Epidermal Growth Factor–induced Enhancement of Glioblastoma Cell Migration in 3D Arises from an Intrinsic Increase in Speed But an Extrinsic Matrix- and Proteolysis-dependent Increase in Persistence

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Epidermal growth factor (EGF) receptor-mediated cell migration plays a vital role in invasion of many tumor types. EGF receptor ligands increase invasiveness *in vivo*, but it remains unclear how consequent effects on intrinsic cell motility behavior versus effects on extrinsic matrix properties integrate to result in net increase of translational speed and/or directional persistence of migration in a 3D environment. Understanding this convolution is important for therapeutic targeting of tumor invasion, as key regulatory pathways for intrinsic versus extrinsic effects may not be coincident. Accordingly, we have undertaken a quantitative single-cell imaging study of glioblastoma cell movement in 3D matrices and on 2D substrata across a range of collagen densities with systematic variation of protease-mediated matrix degradation. In 3D, EGF induced a mild increase in cell speed and a strong increase in directional persistence, the latter depending heavily on matrix density and EGF-stimulated protease activity. In contrast, in 2D, EGF induced a similarly mild increase in speed but conversely a decrease in directional persistence (both independent of protease activity). Thus, the EGF-enhanced 3D tumor cell migration results only partially from cell-intrinsic effects, with override of cell-intrinsic persistence decrease by protease-mediated cell-extrinsic reduction of matrix steric hindrance.

INTRODUCTION

Members of the ErbB receptor tyrosine kinase family and their associated ligands are aberrantly expressed in many cancers, including carcinomas and glioblastomas, and have become a major realm for therapeutic targeting (Yarden, 2001; Bublil and Yarden, 2007). Their overexpression is often correlated with poor prognosis as they play a central role in tumor progression, especially in invasion and metastasis, which lead to cancer-related fatalities (Wells, 2000; Yarden and Sliwkowski, 2001). Tumor invasion requires carefully orchestrated cell motility behavior, which is stimulated by epidermal growth factor (EGF) family ligands (Wells, 1999; Wells *et al.*, 2002). Multiple biophysical processes involved in cell locomotion (Lauffenburger and Horwitz, 1996; Ridley *et al.*, 2003) are regulated by EGF receptor (EGFR) activation (Maheshwari *et al.*, 1999). In tumor cells, a number of key signaling pathways regulating these migration-related biophysical processes are dysregulated (Condeelis *et al.*, 2005).

Glioblastoma multiforme is a particularly serious example of EGFR-related dysregulation of cell motility strongly correlated with poor disease prognosis (VanMeter *et al.*, 2001; Lal *et al.*, 2002; Huang *et al.*, 2007a).

Current models of tumor cell migration suggest that extracellular matrix proteolysis is critical for tumor cell migration in three-dimensional (3D) environments (Wolf and Friedl, 2005; Zaman *et al.*, 2006), although certain cell types in some environments exhibit fascinating exceptions to such models (Wolf and Friedl, 2006). Tumor cells move through their environment by extending their leading edge and concomitantly secreting matrix-metalloproteinases locally that act pericellularly; this matrix-remodeling step allows directed cell locomotion upon cell contraction and rear release (Wolf and Friedl, 2005, 2006). In a wide range of contexts, tumor invasion requires matrix proteolysis mediated by proteinases such as the matrix metalloproteinase (MMP) and a metalloproteinase and disintegrin (ADAM) families (Yana and Seiki, 2002; Mochizuki and Okada, 2007). There are currently more than 20 known members of the MMP family alone, which differ by their preferred extracellular matrix substrate specificity. MMPs are highly up-regulated in invasive tumors (Nakada *et al.*, 2003), and MMP expression is induced by EGF receptor activation in various cancers (Ellerbroek *et al.*, 1998; Rooprai *et al.*, 2000; Alper *et al.*, 2001). Although tumor cell migration can occur without matrix proteolysis in some circumstances (Wolf *et al.*, 2003), various studies have correlated MMP expression and activity with

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Abbreviations used: EGF, epidermal growth factor; EGFR, EGF receptor; MMP, matrix metalloproteinase; PRW, persistent random walk.

tumor invasion, including glioblastoma (VanMeter *et al.*, 2001; Binder and Berger, 2002).

The effects of matrix proteolysis on cell migration processes may involve more than simply providing pathways for transit (Zaman *et al.*, 2007). Although matrix degradation may loosen steric barriers, it can simultaneously decrease the availability of matrix adhesion ligands or even expose new cryptic adhesion sites. Moreover, cell speed in 3D matrices is influenced by matrix stiffness, which in turn is partly determined by the matrix fiber network density and associated network pore size (Zaman *et al.*, 2006). Local matrix degradation can produce a reduction in stiffness that can either increase or decrease cell migration depending on environmental and cellular context. For these reasons as well as others (Martin and Matrisian, 2007), it is perhaps not surprising that the therapeutic success of matrix protease inhibitors has been mixed at best (Matrisian *et al.*, 2003).

