Integrin α-3 β-1’s central role in breast cancer, melanoma and glioblastoma cell aggregation revealed by antibodies with blocking activity

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ABSTRACT
Breast cancer, melanoma and glioblastoma cells undergo cell-mediated aggregation and aggregate coalescence in a transparent 3D Matrigel environment. Cells from normal tissue and non-tumorigenic cell lines do not exhibit these behaviors. Here, 266 monoclonal antibodies (mAbs) demonstrated to interact with a wide variety of membrane, secreted and matrix proteins, have been screened for their capacity to block these tumorigenic cell-specific behaviors in a 3D environment. Remarkably, only six of the 266 tested mAbs exhibited blocking activity, four targeting integrin β-1, one targeting integrin α-3 and one targeting CD44. Colocalization of integrins β-1 and α-3 in fixed cells and in live aggregates suggests that the integrin α-3 β-1 dimer plays a central role in cancer cell aggregation in the 3D environment provided by Matrigel. Our results suggest that blocking by anti-integrin and anti-CD44 mAbs involves interference in cell-cell interactions.

Introduction

Tumors form in a 3D environment

Therefore, in vitro studies of cancer cell behavior should be performed in a 3D model.1-3 We recently reported that both tumorigenic cell lines and fresh tumor cells, when dispersed in a transparent 3D Matrigel environment, divide and undergo directed, cell-mediated cell aggregation and aggregate coalescence (“aggregation and coalescence”).1-3 Non-tumorigenic cell lines and normal cell cultures derived from non-cancerous tissue, do not exhibit these behaviors.1-3 In time, large aggregates formed by tumorigenic cells assume forms consistent with the tumors formed in vivo.1-3 Previously, we demonstrated that the anti-integrin β-1 monoclonal antibody (mAb), AIIß2, blocked aggregation and coalescence in a 3D Matrigel environment,1 in the tumorigenic cell line MDA-MB-435-a6HG6, which was derived from a breast cancer.5 In a subsequent study of cell aggregation and aggregate coalescence by melanoma cell lines and cells cultured from fresh melanomas, a screen of 51 purified mAbs targeting cell surface-associated molecules yielded only two anti-integrin β-1 mAb and one anti-CD44 mAb that blocked aggregation and coalescence.3 Neither affected cell growth. Both of the targeted membrane proteins have been shown to play a number of different roles in cell-cell interactions, cell behavior and cell-matrix interactions. Integrin β-1 forms heterodimers with a number of different integrins.6-17 These dimers facilitate binding to matrix molecules,6-17 transduce signals through regulatory pathways to the actin cytoskeleton18-20 and play direct roles in cell-cell interactions.21-24 They have also been shown to play roles in tumor progression and metastasis.25-27 CD44 plays a role in cell-cell adhesion, cell locomotion and metastasis.28-32 It can function coordinately with integrins.33-37

Here, we expanded our screen of mAbs with the expectation of identifying additional ones that block tumorigenic cell aggregation and aggregate coalescence in a 3D Matrigel environment. A collection of 266 purified and concentrated mAbs obtained from the mAb collection of the Developmental Studies Hybridoma Bank (DSHB) at Iowa, including the original 51 mAbs previously analyzed, were first screened at a very high concentration (540 µg per ml) for their capacity to block cell aggregation and aggregate coalescence of breast cancer and melanoma cell lines in a 3D Matrigel environment. The mAbs, previously validated for one or more cell applications, targeted membrane, secreted and matrix proteins that are in the following categories: cell adhesion receptors, cell signaling receptors, clusters of differentiation surface molecules, general cell surface markers, channel molecules and transporters, growth factors, metabolic proteins, secreted transcription factors and secreted tumor suppressors.

To our surprise, of the 266 mAbs tested in the initial screen, only mAbs against 3 targets blocked cell aggregation and aggregate coalescence in one or both test strains in the initial screen. Four were against integrin β-1, one against integrin β-3 and one against CD44. Because five of six were integrins, we verified all 27 anti-integrin mAbs in the DSHB collection at 0.6 and 0.3 µg per ml, using the breast cancer and melanoma cell lines, as well as an additional glioblastoma cell line. Three of the four anti-integrin β-1 mAbs that blocked cell aggregation and aggregate coalescence did so maximally...
at 0.6 µg per ml, in all three cell lines. One anti-α-3 integrin mAb blocked aggregation and coalescence at 540 µg per ml for two of the three cell lines and for one at 0.6 µg per ml. These results suggest that β-1 plays a fundamental role in the single cell behaviors that mediate cell aggregation and aggregate coalescence in a 3D Matrigel environment, and that it may do so in the integrin α-3 β-1 heterodimer.

All of the anti-integrin mAbs that exhibited blocking activity immunostained all three cell lines. Eighteen mAbs against seven additional integrins stained one or more of the three cell lines, but exhibited no blocking activity in the aggregation and coalescence assay, even though the majority have been shown to block cell-matrix adhesion. Mixtures of mAbs against multiple anti-integrin α mAbs that stain cell and block cell-matrix adhesion had no effect on aggregation and coalescence. Our results not only reveal the specificity of mAb blocking activity for aggregation and coalescence in a 3D Matrigel environment, but also suggest that the anti-integrin mAbs and anti-CD44 mAb block cell aggregation and aggregate coalescence by interfering directly with cell-cell interactions, rather than cell-matrix interactions. Given the complexity at the cellular and behavioral level of cell aggregation and aggregate coalescence in a 3D matrix, it seems remarkable that only 6 of 266 mAbs (four against integrin β-1, one against integrin α-3 and one against CD44) were the only ones that exhibited blocking activity in a 3D environment. These results, however, do not exclude the targets of mAbs with no blocking activity from still being viable blocking targets for mAbs against domains of the target proteins different from those of the mAbs tested.

