, all tissues
are rendered α2 integrin-deficient throughout embryonic and
postnatal development, and thus the MMTV-Neu oncogene is
expressed. By necessity, in α2 integrin-null mammary epitheli-
cells, where potential effects of the integrin on tumor trans-
formation and progression are difficult to define (i.e., as opposed
to deleting the α2 integrin in committed carcinoma cells). Indeed,
in contrast to these findings, targeting α2β1 with either function-
blocking antibodies or shRNA-based strategies has been reported
to block metastatic activity in a number of animal model systems
(50–53). Likewise, in a second in vivo model of cancer progression
using α2-null mice bred into a K14-HPV16 transgenic line, squa-
mous carcinoma cell proliferation and metastatic activities were
decreased in the absence of the α2 integrin (54).

Independent of studies in mouse models, recent studies of
human breast cancer and prostate cancer samples have indicated
that α2 mRNA expression levels can decrease as a function of
increased metastatic burden and decreased survival (49). How-
ever, at the protein level, α2β1 is readily detected at both primary
and metastatic sites in a variety of cancers, including breast (as
described herein) and prostate cancer (52, 55, 56). Although it
may be reasonable to conclude that high levels of α2β1 can po-
tentially retard motile responses by promoting adhesion, lower
levels of the integrin nevertheless may be required to support the
cell–ECM interactions most conducive to invasion and growth.

Nevertheless, it is unlikely that all carcinomas will prove equally
dependent on α2β1, considering that other collagen-binding ad-
hesion molecules, including α6β1, α10β1, α11β1, and discoidin
receptors, have been described previously (30). As such, it should
be stressed that the intent of using carcinoma cell–type I collagen
composites as an antigen for mAb production is not to simply
identify collagen-binding ligands, but rather to generate mAbs
that interfere with cancer cell behavior in an environment similar
to that encountered in vivo. Indeed, our preliminary studies in-
dicate that most of the function-blocking mAbs identified in
screens preformed to date target not type I collagen receptors,
but rather surface molecules with as-yet uncharacterized mech-
nisms of action.

After applying the above-outlined strategy to identify func-
tion-blocking mouse antibodies, these reagents could be lever-
gaged to generate humanized mAbs (14). From a therapeutic
perspective, the broad distribution of the α2β1 integrin in normal
tissues, as well as its ability to ligate other ECM proteins [e.g.,
type IV collagen, laminin, type XXIII collagen (30, 57)], might
raise concerns regarding potential toxicities associated with tar-
geting strategies. However, it is noteworthy that α2-null mice are
viable and fertile, and that α2-integrin-deficient human patients
who present only with mild bleeding diatheses have been iden-
tified (58–61). Interestingly, small-molecule α2β1 inhibitors have
been developed as potential antithrombotic agents (62, 63), but
our preliminary studies indicate that these agents are not as ef-
fective as mAb 4C3, at least in terms of interfering with MDA-
MB-231–type I collagen adhesive interactions (Fig. S8). Thus,
it remains possible that mAb 4C3 exerts unique effects on carci-
noma cell function that might not be recapitulated by small-
molecule inhibitors or α2 integrin silencing. Finally, although our
studies have emphasized potential roles for α2β1 in neoplastic
states, we note that the integrin also has been implicated in fi-
brosis, inflammation, platelet-mediated thrombosis, and angio-
genesis, raising the possibility that similar targeting strategies
might be applied in other diseases as well (64–70).

The experimental approach outlined herein allows for the
rapid identification of new target antigens in an unbiased fashion,
as well as the isolation of murine mAbs suitable for humanization.
Although our initial studies used a well-studied human breast
carcinoma cell line, our approach is similarly amenable to the use
of primary carcinoma cells or cancer stem cells. Indeed, we re-
cently used primary human glioblastoma cancer stem cells to
provide mAb libraries that were also found to exert inhibitory
effects with target identification in process. As such, we antici-
pathe that our phenotypic screening stratagem, using either human
cancer cell lines, primary cancer cells, or even cancer cell–stromal
cell composites (71), as well as more complex ECM-supple-
mented hydrogels to better recapitulate the anticipated changes
that occur in connective tissue composition during tumor pro-
gression (12, 72), will prove valuable in the search for new targets
and therapeutics in neoplastic states.