As noted earlier, there is increasing interest in ErbB inhibitors as cancer therapeutics, with specific focus on reducing motility in order to diminish invasiveness (Willmarth and Ethier, 2006; Rosano *et al.*, 2007; Kumar *et al.*, 2008) in particular for treating glioblastoma (Lal *et al.*, 2002; Lamszus *et al.*, 2005; Huang *et al.*, 2007b). However, the effects of ErbB signaling network activation on cell migration are complex and highly dependent on extracellular matrix conditions (Ware *et al.*, 1998; Maheshwari *et al.*, 1999; Harms *et al.*, 2005; Zaman *et al.*, 2006); even in the absence of chemotactic concentration gradients, EGF stimulation can increase or decrease the speed of migration while simultaneously increasing or decreasing directional persistence of migration, depending on the substratum. These two facets of migration are governed differentially by diverse molecular components and regulatory pathways (Ridley *et al.*, 2003). Most studies, however, have utilized 2D assays and therefore have not integrated the additional effects of EGF-induced matrix proteolysis in 3D. Therefore, there is currently no effective basis to predict the net effect of EGF stimulation on cell migration in 3D environments, in terms of the effects this factor has on intrinsic cell locomotion behavior and its modulation by extrinsic matrix-associated properties.

To address this problem, we utilized confocal imaging to generate time-lapse movies of individual EGF-responsive human glioblastoma cells migrating in 3D matrices and quantitatively analyzed cell tracks to obtain migration parameters, including cell speed and directional persistence across different collagen densities and with varying degrees of protease inhibition. We establish that EGF stimulation leads to increase in overall motility in 3D matrices mediated by an increase in cell speed and a matrix density-dependent increase in apparent directional persistence. In contrast, parallel analysis of cell migration on 2D substrates shows that cell-intrinsic effects of EGF are increased cell speed but decreased directional persistence. Thus, the observed increase in apparent directional persistence in 3D is mediated by cell-extrinsic matrix proteolysis. Detailed biophysical analysis of cell tracks indicated that low matrix proteolysis leads to characteristically unproductive motility confined by matrix steric hindrance. Quantitative modulation of EGF-induced matrix proteolysis using an MMP inhibitor correlated directly with 3D directional persistence in high matrix concentrations. Therefore, the importance of EGF-induced matrix proteolysis for cell migration is highly dependent on matrix properties, where overall increase in cell migration is accomplished by cell speed in low-barrier matrix environments but by protease-mediated directional persistence in high-barrier matrix environments. Our results offer potential

implications for the efficacy of EGFR and MMP inhibitors in treatment of invasive cancers.

MATERIALS AND METHODS

Cell Culture and Stable Transduction of Enhanced Green Fluorescent Protein

U87MG human glioblastoma cells were originally obtained from Webster Caveness (Ludwig Institute for Cancer Research, San Diego, CA) and maintained in DMEM supplemented with 10% FBS. pML2-eGFP retroviral plasmid was used for retroviral packaging, infection, and subsequent FACS sorting of cells as described previously (Bear *et al.*, 2000). Morphology and migration properties of enhanced green fluorescent protein (eGFP)-expressing cells were similar to the parental cells for fewer than 15 passages (data not shown). All experiments were performed with cells with <10 passages.

Cell Culture on 2D Collagen-coated Surfaces and in 3D Collagen Matrices

For 2D migration assays, 24-well Visiplates (Perkin Elmer-Cetus, Waltham, MA) were coated with native bovine dermal type I collagen (Nutragen, Inamed Biomaterials, Fremont, CA) as described previously (Harms *et al.*, 2005). In short, dishes were acid-treated, incubated with varying concentrations of collagen for 2 h, washed with PBS, and blocked with 1% BSA for 1 h. After washing with PBS, GFP-expressing cells were seeded at 2000 cells/well and serum-starved overnight (>16 h) with serum-free DMEM supplemented with 0.5% BSA.

For 3D migration assays, cells were mixed with pH-neutralized collagen of varying concentrations at 200,000 cells/ml in serum-free media. The matrix-cell solution was placed on glass-bottom cell culture dishes (MatTek, Ashland, MA) and polymerized for 1 h at 37°C. The culture was immediately serum-starved overnight (>16 h).

For both 2D and 3D cultures, 8 h before imaging, cells were stimulated with 50 ng/ml human recombinant epidermal growth factor (EGF; Invitrogen, Carlsbad, CA). All experiments were performed with 0.1% DMSO as control for further inhibitor studies. For studies of matrix proteolysis inhibition, a broad inhibitor of matrix metalloproteinases, GM6001 (Biomol International, Plymouth Meeting, PA) was added simultaneously with EGF.

