Curcumin inhibition of the functional interaction between integrin α6β4 and the epidermal growth factor receptor

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Abstract

The functional interaction between integrin α6β4 and growth factor receptors has been implicated in key signaling pathways important for cancer cell function. However, few attempts have been made to selectively target this interaction for therapeutic intervention. Previous studies showed that curcumin, a yellow pigment isolated from turmeric, inhibits integrin α6β4 signaling important for breast carcinoma cell motility and invasion, but the mechanism is not currently known. To address this issue, we tested the hypothesis that curcumin inhibits the functional interaction between α6β4 and the epidermal growth factor receptor (EGFR). In this study, we found that curcumin disrupts functional and physical interactions between α6β4 and EGFR, and blocks α6β4/EGFR dependent functions of carcinoma cells expressing the signaling competent form of α6β4. We further demonstrated that curcumin inhibits EGF dependent mobilization of α6β4 from hemidesmosomes to the leading edges of migrating cells such as lamellipodia and filopodia, and thereby prevents α6β4 distribution to lipid rafts where functional interactions between α6β4 and EGFR occur. These data suggest a novel paradigm in which curcumin inhibits α6β4 signaling and functions by altering intracellular localization of α6β4, thus preventing its association with signaling receptors such as EGFR.
Introduction

Curcumin is a polyphenolic component of turmeric (Fig. 1A), which is widely used as a spice and as a traditional ancient medicine in Indian culture (1). Curcumin has garnered a lot of attention as anti-cancer agent due to its non-toxic chemopreventive effect against virtually all known human cancers (2-3). There is accumulating evidence that curcumin inhibits cancer initiation and progression through regulation of multiple cellular pathways including EGFR/PDGFR (4,5), AKT/mTOR (6,7), NF-kappaB (8), MAPK (9) and STAT pathways (10). Most of these curcumin targets are either signaling partners or downstream effectors of integrin α6β4 (11-13), and our recent study demonstrated that curcumin indeed inhibits α6β4 signaling and functions associated with cancer cell motility and invasion (14). However, the mechanism by which curcumin inhibits α6β4 signaling in aggressive cancer cells remains to be elucidated.

α6β4 integrin is a laminin receptor and it was thought that its primary role was to maintain the integrity of epithelia (15). However, recent evidence has established that α6β4 also plays a pivotal role in functions associated with tumorigenesis and carcinoma progression, suggesting that α6β4 may switch its functions depending on the surrounding microenvironment (15,16). In aggressive carcinoma cells, the host-tumor microenvironment induces growth factor dependent mobilization of α6β4 from hemidesmosomes into the leading edges of migrating cells such as lamellipodia and filopodia (17). The localization of α6β4 to actin filament rich structures such as lamellipodia and filopodia has an important implication for cancer cell motility and invasion, because integrins can harness traction forces through association with F-actin
and their ability to engage the extracellular matrix (18,19). In addition, localization of α6β4 in the leading edge is thought to increase the level of α6β4 in lipid rafts (20). Lipid rafts are sphingolipid and cholesterol rich microdomains of the plasma membrane (21,22). Lipid rafts can act as “signaling platforms” where signaling initiation and amplification occur more efficiently by recruiting signaling receptors into close proximity (20-23). Once α6β4 localizes in lipid rafts, it is assumed that its signaling function is enhanced through interaction with other signaling receptors such as EGFR (20,21).

In this study, we assessed the mechanism by which curcumin inhibits α6β4 signaling. Specifically, we tested the hypothesis that curcumin disrupts functional and physical interactions between α6β4 and growth factor receptors. EGFR was chosen as a model to address this hypothesis because the interaction between EGFR and α6β4 has been implicated in carcinoma cell functions important for progression (17, 24). We found that curcumin inhibits α6β4/EGFR dependent proliferation in A431 squamous carcinoma and MDA-MB-231 breast carcinoma cells, both of which express signaling competent form of α6β4. Additional studies have shown that curcumin reduces α6β4 dependent EGFR phosphorylation and the physical association between these two receptors. Curcumin inhibition of α6β4/EGFR signaling is likely to be due to its prevention of EGF induced mobilization of α6β4 from hemidesmosomes to lamellipodia and filopodia, where α6β4 can functionally interact with EGFR. Consistent with this observation, curcumin decreases the ratio of raft/ non-raft α6β4. Altogether, our studies provide a basis for a novel paradigm in which curcumin inhibits cancer cell functions by disrupting the interaction between integrin and growth factor receptors.
Materials and Methods