Results

The assay for blocking

The assay employed here tested the capacity of a mAb to block tumorigenic cell aggregation and aggregate coalescence (referred to henceforth as “aggregation and coalescence”) in a 3D Matrigel environment, behavioral characteristics that have been demonstrated to be specific to tumorigenic cells. The assay relies on the transparency and gelation characteristics of Matrigel, an extract of mouse basement membrane, which separates epithelial cells from underlying tissue. It is composed primarily of laminin, nidogen (entactin), collagen IV, and proteoglycans. It exists as a clear liquid at 5°C, but gels, raising to 37°C. By mixing tumorigenic cells with Matrigel at 5°C, then raising the temperature to 37°C, cells are dispersed randomly in the 3D transparent gel. After gelation, tumorigenic cells move in a directed fashion into aggregates, which in turn undergo aggregate coalescence. Aggregation and coalescence are not due to random contact, but are rather cell mediated through the formation of pseudopodia and filopodia that form intercell contacts that contract, bringing together cells and aggregates. In the coalescence of aggregates, cells extend from aggregates to form bridges that contract, bringing aggregates together. Normal cells do not undergo aggregation and coalescence, but rather multiply, forming small clonal islands. The preparation to screen for mAbs that block this process is diagrammed in Figure 1(a). A thin Matrigel cushion free of cells was first cast on the bottom of wells in a 96-well tissue culture plate (steps 1 and 2, Figure 1(a)). Cells were then suspended in cold (5°C) Matrigel (liquid), with (test) or without (control) mAb, in growth medium, pipetted onto the cushion and then allowed to gel for 30 minutes at 37°C, resulting in a 3 to 4 mm-thick upper transparent gel layer with independent cells dispersed randomly throughout (steps 3 and 4, Figure 1(a)). These cell cultures were then imaged at three different depths each day over a four day (96 hour) period to assess aggregation and coalescence (steps 5 to 7, Figure 1(a)). When the preparation was cast in the absence of mAbs with cells of the non-tumorigenic cell line MCF-10A, the cells multiplied, forming small clonal aggregates that remained intact after four days (Figure 1(b)). Single MCF-10A cells did not aggregate and the small clonal aggregates did not undergo coalescence, even when making contacts with one another (Figure 1(b)). The three tumorigenic cell lines, MDA-MB-231 (referred to henceforth as MB-231) a breast cancer cell line, HTB-66, a melanoma cell line, and U87, a glioblastoma cell line, multiplied in the absence of mAbs, and single cells aggregated, cells and small aggregates aggregated, and small aggregates coalesced in the 3D Matrigel environment, through active cell interactions involving pseudopods and filopodia as previously described in detail. Cell aggregation began by day two and aggregate coalescence by day three (Figure 1(c–e), respectively).

Initial collection of the 266 mAbs

A collection of 266 mAbs were employed in the initial screen to test for their capacity to block aggregation and coalescence of MB-231 and HTB-66 cells. The entire collection was obtained from the DSHB, a National Resource created by the National Institutes of Health and housed at the University of Iowa. All 266 mAbs were purified and concentrated. The targets and mAbs are listed in Table 1. The validation characteristics and reported applications of all 266 mAbs, obtained from the DSHB website (http://dshb.biology.uiowa.edu/), are assembled in supplemental Table S1. The mAbs were categorized by their targets as follows: cell adhesion receptors, cell signaling receptors, cluster of differentiation, extracellular matrix molecules, general cell surface markers, growth factors, metabolic proteins, secreted transcription factors, channels and transporters, and secreted tumor suppressors (supplemental Table S1).

Initial screen of 266 mAbs

In the initial screen of the 266 mAbs, 123 were tested on both MB-231 and HTB-66 cells, and 143 were tested on one or the other cell line (Table 1). All were tested at a concentration of 540 µg per ml. Only 6 (2.3%) blocked aggregation and coalescence, in one or both cell lines (Table 1). Five were anti-integrins, 4 (P4C10, AIIB2, P5D2, 7E2) targeting integrin β-1 and 1 (P1B5) integrin α-3 (Table 1). Only AIIB2 was previously shown to block aggregation and coalescence in a 3D Matrigel model. The sixth was an anti-CD44 mAb, H4C4 (Table 1), previously shown to block aggregation and coalescence of melanoma cells.
Therefore, 260 (97.7%) of the 266 tested mAbs did not affect cell aggregation and aggregate coalescence. Qualitative examination of images over a four-days period revealed that, in all cases in which a mAb did not block aggregation and coalescence, cells were viable and proliferated.

**Blocking by anti-integrin mAbs**

Because five of the six mAbs that had blocking activity were anti-integrins, we focused on the 27 anti-integrin mAbs in the test collection. We first tested a mAb concentration range between 540 and 0.3 µg per ml (540, 250, 125, 50,
Table 1. Results of the first screen for mAbs that block cell aggregation and aggregate coalescence in a 3D Matrigel environment.

| Antigen                     | mAb#     | Block               |
|-----------------------------|----------|---------------------|
| **Apoptosis**               |          |                     |
| ING1                        | PCRP-    | -                   |
| **Cell Adhesion Receptors** |          |                     |
| β-Catenin-like protein 1    | CPTC-CTNBNL | 1-1                 |
| Cadherin                    | DCAD2    | - n.d.              |
| Cadherin                    | nr1      | - n.d.              |
| Cadherin                    | CPTC-CDH1-3 | n.d.               |
| Cadherin                    | CPTC-CDH2-1 | n.d.             |
| Cadherin                    | CPTC-    | n.d.                |
| Cadherin N                  | PB3      | - n.d.              |
| Fasciclin II                | 1D4      | -                   |
| HNK-1 epitope               | 3H5      | - n.d.              |
| Integrin β-1                | A1B2     | ++                  |
| Integrin β-1                | PAC10    | ++                  |
| Integrin β-1                | PS2D     | ++                  |
| Integrin β-1                | V2E9     | -                   |
| Integrin β-1                | 7E2      | ++                  |
| Integrin β-2                | H52      | -                   |
| Integrin β-2                | P4H9     | -                   |
| Integrin β-2                | TS1      | -                   |
| Integrin β-2                | 10E5     | -                   |
| Integrin β-3                | 7H2      | -                   |
| Integrin β-3                | 9H5      | -                   |
| Integrin β-3                | 15AE10   | -                   |
| Integrin α-2                | P1E6     | -                   |
| Integrin α-2                | P1H6     | -                   |
| Integrin α-2                | 6F1      | -                   |
| Integrin α-3                | P1B5     | ++                  |
| Integrin α-4                | P4G9     | -                   |
| Integrin α-5                | B1G2     | -                   |
| Integrin α-5                | D71E2    | -                   |
| Integrin α-5                | P1D6     | -                   |
| Integrin α-5                | P3G8     | -                   |
| Integrin α-5                | P1F6     | -                   |
| Integrin α-5                | P5H9     | -                   |
| Integrin α-5                | PS17     | -                   |
| Integrin α-6                | P262C24  | -                   |
| Integrin α-6                | P51G10   | -                   |
| Integrin α-7                | 9.11TGA7 | -                   |
| L-CAM                       | 7D6      | -                   |
| Mucin 1                     | CPTC     | - n.d.              |
| Mucin 17                    | MUC1     | -                   |
| NCAM                        | 5A5      | - n.d.              |
| ACAM                        | AG1      | -                   |
| Neurocan receptor           | 7A2      | - n.d.              |
| Neural ganglioside           | 8A2      | -                   |
| Retinal gangliosides         | JONES    | - n.d.              |
| **Cell Signaling Receptor** |          |                     |
| Acetylcholine nicotinic receptor | mAB 35 | -                   |
| Acetylcholine nicotinic receptor | 31-1C10 | n.d.                |
| Acetylcholine nicotinic receptor | 6A12-11C | n.d.               |
| Annexin A1                  | CPTC-ANXA1-1 | n.d.               |
| Annexin A1                  | CPTC-ANXA1-2 | n.d.             |
| Annexin A1                  | CPTC-ANXA1-3 | n.d.             |
| Annexin A1                  | CPTC-ANXA1-4 | n.d.             |
| Crumbs selenoprotein-p receptor | Co4   | - n.d.              |
| Delta extracellular domain   | C594.9B  | - n.d.              |
| EPH receptor B4              | CPTC-    | n.d.                |
| (Continued)
Table 1. (Continued).