2D and 3D Time-Lapse Microscopy Assays

To generate time-lapse movies of cells migrating in 3D matrices, 80 confocal images were taken with 1 μ m spacing in z every 15 min for 10 h using a McBain spinning-disk confocal microscope (McBain Instruments, Simi Valley, CA) equipped with environmental control (37°C, 5% CO₂, humidity). The bottom of the field of view was consistently chosen to be 50 μ m above the glass. The final dimensions of the field of view were 870 $\times$ 660 $\times$ 80 μ m with 1.3 $\times$ 1.3 $\times$ 1- μ m voxels. For 2D migration assays, cells similarly imaged every 15 min for 10 h using fluorescence. The imaged field was 2200 $\times$ 1700 μ m with 1.6 $\times$ 1.6- μ m pixels. All movies with the slightest drifts in x, y, or z-direction as assessed by movement of stationary fluorescent objects were not included for further analysis.

Quantitative Analysis of Cell Migration Tracks

Imaris (Bitplane, Zurich, Switzerland) was used to visualize both the 2D and 3D time-lapse images. The spots function was used to calculate centroids of fluorescent objects and to generate migratory tracks as described previously (Zaman *et al.*, 2006; Harley *et al.*, 2008). All generated tracks were manually verified for accuracy and modified when computational mistakes were present. If a cell exhibits any movement more than its own cell length during the course of the experiment in the field of view, it was identified as motile. Motile cell fraction was calculated as the ratio of number of motile cells to total number of cells tracked. Cells undergoing division or death (as identified as release of fluorescence) were not included in the analysis. 2D and 3D Wind-Rose plots were generated by randomly choosing 30 tracks from the motile population and overlaying the starting coordinates at the origin of the plots in order to graphically represent average cell dispersion during migration. Only tracks longer than 3 h that migrated independently without physical contact with other cells were included in the calculation of cell speed and directional persistence.

Average individual cell speeds (S) were calculated from individual cell tracks by averaging the distances over the time interval. Mean squared displacements (MSD) at various time intervals (t) were calculated using the method of nonoverlapping intervals (Dickinson and Tranquillo, 1993) and directional persistence time (P) was obtained by fitting them to the persistent random walk model (PRW):

$$MSD = 2S^2P[t - P(1 - e^{-t/P})]$$

The random motility coefficient (RMC) for each cell was calculated as $RMC = S^2P$, which bears the units of the diffusion coefficient and quantifies cell dispersion from its starting point.

Confocal Reflection Microscopy

3D matrices were generated without cells by incubating pH-neutralized collagen at varying concentrations for 1 h at 37°C. Confocal reflection images were taken with a Zeiss LSM 510 scanning confocal microscope (Carl Zeiss Microimaging, Thornwood, NY).

Quantitative Measurements of MMP-1 Release and MT1-MMP Expression

Cells were seeded on 10 $\mu\text{g}/\text{cm}^2$ collagen-coated tissue culture dishes in serum-free media. After >16 h, cells were stimulated with 50 ng/ml EGF. For MMP-1 release assay, medium was collected after indicated times and was assayed with Human MMP-1 Fluorokine MAP kit (R&D Systems, Minneapolis, MN). For assaying membrane-bound MT1-MMP expression, membrane-bound proteins were biotinylated using EZ-Link Sulfo-NHS-LC-LC-Biotin (Pierce Biotechnology, Rockford, IL) as described previously (Wolf *et al.*, 2007). Cells were lysed and biotinylated proteins were precipitated using Neutravidin beads (Pierce Biotechnology). Precipitated lysates were subjected to standard Western blot analysis using an anti-MT1-MMP antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and the Odyssey detection system (Li-Cor, Lincoln, NE). Densitometry of bands was performed with the Odyssey software.

Rac1 Activity Measurements

Rac1-GTP levels were measured using the G-LISA Rac1 specific activation assay (Cytoskeleton, Denver, CO). For 2D samples, cells were seeded at 15,000 cells/ cm^2 on 10- $\mu\text{g}/\text{cm}^2$ collagen-coated tissue culture dishes in serum-free media. For 3D samples, cells were seeded at 500,000 cells/ml gel in serum-free media. After >16 h, cells were stimulated with 50 ng/ml EGF and lysed at indicated times. For 3D samples, gels were homogenized in presence of lysis buffer and lysates clarified. Manufacturer instructions were followed for the remainder of the assay protocol. To normalize for total Rac1 levels, we subjected lysates to a semiquantitative Western blot analysis using an anti-Rac1 antibody and an anti-GAPDH antibody (Millipore, Billerica, MA). Bands were detected and analyzed with the Odyssey detection system. Rac1-GTP levels were normalized internally and then to total Rac1 levels of the 2D serum-free levels.