Cell lines and reagents

A431 squamous carcinoma cells, MDA-MB-231 breast carcinoma cells and MCF10A cells were characterized by and purchased from ATCC (American Type Culture Collections) in January 2009. These cell lines have not been tested and authenticated by us. A431 and MDA-MB-231 cells were cultured in DMEM with 1 g/L glucose, L-glutamine and sodium pyruvate formulation, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 g/ml streptomycin. MCF-10A cells were maintained in MEGM containing 13 mg/ml BPE, 0.5mg hydrocortisone, 10 μg/ml hEGF, 5 mg/ml insulin and 100 ng/ml Cholera toxin (Lonza). All cells used were always less than 20 passages from the stock and cultured in humidified incubators at 37°C in 5% CO2. To stably reduce β4 integrin expression, A431 cells were infected with lentiviruses that expressed shRNA targeted against either GFP or the β4 integrin subunit. MDA-MB-231 cells expressing shRNA against either GFP or the β4 integrin were used as previously described (14). To evaluate the effect of curcumin, cells were treated with the indicated doses (see figure legends) of curcumin (Sigma) under serum starvation for 24 hr, followed by stimulation with 10 ng/ml EGF for 15-20 min.

The following reagents were obtained commercially. EGF was purchased from Sigma-Aldrich (Sigma). Integrin β4 (clone H-101) and actin (clone C-11) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz); Akt, p-Akt (Ser473), EGFR, p-EGFR(Tyr1068) and p-EGFR(Tyr1045) antibodies were obtained from Cell Signaling Technology. Phospho-β4 integrin (Y1494) was purchased from ECM.
Biosciences. Flotillin-1 antibody was obtained from BD Transduction Laboratories (BD) and used to detect isolation of lipid rafts. Transferrine receptor antibody was purchased from Invitrogen. Integrin β4 (CD104) antibody was purchased from BD Pharmingen (BD) and used to visualize localization of β4 and EGFR. EGFR (2E9) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz). Alexa-fluor 546 goat anti-mouse IgG and Alexa-fluor 488 goat anti-Rat IgG were purchased from Invitrogen.

**Cell proliferation assay**

The cells were plated in 6-well plates at 1x10^4 cells per well. Once stabilized, the cells were treated with the indicated doses of curcumin and then stimulated with or without 10 ng/ml of EGF. At the times indicated, cells were trypsinized and counted using a hematocytometer.

**Soft agar growth assay**

MDA-MB-231 cells (1 x 10^3) were suspended in a top layer of DMEM (1 ml) containing 0.35% low melt agarose (ISC BioExpress) with or without 10 μM curcumin and plated on a bottom layer of DMEM (2 ml) containing 0.75% agar with or without 10 ng/ml of EGF in six-well plates. The cells were fed twice per week with 0.5 ml DMEM added with each indicated dose of EGF and curcumin. After 3 weeks, the total number of colonies was quantified by counting 50 fields per well using bright-field optics. The average total number of colonies was obtained from counting triplicate wells.

**Isolation of detergent-free lipid rafts**
To isolate lipid raft fractions, we used the procedure described by Macdonald and Pike (25) with minor modifications. A431 cells were maintained in 150 nm dishes until the cells were 70% confluent and treated with or without curcumin overnight. The cells were scraped into cold base buffer (20 mM pH 7.8 Tris-HCl, 250 mM sucrose) to which 1 mM CaCl₂ and 1 mM MgCl₂ had been added. Cells pelleted by centrifugation were resuspended in 1 ml lysis buffer containing protease inhibitors and then lysed by repeated passage through a 22 gauge needle (20 times). After centrifugation at 1000 x g for 10 min, the resulting postnuclear supernatants were collected into 12 ml centrifuge tubes. The pellets were lysed once more with 1 ml lysis buffer and the second postnuclear supernatants were combined with the first. Equal volumes (2 ml) of base buffer containing 50% OptiPrep (Sigma) were mixed with the postnuclear supernatants in the bottoms of 12 ml centrifuge tubes. An 8 ml gradient of 0% to 20% OptiPrep in base buffer was poured on top and the tubes were centrifuged for 3 hr at 52,000 x g using the SW41 rotor in a Beckman ultracentrifuge. Gradients were fractionated into 1 ml fractions obtained from the top. The distribution of various proteins was determined by Western blotting.