| Material | CD11a | Integrin α L | CD11b | Integrin α M | CD11b | Integrin α M | CD11b | Integrin α M | CD11b | Integrin α M | CD11b | Integrin α M | CD11b | Integrin α M |
|----------|-------|-------------|-------|-------------|-------|-------------|-------|-------------|-------|-------------|-------|-------------|-------|-------------|
| Block    | 751/21/1/1 | 13.3 | HSA4 | LM2/1.6.11 | MHM 24 | M1/ | 70.15/11.5/2 | TRA-1-85 | MC-480 | HBS | P2B1 | STR1- | 3F2/D8 | 1D10 | SD2-27 |
| mAb**    | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Block    | MB-231 | HTB-66 | - | - | - | - | - | - | - | - | - | - | - | - | - |

(Continued)

Table 2. (Continued).

| Material | CD11a | Integrin α L | CD11b | Integrin α M | CD11b | Integrin α M | CD11b | Integrin α M | CD11b | Integrin α M | CD11b | Integrin α M | CD11b | Integrin α M |
|----------|-------|-------------|-------|-------------|-------|-------------|-------|-------------|-------|-------------|-------|-------------|-------|-------------|
| Block    | 751/21/1/1 | 13.3 | HSA4 | LM2/1.6.11 | MHM 24 | M1/ | 70.15/11.5/2 | TRA-1-85 | MC-480 | HBS | P2B1 | STR1- | 3F2/D8 | 1D10 | SD2-27 |
| mAb**    | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Block    | MB-231 | HTB-66 | - | - | - | - | - | - | - | - | - | - | - | - | - |

(Continued)

Table 3. (Continued).

| Material | CD11a | Integrin α L | CD11b | Integrin α M | CD11b | Integrin α M | CD11b | Integrin α M | CD11b | Integrin α M | CD11b | Integrin α M | CD11b | Integrin α M |
|----------|-------|-------------|-------|-------------|-------|-------------|-------|-------------|-------|-------------|-------|-------------|-------|-------------|
| Block    | 751/21/1/1 | 13.3 | HSA4 | LM2/1.6.11 | MHM 24 | M1/ | 70.15/11.5/2 | TRA-1-85 | MC-480 | HBS | P2B1 | STR1- | 3F2/D8 | 1D10 | SD2-27 |
| mAb**    | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Block    | MB-231 | HTB-66 | - | - | - | - | - | - | - | - | - | - | - | - | - |

(Continued)
concentration (supplemental Figure S1). We also tested the concentration range of 7E2, which blocked MB-231, but not HTB-66, at 540 µg per ml (Table 1). The minimum inhibitory blocking concentration was 250 µg per ml (Supplemental Figure S1). Using the minimum blocking activity of P4C10 as a standard, we then tested all 27 anti-integrin mAbs at 0.6 and 0.3 µg per ml. All were tested with the 2 original cell lines MB-231 and HTB-66, as well as the glioblastoma cell line U87.60 This latter cell line, which was not used in the initial screen (Table 1), was found to be inhibited by the anti-integrin β-1 mAbs P4C10, AIIB2, P5D2 and 7E2, and by the anti-integrin α-3 mAb P1B5 at 540 µg per ml, as was the case for the MB-231 cell line (Table 2; Figure 2(a–c), respectively). Three of the four anti-integrin β-1 mAbs (P4C10, AIIB2, P5D2) blocked HTB-66 at 540 µg per ml and all three test strains at 0.6 µg per ml (Table 2; Figure 2(a–c), respectively). P4C10 partially blocked MB-231 and U87 at 0.3 µg per ml, and AIIB2 partially blocked HTB-66 at 0.3 µg per ml. 7E2 did not block any of the three cell lines at 0.6 µg per ml (Table 2; Figure 2(d)). The anti-integrin α-3 mAb, P1B5, blocked MB-231 and U87, but not HTB-66, at 540 µg per ml. PIB5 also blocked U87 at 0.6 µg per ml, and partially blocked U87 at 0.3 µg per ml (Table 2; Supplemental Figure S2).