FITC-Collagen Release Assay

Matrix degradation assays were performed with a quenched FITC-labeled DQ-collagen type I (Invitrogen) as described previously (Wolf *et al.*, 2007). In short, parental cells were seeded at 500,000 cells/ml pH-neutralized collagen (3.0 mg/ml) containing 5% (wt/vol) DQ-collagen in a 24-well plate. Cells were serum-starved >16 h and stimulated with 50 ng/ml EGF in presence or absence of GM6001. Matrices were collected after 12 and 24 h and centrifuged. Supernatant was collected, and released fluorescence was read with a SpectraMax M2e fluorescence plate reader (Molecular Devices, Sunnyvale, CA). All fluorescence values were normalized to no-cell control.

Rheometry of Collagen Gels

Elastic moduli of cell-free collagen gels were obtained using the AR2000 rheometer (TA Instruments, New Castle, DE). Collagen was pH-neutralized, placed in a 2.0-cm-diameter and 0.9-mm-height stainless steel washer on top of wetted parchment paper and polymerized for 1 h at 37°C. Washer was gently removed and placed on the rheometer for measurement of storage moduli (G') at 1 Hz and 0.87 μNm oscillatory torque using a 2-cm plate.

Statistical Analysis

All data are shown as mean $\pm$ SEM unless otherwise noted. All experiments had three to five biological replicates unless otherwise noted. For migration experiments, n = number of motile cells. Statistical significance depicted was assessed by two-way analysis of variance (ANOVA) of log-transformed data values to obtain normal distributions as tested by Kolmogorov-Smirnov test. p values represent significance across treatments. One-way ANOVA and two-tailed student's t tests of log-transformed data values were performed where appropriate (Figure 1F and see text). To test significance of deviation of a single data point to trends in Figure 6, B and C, one-way ANOVA was performed on data sets excluding the 1 μM GM6001 with and without the serum-free data point.

RESULTS

EGF-stimulated 3D Cell Migration Results from Increased Cell Speed and Matrix Concentration-dependent Increased Directional Persistence

EGF increases tumor cell invasiveness via enhancement of intrinsic cell motility machinery and induction of MMPs.

Because most previous in vitro studies were performed in 2D assays where matrix-degrading effects of MMPs are not applicable, the combined effects of these two factors on cell migration behavior have not yet been assessed. To quantify the effects of EGF stimulation on cell migration in 3D, we optimized a 3D culture system for cell tracking using highly invasive and EGF-responsive U87MG human glioblastoma cells seeded sparsely in type I collagen matrices. Our use of type I collagen provides a reasonably physiological model for at least some aspects of glioma cell migration: glioblastoma cells secrete high levels of their own extracellular matrix proteins in vivo and in vitro that are present both in normal brain and tumor microenvironment (Han and Daniel, 1995; Nakada *et al.*, 2007) and type I collagen is found in perivascular regions of the brain (Gladson, 1999) where glioblastoma invasion has been often noted (Giese *et al.*, 2003; Bellail *et al.*, 2004).

Because extracellular matrix concentrations influence cell migration by varying ligand density and matrix compliance (Palecek *et al.*, 1997; Ware *et al.*, 1998; Zaman *et al.*, 2006) and the effects of matrix proteolysis have been predicted to depend on these factors (Zaman *et al.*, 2007), cells were seeded in collagen concentrations from 2.0 to 4.0 mg/ml, which corresponded to a 20-fold increase of elastic modulus (Supplementary Figure S1). After serum starvation, cells were uniformly stimulated with saturating levels of EGF for 8 h, and the movements of cells expressing cytoplasmic eGFP were tracked for 10 h using 3D time-lapse confocal microscopy as described previously (Zaman *et al.*, 2006).

Reconstructed real-time movies (Figure 1A and Supplementary Movie S1) showed cells with a range of migratory phenotype; a significant number of cells (50–70%) failed to locomote productively despite actively forming protrusions. This is likely due to heterogeneity in the local matrix network, as well as intrinsic heterogeneity within the cell population. By analyzing the cell tracks of the motile population, we confirmed that EGF had a qualitatively stimulatory effect on U87MG cell migration over the course of the experiment (Figure 1B). As expected, the stimulatory effect was chemokinetic because EGF-induced migration failed to show preferential migration toward the top of the gel (Figure 1B) and addition of EGF elicited an almost immediate protrusion response of cells in the bottom of the matrix (data not shown). We posit that the EGF concentration perceived by these cells is dominated by the exogenous treatment as blocking of the EGF receptor by an inhibitory antibody did not change motility in serum-free media and because TGF- α release by U87MG cells was found to be negligible (data not shown).