**Western blot analysis**

Total cell extracts were prepared according to the methods described in our previous study (14), and separated on 4% to 20% gradient SDS PAGE and transferred to polyvinylidene fluoride membranes (Millipore). The blots were incubated with primary antibodies in TBS-T or TBS-T with 5% w/v nonfat dry milk, then with appropriate
secondary antibodies conjugated to IgG-horseradish peroxidase. Proteins were detected using the pierce ECL Western blotting substrate (Thermo).

**Immunoprecipitation assay**

Cells were counted and plated into 10-cm or 6-well culture dishes. After reaching 50-60% of confluence, cells were treated with curcumin (15 μM in A431 cells and 10 μM in MDA-MB-231 cells) under serum starvation overnight and then stimulated with or without 10 ng/ml EGF for 20 min, prior to cell lysis. Cells were then lysed using lysis buffer (50 mM pH 7.4 Tris–Hcl, 150 mM NaCl, 1% NP-40, 5 mM EDTA, NaF, Na3VO4, Na4P2O7, β-glycerolphosphate, aprotinin, leupeptin, pepstatin A, 100 mM PMSF). The lysates containing 1 mg of protein were precleared with protein A-conjugated agarose beads (Sigma) for 2 hours and then incubated with the appropriate primary antibodies overnight, followed by incubation with protein A-conjugated agarose beads. The beads were washed 4 times with PBS, and then boiled for 5 min in Laemmli sample buffer (BioRad Life Science). The immunoprecipitated proteins were separated by SDS-polyacrylamide gel and probed with specific antibodies against EGFR, β4, and IgG.

**Immunofluorescence staining and confocal microscopy**

A431 cells and MDA-MB-231 cells seeded on cover glasses at a density of 5 × 10³ per well were treated with or without the indicated doses of curcumin overnight before EGF (100 ng/ml) stimulation for 15 min. After these treatments, cells were fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.5% Triton X-100 for 15 min and then incubated with primary antibody against EGFR and β4 integrin overnight at 4 °C. After
washing three times with PBS, cells were incubated with Alexa Fluor 488 or Alexa Fluor 558 labeled secondary antibodies (Invitrogen). Fluorescent images were observed under a Zeiss LSM 510 laser-scanning confocal microscope. Co-localization of α6β4 and EGFR was observed by merging the two fluorescence images in green (488nm) and red (546nm), indicating similar distribution patterns within merged images in yellow.
Results

Curcumin inhibits \( \alpha_6\beta_4/ \)EGFR dependent functions of carcinoma cells that express signaling competent \( \alpha_6\beta_4 \)

Based on the previous studies that curcumin inhibits carcinoma cell functions by inhibiting integrin \( \alpha_6\beta_4 \) signaling (14), we tested the hypothesis that curcumin’s inhibitory effect is mainly observed in cells that express the signaling competent form of \( \alpha_6\beta_4 \). To address this issue, we used A431 squamous carcinoma, MDA-MB-231 breast carcinoma and MCF-10A normal epithelial cells. Both A431 and MDA-MB-231 carcinoma cells express the signaling competent form of \( \alpha_6\beta_4 \) (14, 17), as confirmed by high levels of \( \beta_4 \) phospho-Y1494 signal (Fig. 1B) (an indicator of \( \alpha_6\beta_4 \) signaling competency)(26). Consistent with our previous report (14), curcumin effectively blocked \( \beta_4 \) Y1494 phosphorylation in A431 cells and MDA-MB-231 cells in a dose dependent manner (Fig. 1B). In contrast, MCF-10A cells express levels of \( \beta_4 \) integrin comparable to those of A431 and MDA-MB-231 cells, but the phospho-Y1494 signal was undetectable, suggesting that \( \alpha_6\beta_4 \) in MCF-10A cells is signaling incompetent (Fig. 1B).