### Staining by the anti-integrin mAbs

All 27 anti-integrin mAbs were also tested for their capacity to stain the three cancer-cell lines by indirect immunostaining. Of the 27 mAbs, 23 stained cells of one or more of the test strains (Figure 3(c–d)). Five of the six anti-integrin β-1 mAbs (P4C10, AIIB2, P5D2, P5D8, 7E2) stained all three cell lines (Figure 3(c–d); Figure 4(b)). One of the five, P5D8, stained (Figure 3(d)), but did not block aggregation and coalescence (Table 2; Figure 3(d)). Staining of individual cells was associated with the cell surface, lamellipodia and filopodia (Figure 4(b–c)). Of the four anti-integrin β-1 mAbs that blocked, the three (P4C10, AIIB2, P5D2) that blocked all three cell lines at 0.6 µg per ml have been demonstrated to interact with the β1 domain of integrin β-1 (Figure 3(a–b)).61 The domain that is recognized by 7E2, which blocks two of the three cell lines, has not been determined. The anti-integrin α-3 mAb P1B5, which recognizes the β-propeller of integrin α-3, blocked MB-231 and U87 cells at 540 µg per ml, but not HTB-66 cells (Table 2), but stained all three cell lines (Figures 3(c) and 4(c)). Of the 22 anti-integrin mAbs that did not block, 18 stained one or more of the three test strains (Figure 3(d)). Eleven stained all three cell lines (Figure 3(d)). It should be noted that, of the 27 tested anti-integrin mAbs, 16 have been shown to block cell adhesion to matrix molecules in a variety of previous studies (supplemental Table S2). The 16 mAbs include both mAbs that blocked aggregation and coalescence in a 3D Matrigel environment (P4C10, AIIB2, P5D2, P1B5), and mAbs that did not block aggregation and coalescence in a 3D Matrigel environment (P5D8, P4H9, 10E5, 7H2, 9H5, P1E6, 6F1, P4G9, B11G2, P1D6, P1F6, P5H9, P5G10, P5F2, P5F6) for the mAb P4C10 with MB-231 cells, since it was one of the 3 anti-integrin β-1 mAbs that blocked both cell lines at 540 µg per ml in the initial screen. We found that 0.6 µg per ml was the minimum blocking concentration (supplemental Figure S1). We also tested the concentration range of 7E2, which blocked MB-231, but not HTB-66, at 540 µg per ml (Table 1). The minimum inhibitory blocking concentration was 250 µg per ml (Supplemental Figure S1). Using the minimum blocking activity of P4C10 as a standard, we then tested all 27 anti-integrin mAbs at 0.6 and 0.3 µg per ml. All were tested with the 2 original cell lines MB-231 and HTB-66, as well as the glioblastoma cell line U87.60 This latter cell line, which was not used in the initial screen (Table 1), was found to be inhibited by the anti-integrin β-1 mAbs P4C10, AIIB2, P5D2 and 7E2, and by the anti-integrin α-3 mAb P1B5 at 540 µg per ml, as was the case for the MB-231 cell line (Table 2; Figure 2(a–c), respectively). Three of the four anti-integrin β-1 mAbs (P4C10, AIIB2, P5D2) blocked HTB-66 at 540 µg per ml and all three test strains at 0.6 µg per ml (Table 2; Figure 2(a–c), respectively). P4C10 partially blocked MB-231 and U87 at 0.3 µg per ml, and AIIB2 partially blocked HTB-66 at 0.3 µg per ml. 7E2 did not block any of the three cell lines at 0.6 µg per ml (Table 2; Figure 2(d)). The anti-integrin α-3 mAb, P1B5, blocked MB-231 and U87, but not HTB-66, at 540 µg per ml. PIB5 also blocked U87 at 0.6 µg per ml, and partially blocked U87 at 0.3 µg per ml (Table 2; Supplemental Figure S2).

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Table 2. Blocking activity of 27 anti-integrin mAbs.

| Integrin | mAb* | 540µg/ml | 0.6µg/ml | 0.3µg/ml | Matrix block |
|----------|------|----------|----------|----------|--------------|
|         |      | MB-231   | HTB-66   | U87      | MB-231       | HTB-66     | U87      |           |
| β-1      | P4C10++ | ++       | ++       | ++       | ++           | ++        | ++       | +         |
|          | AIIB2++ | ++       | ++       | ++       | ++           | ++        | ++       | +         |
|          | P5D2++ | ++       | ++       | ++       | ++           | ++        | +        | +         |
|          | P5D8-  | -        | -        | -        | -            | -         | -        | +         |
|          | VE29-  | -        | -        | -        | -            | -         | -        | +         |
|          | 7E2-   | -        | -        | -        | -            | -         | -        | +         |
| β-2      | H52++ | ++       | ++       | ++       | ++           | ++        | ++       | +         |
|          | P4H9-  | -        | -        | -        | -            | -         | -        | +         |
|          | TS1-   | -        | -        | -        | -            | -         | -        | n.d.      |
|          | 10E5-  | -        | -        | -        | -            | -         | -        | +         |
| β-3      | 7H2-  | -        | -        | -        | -            | -         | -        | +         |
|          | 9H5-   | -        | -        | -        | -            | -         | -        | +         |
|          | 15AE10- | -        | -        | -        | -            | -         | -        | n.d.      |
| α-2      | P1E6-  | -        | -        | -        | -            | -         | -        | +         |
|          | P1H6-  | -        | -        | -        | -            | -         | -        | n.d.      |
| α-3      | P1B5++ | ++       | ++       | ++       | ++           | ++        | +        | +         |
|          | P4C9-  | -        | -        | -        | -            | -         | -        | +         |
| α-4      | P1E6-  | -        | -        | -        | -            | -         | -        | +         |
| α-5      | BIIG2- | -        | -        | -        | -            | -         | -        | +         |
|          | D71E2- | -        | -        | -        | -            | -         | -        | n.d.      |
|          | P1D6-  | -        | -        | -        | -            | -         | -        | +         |
|          | P3G8-  | -        | -        | -        | -            | -         | -        | n.d.      |
| α-5/β-5 | P1F6-  | -        | -        | -        | -            | -         | -        | +         |
|          | P5H9-  | -        | -        | -        | -            | -         | -        | +         |
| α-6      | P2G6C4- | -        | -        | -        | -            | -         | -        | +         |
|          | P5G10- | -        | -        | -        | -            | -         | -        | n.d.      |
| α-7      | 9.11TGA7- | -        | -        | -        | -            | -         | -        | +         |

The capacity to block cell aggregation and coalescence was assessed after four days of incubation, according to the protocol diagrammed in Figure 1(a). * TSL, TS1/18.1.2.11.4; * mAb, monoclonal antibody; n.d., not determined, all monoclonal antibodies were purified and concentrated from hybridoma supernatant; -, no inhibition; +, weak inhibition; ++, strong inhibition.

9.11TGA7) (supplemental Table S2). Therefore, the capacity to block adhesion of cells to matrix molecules was accompanied by the capacity to block aggregation and coalescence for only a minority of anti-integrin mAbs.

**Colocalization of β-1 and α-3**

Integrin β-1 pairs with other integrins in the plasma membrane to form integrin receptors. Since the only anti-integrin mAb with blocking activity other than the anti-integrin β-1 mAbs that blocked aggregation and coalescence was the anti-integrin α-3 mAb P1B5, we tested for colocalization by double-labeling experiments. Cells of each of the three test strains were fixed, cotreated with the rat-derived anti-integrin β-1 mAb, AIIB2, and the mouse-derived anti-integrin α-3 mAb, P1B5. Cells were then treated with the secondary fluorescent antibodies, anti-rat IgG H + L-AlexaFluo 488 and anti-mouse IgG H + L-R-Phycerythrin, and analyzed for localization of the alternate fluors by confocal microscopy. AIIB2 and P1B5 overlapped at the plasma membrane for all three cell lines (Figure 5(a)), suggesting that the great majority of integrin β-1 and integrin α-3 are complexed.