EGF-induced chemokinetic migration exhibited significant increase in the population of motile cells across all measured matrix concentrations (Figure 1C). Moreover, the motile fraction showed a significant dependence on matrix concentration ($p = 0.0012$, two-way ANOVA across matrix concentrations). Motile cells were analyzed further for their overall cell dispersion by calculating random motility coefficients for each individual cell, a parameter analogous to the diffusion coefficient of a single molecule. As reflected in Wind-Rose plots (Figure 1B), cell dispersion was increased upon EGF treatment across all matrix concentrations (Figure 1D) and depended on matrix concentration ($p = 0.017$).

Chemokinetic cell movement has often been quantitatively characterized via its translational speed and directional persistence (Cukierman *et al.*, 2001; Kumar *et al.*, 2006). These migratory attributes have been previously shown to be regulated by distinct intracellular mechanisms (Pankov

Figure 1. 3D time-lapse microscopy reveals EGF-stimulation leads to increased 3D U87MG migration in collagen mediated by increase in cell speed and concentration-dependent increase in directional persistence. (A) Representative 3D image of eGFP-expressing U87MG cells seeded in varying concentrations of collagen matrix captured by 3D time-lapse confocal microscopy. Images were taken over 10 h with 15-min intervals and cells tracked using Imaris tracking software. Image dimensions: $870 \mu\text{m} \times 660 \mu\text{m} \times 80 \mu\text{m}$. (B) Representative 3D Wind-Rose plots depicting migratory tracks over 10 h of 30 random cells determined as motile in 3.0 mg/ml collagen matrix. Cells that moved more than one cell length were determined as motile. (C) Ratio of number of motile cells to total number of cells tracked in presence (red) or absence (black) of 50 ng/ml EGF. Cells that moved more than one cell length were determined as motile. (D) Cell dispersion quantified as random motility coefficient. Random motility coefficient of each cell is calculated from cell speed and persistence time of the persistent random walk model. (E) Cell speeds of cells determined as motile. (F) Directional persistence time fitted from the persistent random walk model. High persistence times indicate directed movement, whereas low persistence times indicate erratic movement according to the model. Supplementary Figure S2 shows box-and-whisker and scatter plots of individual cells. Data shown as mean $\pm$ SEM. $n = 3$ –5 biological experiments (C). $n = 18$ –105 motile cells (D–F) out of ~ 100 –250 total cells tracked. p values indicate statistics obtained from two-way ANOVA across treatment conditions.

et al., 2005). To determine the contributions of speed and persistence to overall increase in EGF-induced cell dispersion, we calculated average cell speed and applied the persistent random walk (PRW) model to cell tracks to obtain persistence time (Gail and Boone, 1970; Dunn, 1983; Dickinson and Tranquillo, 1993). Although cell speed describes the rate of translational locomotion, persistence time quantifies the extent of directionality changes over the course of locomotion. High persistence times indicate strongly directional movement and low persistence times are indicative of very random or erratic movement. In the presence of a homogeneous, isotropic migratory environment without physical barriers such as the 3D extracellular matrix or another cell, persistence times obtained from the PRW model may describe the cell's intrinsic inclination for randomness or directionality. However, cells in 3D extracellular matrices are obviously subjected to physical influences, such as steric ob-

structions. Keeping the model assumptions in mind, our purpose in employing the PRW model is merely to quantitatively describe persistence of cell tracks rather than to characterize intrinsic cell motility persistence (Harley *et al.*, 2008).

As expected, cell speed increased significantly ($\sim 33\%$) across all matrix concentrations upon EGF induction (Figure 1E); matrix concentration did not influence cell speed of motile cells ($p = 0.95$). Interestingly, directional persistence (Figure 1F) displayed a matrix concentration-specific response ($p = 0.006$) with a significant increase in response to EGF only at higher matrix concentrations ($p = 0.003$ and $p = 0.04$ for 3.0 and 4.0 mg/ml collagen, respectively) and not at low concentrations ($p = 0.61$ for 2.0 mg/ml collagen). Hence, the EGF-induced increase in cell dispersion is environment specific and is mediated by an increase only in cell speed at low matrix concentrations, but predominantly by an increase in directional persistence in high matrix concentrations.