Methods

MDA-MB-231 cells (American Type Culture Collection) were embedded in
mouse type I collagen hydrogels (21). The cell–matrix composite was then
inoculated into 6-wk-old BALB/c female mice for immunization. MDA-MB-
231–reactive mAbs were isolated and screened for antiproliferative activity
in 3D collagen constructs (21). All procedures are described in more detail in
SI Methods.

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Supporting Information

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SI Methods

Immunogen Preparation and Immunization. Type I collagen was isolated from mouse tail tendons as described previously (1) and dissolved in 0.2% acetic acid at a final concentration of 2.7 mg/mL. Before gelation, the collagen solution was mixed with 10× minimum essential media (MEM) and 0.34 N NaOH at a ratio of 8:1:1 at 4 °C, with MDA-MB-231 cells (1·5 · 10⁶) suspended in 1 mL of this mixture. The carcinoma cell-collagen mixtures were incubated for 1 h at 37 °C to allow for gelation and culture media (MEM supplemented with 10% FCS) added atop the gel. Collagen gel rigidity was assessed in a RFSII rheometer (Rheometric) using dynamic shear mode, parallel plate geometry, and a hydrated chamber as described previously (2).

After a 4-d incubation period, the MDA-MB-231/collagen composites were washed extensively and recovered intact from 12-well plates or, alternatively, after the MDA-MB-231 cells were harvested from the gels by dissolving the collagen hydrogels with collagenase type 3 (Advance BioFacture). MDA-MB-231/collagen composites or isolated MDA-MB-231 cells were inoculated i.p. into 6-wk-old BALB/c female mice, followed by boosts at 2- to 3-wk intervals for 3 mo. Spleens were then removed, and somatic cell hybridization was performed by the University of Michigan Hybridoma Core with P3·63-Ag8.653 mouse myeloma cells as the fusion partner (3).

Whole-Cell ELISA. Supernatants from hybridoma clones were assayed in a whole-cell ELISA format. MDA-MB-231 cells (1·10⁶) were added to 96-well V-bottom PVC plates (Corning), and cell pellets were incubated for 1 h at 4 °C with 50 μL of media supernatant from individual hybridoma cultures. After washing, MDA-MB-231 cells were then resuspended in PBS with an HRP-conjugated secondary antibody directed against mouse immunoglobulins (Pierce) for 1 h at 4 °C. The cells were then washed three times with PBS, and HRP activity was detected with a 3,3′,5,5′-tetramethylbenzidine substrate (Thermo Scientific).

Hybridomas giving rise to anti–MDA-MB-231–reactive mAbs were subcloned by limiting dilution and then reassayed for activity to ensure the isolation of monoclonal populations. Positive hybridomas were then used to generate ascites fluid by injection into mouse peritoneal cavities. The resulting ascites fluid was cleared of debris by centrifugation, and antibodies were purified using either Melon Gel Purification Resin or Protein G Resin (Thermo Scientific). mAb isotype was determined using the Rapid ELISA Mouse mAb Isotyping Kit (Pierce). A control IgG1 mAb (3H5) was raised against dengue virus antigen (4). After i.p. injection, ascites fluid generated from the hybridoma cell line [American Type Culture Collection (ATCC)] was purified by protein G affinity chromatography. Both the control mAb 3H5 and the mAb 4C3 preparations were endotoxin-depleted by DeToxi-Gel column chromatography (Pierce) before use.

Cell Proliferation and Apoptosis Assays. For screening mAb anti-proliferative activity, MDA-MB-231 cells were embedded in type I collagen (10² cells in a final type I collagen concentration of 2.2 mg/mL) or Matrigel (5 mg/mL) in the absence or presence of mAb 4C3 at the indicated concentrations, and then plated in 24-well plates in MEM/10% FCS. In selected experiments, the ability of mAb 4C3 to affect the proliferative responses of human squamous cell carcinoma (74B), ovarian carcinoma (ES2), or fibrosarcoma (HT1080) cells (all obtained from ATCC) was assessed. Cell number was quantified by hemocytometry or by the Cell-Titer Glo Kit (Promega). Caspase 3 and caspase 7 activity was evaluated with the Caspase-Glo 3/7 Kit (Promega).