To demonstrate colocalization of integrin β-1 and integrin α-3 in live cells undergoing aggregation and coalescence in the 3D Matrigel model, we used a different strategy than that for single cells. Because Matrigel is mouse-derived, using a mouse anti-IgG as a secondary antibody caused background staining of the matrix, resulting in imaging problems. The anti-integrin β-1 mAb P4C10 was therefore linked to the AnaTag HiLyte Fluor 750 and the anti-integrin α-3 mAb P1B5 was linked to the AnaTag HiLyte Fluor 488. The respective emission wave lengths were 750 and 488 nm, allowing imaging of the same live preparation to be performed independently for the two fluors. U87 cells were treated simultaneously for 4 days in a 3D Matrigel preparation with the fluor-linked anti-integrin β-1 mAb and the fluor-linked anti-integrin α-3 mAb. Live small clonal aggregates, containing two to four cells, exhibited overlapping staining patterns, again demonstrating colocalization (Figure 5(b)).

**mAb-blocked cells are viable**

Blocking by mAbs of aggregation and coalescence was interpreted as interference of cell-cell, cell-aggregate and aggregate-aggregate interactions. However, blocking could also be due to toxicity and cell death. For all 3D analyses, images were taken every day for a four-day period at different depths to assess cell toxicity and cell multiplication. In all 260 cases in which mAbs did not block aggregation and coalescence, cells were motile, extended pseudopods and filopodia, underwent cell-mediated aggregation, and underwent cell division. In addition, we monitored over time individual U87 cells treated with 30 µg per ml of anti-integrin β-1 mAbs that blocked aggregation and coalescence in the 3D Matrigel preparation. In particular, we looked for cell cleavage and formation of clonal aggregates resulting from cleavage. In the presence of the three tested mAbs, P4C10, P5D2 and 7E2, cells divided (Figure 6(a)), formed clonal aggregates as demonstrated in Figure 6(b) for P5D2 (Figure 6(b)). We next tested whether cells treated with mAbs that blocked aggregation and coalescence were metabolically
**Figure 2.** Inhibition by anti-integrin β-1 mAbs of cell aggregation and aggregate coalescence in a 3D Matrigel environment at 540, 0.6 and 0.3 µg per ml. Control cultures were untreated. (a). P4C10. (b). AIIB2. (c). P5D2. (d). 7E2. The scale bar for all micrographs is provided in panel A.
Figure 3. Two-dimensional model of integrin α-3 β-1, known mAb binding sites, and staining patterns of the 23 anti-integrin mAbs that exhibited cytostaining in the three tested cell lines.

(a) Models of integrin α-3 and integrin β-1. (b) Binding sites of anti-integrin α-3 and anti-integrin β-1 mAbs that exhibited blocking activity in one or more of the cell lines tested. (c) Binding sites and cell line staining patterns of anti-integrin mAbs that block. (d) Binding sites and cell line staining patterns of anti-integrin mAbs that do not block. mAb, monoclonal antibody; +++, intense staining; ++, moderate staining; +, low staining; -, no staining.
active (Figure 6(b)). Untreated control cultures and test cultures treated with P4C10, AIIB2 and P5D2, were analyzed for 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction after four days of incubation. Formazan crystal formation reflects metabolic activity. As was the case for the untreated control cells, the mAb-treated preparations exhibited MTT reduction (Figure 6(c)), demonstrating viability.

Addition of anti-integrin β-1 mAbs after aggregation and coalescence

The employed assay assessed the capacity of a mAb to block aggregation and coalescence, but did not test whether mAbs that blocked could rapidly dismantle an aggregate. To this end, we incubated 3D Matrigel preparations of U87 cells for four days in the absence of mAbs, during which they formed large aggregates, then added each of the five anti-integrin mAbs with blocking activity (P4C10, AIIB2, P5D2, 7E2, P1B5) at 5 μg per ml. Seven days after addition of the antibodies, the preparations were imaged. For all five mAbs, a minority of cells did exit the tested aggregates, but the main aggregates remained relatively intact (supplementary Figure S3).

Multiple mAb treatment

Although the results suggest that the integrin α-3 β-1 is involved in cell-cell interactions basic to aggregation and coalescence, there is no explanation for why so many mAbs that do not block aggregation and coalescence do block cell-matrix adhesions and stain the cell lines, suggesting they are expressed. This raises the question of why they are expressed, why the mAbs block cell-matrix adhesion, but do not block aggregation and coalescence. One explanation may be that the integrins are functionally redundant for cell-matrix adhesion, and applying simply one such mAb may not significantly affect cell behaviors. If redundant, then adding three such mAbs should have an additive effect. We therefore tested a combination of the three mAbs that stained HTB-66 cells, and have been shown to block cell-matrix adhesion, but which did not individually block aggregation and coalescence. The three mAbs were P1D6, P1E6 and P5G10, which target α-2, α-5, and α-6, respectively. The combination was tested at 10 μg per ml, 30 μg per ml and 125 μg per ml, of each mAb. None of the three combinations at low, medium or high concentrations blocked aggregation and coalescence (Supplemental Figure S4). Finally, we tested the effects of a combination of the anti-integrin α-3 mAb, P1B5, and anti-integrin α-6 mAb, P5G10. These α-integrins have been shown to work coordinately in adhesion, migration and growth of epithelial cells and integrin

Figure 4. Indirect immunostaining with anti-integrin β−1 and α−3 mAbs.
(a) Controls in which no mAb was added to cells of the three cell lines. (b) Staining by anti-integrin β−1 mAbs P4C10, AIIB2, P5D2 and 7E2. (c) Staining by the anti-integrin α−3 mAb P1B5. Arrows indicate regions that are enlarged in the adjacent panel to the right. The scale bar for all images is presented in panel A, DIC image, MB-231.
Figure 5. Colocalization of integrin β-1 and integrin α-3 in both fixed and live cells.
(a) Indirect double staining of fixed MB-231, HTB-66 and U87 cells for integrin β-1, using the rat-derived mAb AIIB2 and the fluor-tagged secondary antibody, anti-rat IgG Alexa Fluor 488 (green) and for integrin α-3, using the mouse-derived mAb P1B5 and the fluor-tagged secondary antibody, anti-mouse IgG Phycoerythrin (red). AIIB2, P1B5 and merged images are presented for each cell line. (b) Staining of live U87 clonal cell islands in a 3D Matrigel environment with fluor-tagged anti-integrin β-1 mAb P4C10-HiLyte (750 nm) and fluor-tagged anti-integrin α-3 mAb P1B5-HiLyte (488 nm).