Figure 2. EGF-induced barrier-free, 2D migration exhibits increased cell speed but decreased directional persistence. (A) Representative 2D Wind-Rose plots depicting migratory tracks over 10 h of 40 random cells determined as motile on $10 \mu\text{g}/\text{cm}^2$ collagen-coated 2D surfaces. (B–E) Migration parameters for 2D cell movement obtained from 2D time-lapse microscopy analysis of U87MG cells migrating on collagen-coated surfaces of varying densities in presence (red) or absence (black) of 50 ng/ml EGF. (B) Motile fraction, (C) random motility coefficients, (D) cell speeds, and (E) persistence times. Cells that moved more than one cell length were determined as motile. Parameters have been obtained analogously to Figure 1, C–E. Supplementary Figure S3 shows box-and-whisker and scatter plots of individual cells. Data shown as mean $\pm$ SEM. (B) $n = 3$ biological experiments. (C–E) $n = 83$ –118 motile cells. p values indicate statistics obtained from two-way ANOVA across treatment conditions. (F) Time course of Rac1-GTP levels of U87MG cells on collagen-coated 2D surfaces (gray) or in 3D collagen matrix (black) after stimulation with 50 ng/ml EGF using commercially available Rac-GTP ELISA normalized to total Rac1 levels measured by semiquantitative immunoblot. Data shown as mean $\pm$ SEM. $n = 2$ biological replicates.

Cell-intrinsic Response to EGF Treatment Is to Increase Cell Speed, But Decrease Directional Persistence

We hypothesized that the increase in cell speed and matrix density-dependent increase in directional persistence results from a combination of EGF-stimulated cell-intrinsic and matrix proteolytic effects. To determine the cell-intrinsic effects of EGF stimulation on cell speed and directional persistence in absence of matrix proteolysis, we performed a conventional 2D time-lapse microscopy assay to track migration of the same U87MG cells on collagen-coated surfaces in presence and absence of saturating levels of EGF. As expected, in absence of a much more viscous 3D surrounding, cells exhibited much greater motility and displacements (Figure 2, A and B). However, interestingly, 2D Wind-Rose plots showed that cell displacements were comparable and motile cell population were essentially unitary upon EGF treatment even across a 1000-fold variation of matrix coating density (Figure 2, A and B). Quantification of motile tracks showed that cell dispersion was not increased upon EGF stimulation (Figure 2C). Parsing cell tracks into cell speed and directional persistence demonstrated that EGF stimulation led to a slight, but significant increase in cell speed in U87MG cells on 2D collagen-coated surfaces, whereas a significant decrease in persistence was observed across most matrix densities (Figures 2, D and E). This phenomenon was observed previously in fibroblasts on 2D matrix-coated surfaces (Ware *et al.*, 1998) and was explained by the ability of homogeneous EGF stimulation to induce protrusions from all cell edges, which promotes a lower probability of forming a

stable asymmetry for continued directional movement while inducing a greater motility (Ware *et al.*, 1998). Therefore, the cell-intrinsic response to EGF stimulation is to increase cell speed and decrease directional persistence. Cell speed was increased by EGF stimulation both in 2D and 3D environments, suggesting that the EGF-induced cell intrinsic response holds true in 3D matrices. Interestingly, however, directional persistence was increased only in the 3D matrix environment and decreased in a 2D barrier-free environment.

Rac1 has been proposed as a candidate switch for persistence, and its activity was inversely correlated with persistence (Pankov *et al.*, 2005). We therefore asked whether Rac1 activity is differentially regulated in 2D and 3D environments and is responsible for the observed difference in EGF-stimulated directional persistence. We measured Rac1 activity of cells upon EGF stimulation in 2D and 3D environments to assess whether EGF stimulation had differential effects on cell-intrinsic regulators of persistence. It is well established that EGF transiently stimulates Rac1 activity with a return to baseline activity observed after ~ 30 min (Wang *et al.*, 2006; Yip *et al.*, 2007). Quantitative time courses of Rac1 activity displayed similar kinetics in both 2D and 3D environments with an early increase followed by a return to basal levels within an hour, although Rac1 activity was slightly lower in 3D environments (Figure 2F). Therefore, the 3D environment does not significantly alter Rac1 response to EGF stimulation, and absolute activity levels of Rac1 may not explain regulation of EGF-induced directional persistence. We infer that the cell-intrinsic response to EGF stimulation is decreased directional persis-

Figure 3. EGF stimulation increases MMP release and 3D matrix degradation. (A) MMP-1 release was measured using a quantitative immunoprecipitation bead-based MMP-1 detection assay. Cells were seeded on surfaces coated with 10 $\mu\text{g}/\text{cm}^2$ collagen and serum-starved. Media was collected at various times after changing to serum-free media (SF) or to media containing 50 ng/ml EGF. (B) Cell surface MT1-MMP expression was measured by semiquantitative Western blot for cell surface MT1-MMP. Cells were seeded on collagen I and serum-starved. Cell surface proteins were biotinylated, and cells were lysed at indicated times after changing media to serum-free or EGF-containing media. Cell surface proteins were precipitated with streptavidin beads and prepared for SDS-PAGE. (C) Bulk matrix degradation by U87MG cells in 3D collagen gels were measured using a FITC-collagen dequenching assay. Cells were embedded in a matrix consisting of 5% quenched FITC-labeled collagen and serum-starved. FITC-collagen released into media was quantified at indicated times after changing media to serum-free or EGF containing media. 1 μM GM6001 (GM), a broad matrix metalloproteinase inhibitor, was used as negative control.

tence, perhaps partially mediated by a temporal increase in Rac1 activity. The increase in persistence in 3D matrices likely arises from mechanisms independent from the cell intrinsic response, such as matrix proteolysis mediated by expression of proteinases.