Affymetrix Expression Profiling and Analysis. Total mRNA was collected and purified using the RNEasy Mini Kit (Qiagen) (5). Sample quality was confirmed with a Bioanalyzer 2100 (Agilent), and all samples were profiled on Affymetrix Mouse MG-430 PM expression array strips. Expression values for each probe set were calculated using the robust multiarray average system (5) and filtered for genes with a fold change greater than twofold. Heat maps of selected gene lists were generated using Gene Cluster 3.0 and TreeView 1.6 (5). Gene Ontology analysis was performed using MetaCore from Thomson Reuters (version 6.11, build 41105).

Chick Xenograft. RFP-transduced MDA-MB-231 were injected with a control IgG or 4C3 into the allantoic vein of 11-d-old immune-incompetent chicken embryos (6). After a 6-d incubation period, vessel lumens were visualized by injecting chicks with GFP-labeled isoelectric-B4. Confocal imaging of second harmonic-generated signals was used to analyze collagen fiber microstructure as described previously (7). After an additional 1 h of incubation time, embryos were harvested, whole-mount tissue preparations were obtained distal to the injection site, and carcinoma cells were identified by florescent microscopy. For quantification, MDA-MB-231 cells expressing firefly luciferase were injected in an identical fashion with control mAb 3H5 or mAb 4C3 in tandem with the carcinoma cells or at 24 h after the carcinoma cell inoculation. For imaging, eggs were injected i.v. with 100 μL of luciferin (40 mg/mL in PBS) at 10 min before removal of the lower chioroallantoic membrane. Membranes were washed with PBS and imaged for bioluminescence with a Xenogen IVIS 200 imaging system.

Mouse Xenograft Model. Luciferase-labeled MDA-MB-231 cells (1·10⁵) were injected via the intracardiac route with either 10 mg/kg of mAb 4C3 or a control IgG1 twice weekly for 4 wk, and tumor progression was monitored by whole-body bioluminescent imaging as described previously (8). In selected experiments, cells were alternatively injected orthotopically in the fourth mammary gland with or without mAb 4C3. Micro-CT analysis of bone lesions were imaged at an isotropic voxel resolution of 18 μm using Explore Locus SP (GE Healthcare), and calibrated 3D images were reconstructed (7).

Immonoaaffinity Purification and Mass Spectrometry of Target Antigen. To identify the mAb 4C3 ligand, RIPA lysates of MDA-MB-231 cells (1 mg/mL) were precleared with 5 μg of control mouse IgG1 and protein A/G beads (Santa Cruz Biotechnology). mAb 4C3 (5 μg) was then incubated with protein A/G beads overnight at 4 °C. After the beads were pelleted and washed with RIPA buffer, attached proteins were solubilized in Laemmli sample buffer and resolved on 10% SDS/PAGE gels (Bio-Rad). The immunoprecipitated protein was visualized by silver staining (Pierce), with the band excised and subjected to in-gel digestion with porcine trypsin. Gel digests were analyzed by LC/MS/MS on a Thermo Scientific LTQ Orbitrap XL mass spectrometer. Peptide ion data were searched and identified using Mascot and Scaffold at the University of Michigan’s Protein Structure Facility. To verify the indentified ligand, immunoprecipitated protein was resolved on SDS/PAGE gel, transferred to a nitrocellulose membrane, and immunoblotted with a second
antibody against directed human integrin α2 (sc-74466; Santa Cruz Biotechnology).