P1B5 blocks cell-matrix adhesion and P5G10 appears to bind to the same matrix protein domain as the anti-integrin α-6 mAb GoH3, which blocks cell matrix protein interactions. GoH3 is not in the DSHB collection, and was therefore not tested here. A mixture of the two mAbs P1B5 and P5G10 at 30 µg per ml each had no effect on HTB-66 aggregation and coalescence (supplemental Figure S5).
Anti-CD44 mAbs

CD44 is a plasma membrane glycoprotein that is spliced, post-transcriptionally into a number of isoforms. It plays a variety of roles in metastasis and tumorigenesis. We previously reported that the anti-CD44 mAb, H4C4, blocked cell aggregation and aggregate coalescence in melanoma cells. H4C4 recognizes the N-terminus of CD44, which is extracellular and in the hyaluronan binding domain. In the initial screen, we found that H4C4 was the only mAb of four anti-CD44 mAbs that exhibited blocking activity for both MB-231 and HTB-66 cells (Table 1, Figure 7(a)). However, even at 540 µg per ml, it did not block aggregation and coalescence of glioblastoma-derived U87 cells (Figure 7(a)). H4C4 did stain cells of all three cell lines (Figure 7(b)). At 0.6 µg per ml, H4C4 blocked MB-231 and HTB-66 aggregation and coalescence (Figure 7(a)). H4C4 has also been shown to block cell adhesion to hyaluronic acid. It is noteworthy that CD44 has been shown to cooperate with integrins in cell matrix adhesion, and signaling.

Discussion

In the initial 3D screen performed at 540 µg per ml of mAb, only six of the 266 mAbs (2.3%) blocked aggregation and coalescence of one or both of the cell lines, MB-231 and HTB-66. Five of the six were against integrins and one against CD44. In previous studies, it was demonstrated that the anti-integrin mAb AIIB2 against integrin ß-1 blocked aggregation and coalescence, that the mAb P1B5 against integrin α-3 caused cells to exit aggregates and that the mAb H4C4 against CD44 blocked aggregation and coalescence. Weavers et al. also observed that AIIB2 destabilized aggregates of the breast cancer-derived cell line T4-2, in a 3D Matrigel environment. What seems remarkable in the present screen of 266 mAbs is the paucity of mAbs that blocked aggregation and coalescence in a 3D environment. Thus, 260 of the tested mAbs had no effect on cell multiplication, viability, cell polarization, or cell-mediated aggregation and coalescence in a 3D Matrigel environment.

Of the 27 anti-integrin mAbs that were tested, only five blocked aggregation in one or more of the three test cell lines, four against integrin ß-1 and one against integrin α-3. The three anti-integrin ß-1 mAbs that blocked all three cell lines (P4C10, AIIB2, P5D2) had been shown previously to bind to the amino acid sequence NKGEVFULLVGK (207 to 218) of the ß-1 domain. All three of these mAbs blocked maximally at 0.6 µg per ml and stained cells of all three cell lines intensely at 10 µg per ml. In addition, P4C10 and P1B5
were demonstrated to stain living cells forming aggregates in a 3D Matrigel environment. The fourth anti-integrin β-1 mAb that blocked aggregation, 7E2, did so only for the cell lines MB-231 and U87, but not the cell line HTB-66, at 540 µg per ml. However, 7E2 stained the cell surface of all three cell lines at 10 µg per ml. Unlike P4C10, AIIB2 and P5D2, the 7E2 binding domain has not been determined. 7E2 was generated against Chinese hamster ovary cells, and had not been previously reported to interact with human cells.

One anti-integrin β-1 mAb, P5D8, which did not block, still stained all three cell lines intensely. The target domain of P5D8 has also not been determined. A last anti-integrin β-1, V2E9, neither blocked nor stained any of the three cell lines. It was generated against chicken cell integrin β-1, and had not previously been tested against human integrin β-1.

The anti-integrin α-3 mAb, P1B5, was the only anti-integrin mAb other than the four anti-integrin β-1 mAbs of the 27 anti-integrin mAbs tested that blocked aggregation and coalescence. P1B5 has been demonstrated to bind to the β-propeller of integrin α-3. Although P1B5 blocked aggregation and coalescence of U87 at 0.6 µg per ml, it blocked MB-231 only at a very high concentration (540 µg per ml) and did not block HTB-66 cells even at the very high concentration. P1B5 did, however, stain all three cell lines at the same intensity at 10 µg per ml. Moreover, double-labeling experiments with AIIB2, against β-1, and P1B5, against α-3, revealed colocalization in all three cell types, both in fixed cell preparations and in live cell aggregates in the 3D Matrigel matrix. These results suggest that the integrin α-3 β-1 dimer plays an integral role in the cell-cell interactions that drive aggregation and coalescence in a 3D environment, although the absence of blocking activity by P1B5 in HTB-66 cell preparations, even at 540 µg per ml, and the absence of blocking activity by P1B5 at 0.6 µg per ml in MB-231 cell preparations, are puzzling. One possible explanation is that integrin α-3 plays a role as a modulator of integrin β-1 activity, but it is integrin β-1 that plays the crucial role in the cell-cell interactions that drive cell aggregation and aggregate coalescence. It is more puzzling that blocking of aggregation and coalescence was not obtained with the majority of anti-integrins tested, since many of the target integrins, such as integrin α-6, partner with integrin β-1 in adhesion to matrix molecules.