Based on previous reports that \( \alpha_6\beta_4 \) synergizes with growth factor receptors to enhance carcinoma cell functions (16, 27-28), we assessed the effect of curcumin on \( \alpha_6\beta_4 \) dependent cell proliferation in response to growth factor (EGF) stimulation in these three cell lines. These cells were treated with curcumin prior to stimulation with or without EGF. Proliferation of signaling competent \( \alpha_6\beta_4 \) expressing A431 cells and MDA-MB-231 cells was significantly enhanced by EGF treatment (Fig. 1C). EGF dependent carcinoma cell proliferation requires the presence of \( \alpha_6\beta_4 \), as knockdown of
β4 integrin expression by shRNA effectively blocks EGF stimulation of carcinoma cell proliferation (data not shown). Curcumin also blocks EGF dependent carcinoma cell proliferation in a dose dependent manner, mimicking the loss of β4 integrin expression (Fig. 1C). In contrast, signaling incompetent α6β4 expressing MCF-10A cells responded neither to EGF stimulation nor to curcumin treatment. These results suggest that the presence of the signaling competent form of α6β4 sensitizes carcinoma cells to curcumin inhibition.

We then assessed the effect of curcumin treatment on α6β4/EGFR dependent anchorage independent growth, which is another important component of carcinoma progression, by measuring colony formation on soft agar (Fig. 2). As shown in Fig.2, colony formation in soft agar requires the presence of α6β4, as knockdown of β4 expression by shRNA reduced colony formation by 60% compared with that of GFP shRNA expressing control cells. EGF stimulation in MDA-MB-231 cells further increased the colony formation and size in soft agar (Fig. 2). Curcumin effectively blocked colony formation in soft agar, with levels similar to those of MDA-MB-231 cells whose β4 integrin is knocked down by shRNA in spite of EGF treatment (Fig. 2). No colony formation in soft agar was observed in MCF-10A cells, which express the signaling incompetent form of α6β4 (data not shown). These results suggest that curcumin prevents anchorage independent growth mediated by cooperative signaling between α6β4 and EGFR.

**Curcumin blocks α6β4-dependent EGFR activation**

Curcumin inhibition of α6β4/EGFR mediated proliferation and anchorage
independent growth indicates its possible intervention in the crosstalk between these two receptors. To test this hypothesis, we examined whether curcumin blocks α6β4 dependent EGFR activation. As shown in Fig. 3A, the presence of α6β4 in A431 cells and MDA-MB-231 cells contributes to higher steady state levels of EGFR Y1068 and Y1045 phosphorylation (indicator of EGFR activation) (29, 30), as the knockout of α6β4 expression by shRNA significantly reduced the phosphorylation of these residues. These data suggest that α6β4 amplifies EGFR signaling through crosstalk even in the absence of ligand stimulation. EGF stimulation further increases the levels of EGFR Y1068 and Y1045 phosphorylation, but not in carcinoma cells deficient in α6β4 expression by shRNA (data not shown). We next examined whether curcumin inhibits α6β4 dependent EGFR activation. As shown in Fig. 3B, curcumin effectively blocked phosphorylation of Y1068 and Y1045 of EGFR, and Y1494 of β4 integrin, in MDA-MB-231 cells and A431 cells when these cells were treated with curcumin prior to EGF stimulation for 20 min. The inhibitory effect of curcumin on phosphorylation of these tyrosine residues was similar to that resulting from knockdown of β4 integrin expression (Fig. 3). In addition, there is a nice correlation of phosphorylation levels between EGFR’s Y1068/Y1045 and β4 integrin’s Y1494 (Fig. 3B), suggesting that these two receptors are functionally linked. These findings indicate that the inhibitory effect of curcumin on α6β4 signaling could occur at least in part as a result of disruption of the functional interaction between these two receptors.