**Tissue Histochemistry.** Formalin-fixed, paraffin-embedded tissue blocks from deidentified patient samples (Institutional Review Board protocol HUM000503390) were sectioned (5 μm) and placed on charged slides. The slides were deparaffinized in xylene and rehydrated through graded alcohols. Heat-induced epitope retrieval was performed in a Decloaking Chamber (Biocare Medical) with Target Retrieval Solution at pH 6.0 (Dako). Slides were incubated in Peroxidized (Biocare Medical) for 5 min to quench endogenous peroxidases, and then incubated for 1.5 h at 25 °C with rabbit monoclonal anti-α2 integrin (CD49b; Abcam LTD/Epitomics) diluted 1:200. (This mAb produces superior staining than mAb 4C3.) Antibody was detected with anti-rabbit Envision™ HRP-Labeled Polymer (Dako) for 30 min at 25 °C. HRP staining was visualized with the DAB™ Kit (Dako). Slides were counterstained in hematoxylin, blued in running tap water, dehydrated through graded alcohols, cleared in xylene, and then mounted with Permount (Fisher Scientific).

**Statistical Analysis.** All results are presented as the mean ± SEM of three or more experiments as indicated in the text. Significance was determined using the Student t test.


Fig. S1. MDA-MB-231 breast carcinoma cells, embedded in 3D type I collagen hydrogels (1), are used to immunize recipient mice (2). Hybridoma cultures are generated (3), and mAbs are tested for their ability to inhibit proliferative responses of MDA-MB-231 cells in 3D culture (4). The ability of selected mAbs to inhibit MDA-MB-231 proliferative responses are determined in xenograft models in vivo (5), and the antibody targets are identified by immunoaffinity isolation and mass spectroscopy (6).
Fig. S2. (A and B) MDA-MB-231 cells (1 × 10^5/well) were cultured in 24-well tissue culture plates under 2D conditions in DMEM/10% FCS with or without mAb 4C3 (10 μg/mL), without affecting cell shape at day 3 (A) or proliferation (B). Results are expressed as the mean ± SEM of three experiments. (C) MDA-MB-231 cells (1 × 10^5) were embedded in Matrigel in the presence of a control IgG1 or mAb 4C3 (10 μg/mL each) for 3 d or 4 d without affecting cell shape or cell number. Results are representative of three or more experiments.
Fig. S3. (A) Human squamous cell carcinoma (74B; $2 \times 10^5$), ovarian cell carcinoma (ES2; $5 \times 10^5$), or fibrosarcoma (HT1080; $2 \times 10^5$) cell lines were cultured in 3D type I collagen hydrogels for 2 d in the presence of a control IgG1 (10 μg/mL) or mAb 4C3 (10 μg/mL). Phase-contrast micrographs highlight the ability of mAb 4C3 to block cell shape changes. Results are representative of three or more experiments. (B) Cell proliferation in 3D collagen was inhibited as a function of mAb 4C3 concentration as assessed by cellular ATP levels, with IC_{50} values reported as mean ± SEM (n = 3).

Fig. S4. Peptide mapping of the mAb 8F10-binding sites in an overlapping series of peptides (each 10-aa long) that span the α2 integrin subunit. Asterisk indicates decapeptide epitopes localized within the α-1 domain.
Fig. S5. $\alpha_2$ integrin staining of four additional biopsy specimens from human breast cancer patients with bony metastases. Asterisks indicate bone, and arrows indicate metastatic breast carcinoma cells.

Fig. S6. Breast tissue biopsy specimens harvested from primary sites highlighting strong $\alpha_2$ expression in breast carcinoma tissues (black arrows) with additional, but weaker, staining outlining normal myoepithelial cells. In case 1, the tumor embolus as well as the lymphatic endothelium are positive for $\alpha_2$ expression.
Luciferase-expressing MDA-MB-231 cells (5 × 10⁶) were orthotopically injected into nude mouse recipients and either vehicle 250 μg mAb 4C3/mouse (∼10 mg/kg) or 500 μg mAb 4C3/mouse (∼20 mg/kg) given i.p. 3 times weekly, and tumor volume (A) and luminescence (B) were monitored as described. Results are expressed as mean ± SEM (n = 4).

MDA-MB-231 cell adhesion to type I collagen hydrogels was assessed after a 1-h culture period with control IgG1 (10 μg/mL), mAb 4C3 (10 μg/mL), or the indicated concentrations of the small molecule α₂β₁ antagonist, TC-I 15 (1). Results are expressed as mean ± SEM (n = 3).