EGF Stimulates MMP Expression and Matrix Proteolysis, Which Is Required for Cell Migration in 3D While Preserving Cell-intrinsic Migration Responses to EGF

Before further exploring the underlying mechanism for increased directional persistence at high matrix concentrations, we assessed EGF-induced MMP expression and matrix proteolysis in 3D. We confirmed that EGF stimulates U87MG cells to express MMP specific to the type I collagen used in this study (Figure 3, A and B). The earliest measurable differences were observed at 8 h for MMP-1 with large differences measurable within 24 h after stimulation. Thus, MMP up-regulation could underlie the observed effects on EGF-induced 3D migration, which were performed 8–18 h after stimulation.

We further confirmed the effects of MMP-1 and MT1-MMP up-regulation on matrix proteolysis via a fluorometric matrix proteolysis assay using a quenched FITC-labeled collagen (DQ-collagen) as previously described (Wolf *et al.*, 2003, 2007). Cells were embedded in matrices using 5% DQ-collagen in conditions similar to the migration assays. Although even serum-free cells exhibited considerable bulk

matrix proteolysis, EGF induced a significant increase in degradation by 24 h (Figure 3C).

To illustrate the importance of matrix proteolysis on EGF-induced 3D migration of U87MG cells in collagen, we measured 3D migration in presence of GM6001, a broad MMP inhibitor, which has also been shown to inhibit activity of urokinase plasminogen activator (uPA; Fukuda *et al.*, 2004). Treatment with 1 μM GM6001 was sufficient to significantly decrease matrix degradation below the baseline for serum-free levels (Figure 3C). This level of inhibition was sufficient to decrease 3D motility by 10-fold (Figure 4A), indicating that MMP activity is essential for U87MG migration in 3D collagen. In contrast, even 5 μM GM6001 was not sufficient to affect migratory behavior of U87MG cells on 2D substrates, because cell speed and directional persistence was not significantly changed in presence of GM6001 (Figure 4B). Inhibition of matrix proteolysis thus seems not to affect the cell-intrinsic responses to increase cell speed and decrease directional persistence upon EGF stimulation. These results support a model in which MMPs act as cell-extrinsic factors required for 3D migration.

Low Matrix Proteolysis Results in Cell Migration Characteristic of Matrix Confinement

To investigate the biophysical basis for EGF-induced matrix proteolysis as a cell-extrinsic factor in regulating direction-

Figure 4. Inhibition of MMPs does not affect 2D migration, but abrogates 3D migration. U87MG cells were seeded in 3.0 mg/ml collagen matrices (A) or on 2D surfaces (B) coated with 10 $\mu\text{g}/\text{cm}^2$ collagen were stimulated with 50 ng/ml EGF in presence or absence of 1 or 5 μM of a broad MMP inhibitor GM6001 for 3D and 2D experiments, respectively, for 8 h and then tracked for 10 h. (A) Cells that moved more than its cell length were determined as motile. $n = 3$ –5 biological experiments, $p = 0.002$. (B) 2D cell speed (left) and persistence (right) were calculated for motile cells. Data shown as mean $\pm$ SEM.

Figure 5. Concentration-dependent matrix-confined movement governs low persistent movement in unstimulated cells. (A) Confocal reflection micrographs of collagen matrices of varying concentrations. (B) Representative time-lapse images of an U87MG cell migrating with low persistence time in serum-free cells. White sphere and yellow line indicate the centroid of the cell and the migratory track, respectively. Dimensions of the box: $122 \times 112 \times 80 \mu\text{m}$. (C) Scatter plot for goodness of fit (R^2) of cell migration tracks to the persistent random walk model. Only cells migrating in untreated, serum-free (top, black) or in 50 ng/ml EGF treatment (bottom, red) with persistence (P) < 50 min were plotted. Lines indicate mean of the distribution and the numbers on right indicate the percentage of cells with $R^2 < 0.6$ (threshold chosen as poor fit).