Curcumin blocks EGF dependent mobilization of α6β4 from hemidesmosomes into filopodia and lamellipodia
To gain additional evidence of curcumin disruption of the interaction between α6β4 and EGFR, we assessed the effect of curcumin treatment on the intracellular localization of α6β4 and EGFR in MDA-MB-231 cells and A431 cells using confocal microscopy (Fig. 4). Under normal conditions (no EGF stimulation and no curcumin treatment: Fig. 4, a and d), a significant portion of α6β4 localized in the HDs (see arrow), whereas some portion of α6β4 localized at the leading edges. As reported previously (17), EGF stimulation for 15 min destabilized the HDs and induced the mobilization of α6β4 into the leading edge of MDA-MB-231 cells and A431 cells (Fig. 4, b and e). Overall, there is a good correlation of localization between α6β4 and EGFR. Their colocalization pattern is more obvious at the leading edges upon EGF stimulation (Fig. 4, b and e). In contrast, treatment of curcumin for 24 hrs prior to EGF stimulation effectively blocks EGF induced α6β4 translocation from the HDs into the leading edges of MDA-MB-231 cells and A431 cells (Fig. 4, c and f). Curcumin also blocks EGF dependent disassembly of HDs as the majority of α6β4 remains localized at HDs in spite of EGF treatment (Fig. 4, c and f).

We then examined whether prevention of α6β4 localization at the leading edges by curcumin has any impact on the physical association between α6β4 and EGFR. We performed co-immunoprecipitation assays with specific β4 and EGFR antibodies. As shown in Fig. 5, curcumin treatment reduced the amount of co-immunoprecipitated α6β4 with EGFR in MDA-MB-231 cells (Fig. 5A) and in A431 cells (Fig. 5B). Taken together, these results indicate that curcumin prevents the interaction between α6β4 and EGFR, possibly by altering the intracellular location of α6β4.
Curcumin prevents localization of α6β4/EGFR to lipid rafts

Mobilization of α6β4 from HDs to leading edges of migrating carcinoma cells is thought to increase the localization of α6β4 into lipid rafts (16), where lots of other signaling receptors are located in the near vicinity so that α6β4 acts as a signal amplifier of these receptors (20,21,31). Therefore, we tested whether curcumin mediated α6β4 sequestration in HDs affects localization of α6β4 in lipid rafts (Fig. 6). We monitored the distribution of α6β4 and EGFR in the raft vs. non-raft fractions of A431 carcinoma cells using the detergent-free fractionation method as previously described (25). Fractions 1 and 2 represent the lipid raft fractions as confirmed by the lipid raft marker, Flotillin-1, whereas fractions 3 or higher represent non-lipid raft fractions as indicated by transferrin receptor signals (Fig. 6A). Densitometric analysis from Fig. 6A Western blot analysis showed that the steady state raft/non-raft ratios of α6β4 and EGFR are 1.17 and 1.32, respectively, in A431 cells, whereas curcumin treatment effectively lowered this ratio to 0.66 and 0.53, respectively (Fig. 6A). These results suggest that curcumin mediated prevention of sub-cellular movement of α6β4 in HDs negatively affects lipid raft localization of this integrin.

We then investigated the effect of curcumin on the physical association between α6β4 and EGFR in raft vs. non-raft fractions. Co-immunoprecipitation assays showed that physical association between these two receptors is detected equally in both raft and non-raft fractions in unstimulated A431 cells, whereas EGF stimulation significantly increased their physical association, especially in raft fractions (Fig. 6B). Curcumin treatment effectively reduced the amount of overall co-immunoprecipitation between these two receptors by selectively affecting their association in raft fractions regardless of
EGF stimulation (Fig. 6B). It is interesting to note that physical association between α6β4 and EGFR in non-raft fractions is not affected by curcumin treatment (Fig. 6B). These data suggest that curcumin inhibition of the physical association between these two receptors is likely to be due to its prevention of their localization at the lipid rafts.
Discussion