Indeed, 16 of the 27 anti-integrin mAbs we tested have been previously shown to block adhesion to matrix molecules (supplemental Table S2). Eleven of these mAbs that blocked adhesion to matrix molecules did not block aggregation and coalescence. These 11 mAbs targeted β-1 (P5D8), β-2 (10E5), β-3 (7H2, 9H5), α-2 (P1E6, 6F1), α-4 (P4G9), α-5 (BIIG2, P1D6), α-5/β-5 (P1F6, P5H9), α-6 (P5G10) and α-7 (9.11TGAT) (Table 2). In some cases, mAbs from sources other than the DSHB have been used more routinely than

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**Figure 7.** The anti-CD44 mAb H4C4 blocks aggregation and coalescence of only two of the three cell lines, but stains all three with equal intensity. (a) Blocking of aggregation and coalescence by H4C4 at 540, 0.6 and 0.3 µg per ml. (b) Immunostaining of the three cell lines by H4C4.
mAbs used here, but in at least once case, the DSHB anti-integrin α-6, PSG10, and the more generally used mAb GoH3, the matrix protein binding site facilitating cell-matrix adhesion, appears to be shared. Based on our results, one might conclude that cells express a majority of integrins that do not play a role in aggregation and coalescence in 3D, and that only integrin α-3 β-1 is essential. Since so many of the mAbs employed here have been shown to block binding of cells to matrix molecules, and our staining experiments have revealed that a majority of these integrin targets are expressed in one or more of the tested cell lines, one might conclude that matrix adhesion is not necessary in the 3D model. However, the integrin dimers that could be formed by the β and α-integrins may be functionally redundant. Hence, blocking one or even two different integrins may be insufficient for blocking matrix binding. It seems reasonable to assume that the intricate cell and aggregate translocation behaviors basic to cell aggregation and coalescence may require adhesion to the 3D Matrigel matrix. However, treatment with mixtures of mAbs against integrins α-2, α-5 and α-6, and mixtures of mAbs against integrin α-3 and α-6 even at individual mAb concentrations of 125 µg per ml, had no effect on aggregation and coalescence in a 3D environment. This leads to the tentative conclusion that it may be specifically the integrin α-3 β-1 dimer that plays a central role in these 3D behaviors.

In conclusion, we used the capacity to block cancer cell aggregation and aggregate coalescence in a 3D environment as an assay to screen a collection of 266 mAbs, the majority of which targeted cell surface or secreted molecules. We used this assay for several reasons. First, it has been noted repeatedly that cancer cells form tumors in a 3D environment and in many cases in a basement membrane matrix. Matrigel, used to establish the in vitro 3D matrix, represents reconstituted basement membrane. The crux of this point is that tumor development occurs in a 3D environment, not on a 2D substrate. Second, we have shown that cell aggregation and aggregate coalescence are characteristics of tumorigenic cells, not normal cells, just as is the case for resistance to signals that inhibit cell multiplication, growth factor independence, self-signaling for cell multiplication, invasiveness and metastasis, tumorigenesis in animal models, loss of contact inhibition, and additional characteristics. And third, tumorigenic cell aggregation and aggregate coalescence reflect aspects of tumorigenesis. This is most obvious in field cancerization, in which multiple tumorigenic loci coalesce, contributing to the growth and heterogeneity of developing tumors as is most obvious in histological sections of developing melanomas. Our results are most remarkable for the paucity of blocking mAbs and the specificity of the subset of anti-integrin mAbs, as well as the anti-CD44 mAb that blocks aggregation and coalescence. Our results demonstrate that of 27 anti-integrin mAbs, only five exhibited blocking activity and only three, all against the same β-1, blocked in all three test strains. Of the five, four targeted integrin β-1 and one integrin α-3. Because integrins function in pairs and because of colocalization of β-1 and α-3 on the cell surfaces all three test strains, we have put forward the hypothesis that integrin α-3 β-1 plays a central role in tumorigenic cell aggregation and aggregate coalescence in a 3D environment. Moreover, the same may be true for CD44. Variants of CD44 have been shown to cooperate with integrin β-1 in osteopontin binding.

It must be emphasized, however, that cancer cell aggregation and aggregate coalescence in a 3D environment are complex in vitro behaviors specific to tumorigenic cells. Many of the tested mAbs that bind to proteins may target domains of surface molecules that do not interfere with protein function, but that does not exclude these molecules as potential blocking targets in future screens of mAbs. We have also suggested the possibility that redundancy may exist among the different integrins, and this may explain some of the negative results obtained, but the results of experiments to test this hypothesis have not borne this hypothesis out. We have also not tested whether any of the mAbs in the collection interfere with the differentiation of aggregates after prolonged incubation in a 3D environment. Therefore, it seems likely that an expanded screen in a 3D environment, the use of multiple mAbs targeting functionally redundant integrins, and the effects on the differentiation of aggregates, will reveal additional mAbs and target integrins that play central roles in cancer cell aggregation and aggregate coalescence. Finally, the identified mAbs will be tested in mouse models to assess their effectiveness in blocking tumorigenesis in vivo.

Material and methods

Cell lines

The three cell lines used in the screen were MB-231, derived from a breast carcinoma, HTB-66, derived from a malignant melanoma and U87, derived from a primary glioblastoma. The three were obtained from the American Type Culture Collection (ATCC). MB-231 cells and the non-tumorigenic cell line MCF-10A were cultured in DMEM/F12 medium (Thermo Fisher Scientific) supplemented with 5% horse serum (Thermo Fisher Scientific) and human recombinant epidermal growth factor (Cat.# E-9644), insulin (Cat.# I-9278), hydrocortisone (Cat.# H-0135), cholera toxin (Cat.# C-8052, Sigma Aldrich, and penicillin/streptomycin (Thermo Fisher Scientific). HTB-66 and U87 cells were cultured in MEM medium supplemented with 1% sodium pyruvate, 1X non-essential amino acids (all Thermo Fisher Scientific), 10% fetal bovine serum (Atlanta Biologicals), and penicillin/streptomycin (Thermo Fisher Scientific).

Monoclonal antibodies

The 266 mAbs used in this study were obtained from the DSHB (www.dshb.biology.edu) and are listed with their target antigens in Table 1. Characterization of these mAbs, including their capacity to be used for western blot analysis, immunoprecipitation, immunohistochemistry, immunocytostaining, FACS analysis, ELISA analysis, and functional blocking are provided in supplemental Table S1. Hybridomas were cultured in Hyclone ADCF mAb medium (GE Healthcare, Cat.# SH30349.02), containing 1% glutamax, 1% sodium pyruvate, 1% penicillin streptomycin (all Thermo Fisher Scientific) supplemented with 0.1% gentamycin (IBI Scientific, Cat.#
IB02030), at 37°C in 7.5% CO₂. In all cases, supernatants were harvested from hybridoma cultures that had reached concentrations of 1.5 to 2.0 × 10⁶ cells per ml. The mAbs were affinity purified using a Protein G HP spin trap column (GE Healthcare, Cat.#28903134) and concentrated using an ultra-centrifugal filter (EMD Millipore, Cat.# UFC905024). In some cases, Protein G in the column was replaced with Protein L resin (GenScript Cat.# L00239).