It is widely assumed that α6β4 plays a pivotal role in carcinoma progression through functional and physical interactions with specific growth factor receptors (16,17). However, therapeutic intervention targeting these interactions has not been explored. Based on our previous report that curcumin inhibits α6β4 dependent breast cancer cell motility and invasion (14), we tested the hypothesis that curcumin inhibits α6β4 signaling by intervening in the interaction between α6β4 and growth factor receptors in the current study. We used two carcinoma cell lines, MDA-MB-231 and A431, which over express both α6β4 and EGFR (14, 17, 32). Interactions between these two receptors have been shown to be contributed to the malignant behaviors of these carcinoma cells (29, 33). Using these two carcinoma cell lines, we demonstrated for the first time that curcumin inhibits the functional and physical interactions between integrin and growth factor receptors. More specifically, curcumin effectively blocked EGFR/α6β4 induced carcinoma cell proliferation and anchorage independent growth. The mechanism involves prevention of α6β4 mobilization from the hemidesmosomes into the leading edges so that α6β4 no longer effectively interacts with EGFR in the membrane. Additional studies have shown that curcumin reduced the amount of α6β4 localized in lipid rafts where signaling molecules such as EGFR are concentrated. Altogether, our studies provide the evidence for a novel paradigm by which curcumin selectively inhibits carcinoma functions by altering sub-cellular localization of α6β4 and therefore blocking its signaling competency.

The detailed mechanism(s) by which curcumin inhibits interaction between α6β4 and EGFR remains to be determined. Our study suggests that either sequestration of
α6β4 in the HDs or prevention of its lipid raft localization by curcumin could be major mechanisms. Earlier studies demonstrated that, in response to EGF of cancer cells, α6β4 in hemidesmosomes mobilizes to lamellipodia and filopodia. This is where α6β4 interacts with growth factor receptors, leading to interaction with the actin cytoskeleton important to cancer cell invasion and metastasis (17,31). Phosphorylation of key serine residues (S1356, 1360,1364, 1424) of β4 integrin by PKC-α is involved in HD disassembly and the mobilization of α6β4 from HDs (34-36). Therefore, it is possible that curcumin may affect one of the signaling pathways responsible for the phosphorylation of these Ser residues. Alternatively, palmitoylation of membrane proximal Cys residues of β4 integrin is required for lipid raft localization of α6β4 (20). Based on our data, which show that curcumin shifts α6β4 from lipid raft to non-lipid raft fractions (Fig. 6), it is also possible that curcumin may block palmitoylation of the β4 integrin subunit. These possibilities are currently under investigation.

The selective inhibitory effects of curcumin, as well as those of other phytochemicals, on cancer cell functions without toxicity to normal cells are well known, but there has been little explanation of the mechanisms. Our data, which show that carcinoma cells expressing the signaling competent form of α6β4 are more sensitive to curcumin, may provide the clue that explains the selective inhibitory effects of curcumin. This could be due to the fact that only the signaling competent form of α6β4 localizes into lipid rafts where the functional interaction with growth factor receptors such as EGFR occurs. The selective inhibitory effect of curcumin on carcinoma cells could result from carcinoma cells depending more on lipid raft signaling as curcumin blocks trafficking of signaling molecules such as α6β4 into the lipid rafts. In contrast, the
signaling incompetent form of $\alpha_6\beta_4$ is mainly localized in hemidesmosomes (HDs) and simply provides tissue integrity in normal epithelia (37). Our studies show that curcumin does not affect HD stability or $\alpha_6\beta_4$ localization in HDs. Therefore, signaling incompetent $\alpha_6\beta_4$ expressing cells would be less sensitive to curcumin treatment. While this hypothesis needs to be tested on a larger scale in future studies, it is highly encouraging that this study provides the mechanistic basis for understanding the selective inhibitory effect of curcumin on cancer cells, but not normal cells.

The current paradigm for the most effective treatment of cancer is to combine multiple drugs against multiple targets. In this regard, our finding that curcumin has an inhibitory effect on the interaction of the two receptors suggests a novel possibility, that a multi-modality approach such as one involving curcumin and EGFR inhibitors (i.e. Gefitinib and Eriotinib) (38) could provide the design for effective multi-target agents for clinical chemotherapy. In conclusion, our study demonstrated that the chemopreventive effect of curcumin in carcinoma cells derives from its ability to prevent $\alpha_6\beta_4$ mobilization into the leading edge and lipid rafts, which blocks cooperative interaction between $\alpha_6\beta_4$ and EGFR. Based on previous reports that functional interactions between $\alpha_6\beta_4$ and growth factor receptors are characteristic of aggressive carcinoma cells, our studies provide the basis for a novel paradigm that explains how curcumin selectively inhibits cancer cell function without affecting normal cells.
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Figure Legends

Figure 1. Curcumin inhibits α6β4/EGFR dependent cancer cell proliferation. A, chemical structure of curcumin. B, MDA-MB-231, A431, and MCF-10A cells were treated with the indicated doses of curcumin for 24 hr prior to lysis with RIPA buffer. Equal amounts of extracts from each sample were used for Western blot analysis using antibodies against β4 integrin, phospho-β4-integrin (Y1494) and β-actin. C, MDA-MB-231 cells, A431 cells and MCF10A cells were serum starved, followed by treatment with different doses of curcumin (5, 10 μM) and then stimulated with or without EGF (10 ng/ml). The cell numbers were counted using a hemacytometer over the following 1, 3 and 5 day period. The statistical analysis was done using the two-tailed Student’s t-test. P<0.01(*) and P<0.05(**) compared with the results of the control cell lines.

Figure 2. Curcumin inhibits α6β4/EGFR mediated anchorage-independent growth in response to EGF. DMA-MB-231 cell lines (parental, β4 shRNA infectants) were grown in 0.7% agar containing low serum media with or without curcumin (10 μM) in the absence or presence of 10 ng/ml EGF for 3 wk. Representative images of soft agar assays were captured at x10 magnification. Bottom panel, quantification of the soft agar assays. Columns, mean of three representative experiments performed in triplicate; bars, SD. Significant differences were evaluated using the Student’s t-test. *, P<0.01. **, P<0.05.

Figure 3. Curcumin disrupts α6β4 integrin-dependent EGFR activation. A, equal extracts from the lysates of GFP and β4 shRNA infectants of A431 and MDA-MB-231 cells were
used for Western blot analysis using antibodies against phospho-β4-integrin (Y1494), phosphor-EGFR (Y1068), phospho-EGFR (Y1045) or β-actin. B, A431 cells and MDA-MB-231 cells were pre-treated with 15 μM and 10 μM curcumin respectively under serum starvation prior to EGF stimulation (10 ng/ml) for 20 min and then lysed using RIPA buffer. Equal amounts of protein were isolated from extracts of these cells for Western blot analysis using antibodies against phospho-β4-integrin (Y1494), phosphor-EGFR (Y1068), phospho-EGFR (Y1045) or β-actin. The Western blot results presented were carried out at least 3 times.

Figure 4. Curcumin blocks EGF dependent mobilization of α6β4 from hemidesmosomes to the leading edge of cancer cells. MDA-MB-231 cells and A431 cells were plated on cover glasses and fixed with 0.5% Triton-X and double stained using the anti-β4 antibodies (green) and anti-EGFR antibodies (red) followed by Alexa-labeled secondary antibodies. Control (a,d). Stimulation with EGF in serum-free media for 15 min (b,e). Pretreatment with curcumin (15 μM in A431 cells and 10 μM in MDA-MB-231 cells) for 24 hr before stimulation with 100 ng/ml EGF for 15 min (c, f). The localization of β4 and EGFR was determined using the confocal microscopy. Arrows indicate hemidesmosomes. Scale bar, 10 μm.

Figure 5. Curcumin interrupts the physical association between β4 and EGFR. A, MDA-MB-231 cells and B, A431 cells were treated with or without curcumin overnight before stimulation with 10 ng/ml EGF. The cell lysates were immunoprecipitated with EGFR,
β4 or control IgG antibodies. Western blotting was performed on samples with integrin β4, EGFR antibodies and IgG antibodies.