**Coalescence assay**

The screen for inhibition of coalescence by mAbs in a 3D Matrigel environment was described previously and is diagrammed in Figure 1(a). To each well in a 96-well plate (Corning Inc.), 50 µl of Matrigel in growth factor medium (Corning Inc., Cat.# 354234) at 5°C was added to form a cushion. The cushion polymerized by incubating for 30 minutes at 37°C. Cells suspended in the respective growth medium at 3 × 10⁶ cells per ml were strained through a 70 µm strainer to ensure single cell suspensions. The purified mAb was added to 60 µl of medium to achieve the noted final concentration and added to 80 µl of cells. Sixty µl of medium lacking mAb was added as a control. To this mixture, 30 µl of Matrigel in growth medium at 5°C was added to achieve a final volume of 170 µl. Eighty µl of the cell/Matrigel mixture was immediately distributed over the Matrigel cushion in each tissue culture dish well, and the mixture was incubated at 37°C in 5% CO₂ for 30 minutes, resulting in Matrigel gelation. All mAb preparations were tested in duplicate. Coalescence was monitored in each 3D Matrigel preparation over a 4 day period every 24 hours using an Axiovert 100 inverted microscope (Carl Zeiss, Inc.), equipped with a 10x phase contrast objective and an XCD-V50 digital camera (Sony Corp.).

**Immunofluorescent staining of fixed cells**

Cells were dispersed on a cover slip in a 20 mm petri dish and grown overnight in growth medium. Cells were fixed in 3.8% paraformaldehyde (Electron Microscopy Science), washed with Tris buffer solution, TBS pH7.6 (Thermo Fisher Scientific) and blocked with 1% bovine serum albumin in TBS, pH7.6, for one hour. Preparations were then washed and treated with 10 µg per ml of mAb. For containing experiments, cells were treated with 5 µg per ml of each mAb, then washed three times. Single mAb-stained preparations were treated with affinity-purified fluorescent anti-rat IgG H + L Alexa Fluor 488 antibody (Thermo Fisher Scientific, Cat.# A-11006) or with the affinity-purified fluorescent anti-mouse IgG H + L Alexa Fluor 488 antibody (Jackson Immunoresearch, Cat.# 115-545-003) at a 1:1000 dilution for 1 hour. Double mAb stained preparations were treated with the secondary anti-rat IgG H + L Alexa Fluor 488 antibody (Thermo Fisher Scientific, Cat.# A-11006) and secondary affinity purified anti-mouse IgG H + L R-Phycocerythrin antibody (Thermo Fisher Scientific, Cat.# P852) at a 1:1000 dilution for 1 hour. Cells were imaged with a EOS Rebel T3i/EOS 600D camera (Canon) attached to a Nikon TE2000 inverted epifluorescence microscope (Nikon Instruments).

**Immunostaining of live aggregates in matrigel**

Wells of a µ-slide Angiogenesis 15-well chamber slide (ibidi USA, Inc., Cat.# 81506) were coated with 8 µl of phenol red-free Matrigel (Corning Inc., Cat.# 356237) to provide a Matrigel cushion. To avoid cross-reaction with the mouse-derived Matrigel, the secondary antibody step was omitted. Instead, purified mAbs were directly labeled with the AnaTag HiLyte 750 nm or 488 nm Labeling Kit (ANASPEC, Cat.# AS72043 and AS72047) according to the manufacturer’s instruction. Eighty µl of cells at a concentration of 3 × 10⁶ per ml in growth medium lacking phenol red (HyClone) were mixed with labeled antibody at a final concentration of 5 µg/ml each in 30 µl of phenol red-free Matrigel. Ten µl of this mixture was applied to the Matrigel cushion. After polymerization at 37⁰C, in 5% CO₂, for 30 min, 20 µl of phenol red-free medium was added, and the preparation incubated in the dark for 4 days at 37⁰C, in 5% CO₂. The cell preparations were imaged using a Leica SP8 STDE confocal microscope. Image processing for analysis was done using Fiji software.

**Cell viability by MTT reduction assay**

Cells were cultured in the described 3D assay preparation in phenol-free Matrigel (Corning, Cat.# 356237) with or without mAb. The final mAb concentration was 50 µg/ml. On Day 4, 5 µl of 5 mM MTT (Thermo Fisher Scientific, Cat.# M6494) was added directly to the wells, to a final concentration of 100 µM. After 6 hours of incubation, cells were imaged and scored for formazan formation using a EOS Rebel T3i/EOS 600D camera (Canon) attached to a Nikon TE2000 inverted epifluorescence microscope (Nikon Instruments).

**Cell division in 3D matrigel model**

A glass-windowed, 65 mm Petri dishes (Cellvis, D60-3–0-N), custom modified in house for DIC microscopy, was employed as previously described. In brief, a Matrigel cushion was cast on the glass bottom of a Petri dish. Cells in liquid Matrigel (5°C) were filtered through a 70 µm cell strainer. Each mAb was added to a final concentration of 540 µg/ml. A 750 µl aliquot of the cell/Matrigel/mAb mixture was then cast on the Matrigel coated glass window of the Petri dish. The 1.5 mm-thick preparation was incubated for 30 minutes at 37°C in 5% CO₂ to allow gelation, and medium added. The preparation was positioned on the motor-driven stage of a Zeiss Axio Imager M2 microscope (Carl Zeiss Inc.) equipped with DIC optics and an AxioCam 503 mono color CCD camera (Carl Zeiss Inc.). The camera, the motor, and the microscope were computer-synchronized. The microscope was housed in an incubator, set at 37°C in 5% CO₂. A collection of 150 DIC optical sections were obtained in a 45-second period and repeated every 10 minutes for 4 days. Optical sections were directly saved as jpeg images and processed using 3D-DIAS 4.2.
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Disclosure Interest

The authors report no conflict of interest.

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| DSHB         | Developmental Studies Hybridoma Bank |
| DMEM         | Dulbecco’s modified Eagle medium |
| ELISA        | enzyme linked immunosorbent assay |
| FACS         | fluorescent activated cell sorting; mAb, monoclonal antibody |
| mAb          | monoclonal antibody |
| MB-231       | MDA-MB-231 |
| MEM          | modified Eagle’s medium |
| NEA          | Anon-essential amino acids |
| 2D           | 2 dimensional |
| 3D           | 3 dimensional |

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