**Figure 6.** Curcumin prevents α6β4 distribution to lipid rafts. A, A431 cells were treated with or without curcumin (15 μM). Isolation of lipid rafts from the cells was done using the detergent-free method as described in the Materials and Methods section. Twelve fractions were collected by an Optiprep gradient producer. Each fraction was analyzed by Western blotting using the indicated antibodies. Flot (Flotillin-1) was used as a lipid raft marker and Trf (Transferrin receptors) was used as a non-lipid raft marker. The Western blotting presented here was carried out at least 3 times. B, A431 cells were treated with or without 15 μM curcumin prior to stimulation with or without 10 ng/ml EGF for 20 min. The lipid raft fractions (1-3) and non-lipid raft fractions (4-6) from these cells were immunoprecipitated with β4 antibodies and probed with EGFR antibodies. Densitometric analysis was performed to measure the relative intensity of the bands from Western blotting analysis.
Figure 1

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\begin{align*}
\text{Curcumin (μM)} & \quad 0 \quad 5 \quad 10 \quad 15 \quad 20 \\
\text{P-β4 (Y1494)} & \\
\beta4 & \\
\text{MDA-MB-231} & \\
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B

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\begin{align*}
\text{Curcumin (μM)} & \quad 0 \quad 5 \quad 10 \quad 15 \quad 20 \\
P-β4 (Y1494) & \\
\beta4 & \\
\text{A431} & \\
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\text{Cell numbers (10^3)} & \quad 0 \quad 200 \quad 400 \quad 600 \\
\text{Days in culture} & \quad 1 \quad 3 \quad 5 \\
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\begin{align*}
\text{Cell numbers (10^3)} & \quad 0 \quad 200 \quad 400 \quad 600 \\
\text{Days in culture} & \quad 1 \quad 3 \quad 5 \\
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\begin{align*}
\text{Cell numbers (10^3)} & \quad 0 \quad 200 \quad 400 \quad 600 \\
\text{Days in culture} & \quad 1 \quad 3 \quad 5 \\
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Figure 2

![Image of colony counts for MDA-MB-231 cells under different conditions (Cont, β4 shRNA, EGF 10ng/ml, EGF+Cur). The graph shows a significant increase in colony counts for the EGF 10ng/ml condition compared to other conditions.](image-url)
**Figure 3**

### A

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</table>

**Curcumin(15μM)**

- | - | + |
- | 0 | 10 |
| EGF(ng/ml) | P-β4 (Y1494) |
| 0 | 10 |
| P-EGFR (Y1068) |
| 0 | 10 |
| P-EGFR (Y1045) |
| 0 | 10 |
| Actin |

**Curcumin(10μM)**

- | - | + |
- | 0 | 10 |
| EGF(ng/ml) | P-β4 (Y1494) |
| 0 | 10 |
| P-EGFR (Y1068) |
| 0 | 10 |
| P-EGFR (Y1045) |
| 0 | 10 |
| Actin |
Figure 4

A

**A431 cells**

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<th>a.</th>
<th>β4</th>
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<th>Merge</th>
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**MDA-MB-231 cells**

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Figure 5

A

Curcumin (10μM) - +

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<tr>
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<tr>
<td>IP : EGFR</td>
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<tr>
<td>IgG</td>
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B

Curcumin (15μM) - +

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<tr>
<td>IP : β4</td>
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Figure 6

(A) Lipid Raft versus Non-lipid Raft Fraction:

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</tbody>
</table>

(B) Fraction: Raft Non Raft Non Raft Non Raft Non

IP: β4  
IB: EGFR

Cont Cur (15μM) EGF (10ng/ml) EGF + Cur

Graph: Co-IP signals between Rafts vs. Non-Rafts fractions

- Raft
- Non-Raft

0 5000 10000 15000 20000

Cont Cur EGF EGF + Cur
Molecular Cancer Therapeutics

Curcumin inhibition of the functional interaction between integrin α6β4 and the epidermal growth factor receptor

Young Hwa Soung and Jun Chung

Mol Cancer Ther Published OnlineFirst March 9, 2011.

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