ally persistent migration in 3D matrices, we examined the relationship between matrix properties and cell migration more closely. An obvious function of matrix proteolysis is to clear steric barriers for successful cell migration. Confocal reflection micrographs of matrices of varying concentrations confirmed that matrix pore size decreases as density of matrix fibers increases with increasing concentration (Figure 5A). Although cell speed was relatively constant across matrix concentrations (Figure 1E), the motile fraction, cell dispersion, and directional persistence all decreased with increasing matrix concentration, especially in serum-free conditions (Figure 1, C, D, and F). In addition, the increase in 3D persistence at higher matrix concentration was mainly a result of a sharp decrease in the persistence in serum-free condition, suggesting a role of matrix steric hindrance in regulating cell migration at low levels of matrix proteolysis. When examining the real-time cell tracks in detail, we found qualitatively that the untreated, high matrix concentration conditions contained an increased population of cells that displayed movement characteristic of matrix confinement. Figure 5B shows an example of such cell displaying back-and-forth movements within a confined space over the course of the experiment. This type of movement may be categorized as “erratic” and may display low persistence times, but does not display a true random walk for it is influenced by external confinement. Persistence time for each cell track is obtained from a nonlinear model fit (see Equation 1 in *Materials and Methods*) under the assumption that each cell track arises without external physical influence as mentioned above. Goodness of fit of the cell track data to this model therefore serves as a quantitative indication for cell migration under these assumptions. We calculated the

goodness of fit (R^2) of cells with low persistence times ($P < 50$ min) across all matrix concentrations in an attempt to quantify matrix-confined movement for low persistent cells. Randomly migrating cells in high matrix concentrations and serum-free condition exhibited poor fits to the persistent random walk model, where the fits became poorer with increasing matrix concentration (Figure 5C). When arbitrarily defining a threshold for poor fit at $R^2 < 0.6$, 40–46% of these cells showed poor fit to the PRW model, whereas only 9–20% of randomly migrating cells in EGF showed a poor fit. These analyses suggest that low persistence under the two stimulation conditions are distinct in nature, with serum-free cells exhibiting migratory paths indicative of movement sterically hindered by the 3D matrix. Further, we conclude that EGF-induced increase in 3D directional persistence mediated by cell-extrinsic proteolysis arises from the ability to overcome steric hindrances posed by the 3D matrix.

3D Directional Persistence Correlates with Matrix Degradation Activity

To establish direct correlation between directional persistence and matrix degradation, we set out to modulate matrix degradation activity with GM6001 quantitatively. Via the matrix degradation assay described above, the degree of bulk matrix degradation was measured in presence of EGF and varying levels of GM6001. The decrease in bulk matrix degradation correlated well with increasing levels of GM6001 (Figure 6A; $R^2 = 0.96$ for linear fit). Matrix degradation measured in serum-free condition corresponded to levels between 0.01 and 0.1 μM GM6001.

Figure 6. Modulation of MMP activity results in correlated modulation of 3D directional persistence. (A) Bulk matrix degradation of U87MG cells was quantified in presence of varying concentrations of GM6001 and 50 ng/ml EGF as described above. Horizontal dashed lines indicate mean values of EGF control and serum-free control from Figure 4C. $R^2 = 0.96$ for logarithmic fit. (B–D) Motile fraction, cell speed, and persistence times of cells migrating in 3.0 mg/ml collagen matrices in presence of various concentrations of GM6001 and 50 ng/ml EGF. Migration data were plotted against FITC-collagen release values determined in A. Data point in gray for B and C indicates migration data in serum-free condition at 3.0 mg/ml matrix concentration from Figure 2. Data shown as mean $\pm$ SEM. $n = 3$ biological samples for A, $n = 3$ –5 biological experiments for B, and $n = 7$ –64 motile cells for C and D. $R^2 = 0.92$ for linear fit in D. Supplementary Figure S3 shows box-and-whisker and scatter plots for single cell migration parameters.

3D migration experiments were then performed using varying levels of GM6001, and motile fraction, cell speed, and persistence times were deduced as described above. Instead of plotting parameters against inhibitor levels, we plotted them against the experimentally determined matrix degradation activity in presence of GM6001 to illustrate more directly the effects of matrix degradation on 3D cell migration (Figure 6, B–D). Motile cell fraction and cell speeds displayed similar trends: both parameters were low at the lowest matrix degradation measured, but assumed maximum values once a threshold of matrix degradation was reached (Figure 6, B and C; $p = 0.13$ and $p = 0.32$, respectively, from one-way ANOVA of data points, $<1 \mu$ M GM6001). Serum-free conditions (gray data points) for both motile fraction and cell speed were close to the EGF-induced data points (black data points), but deviated significantly from the trend ($p = 0.0008$ and $p = 0.044$, respectively; see *Materials and Methods* for statistics). A better correlation to matrix degradation was observed with directional persistence for all data points including the serum-free condition

(Figure 6D, $R^2 = 0.92$). Small modulation of matrix degradation at 0.001 and 0.01 μ M GM6001 affected directional persistence significantly, indicating the importance of matrix proteolysis in generating directional persistence. Scatter plot of goodness of fit (R^2) to the PRW model in presence of the inhibitors show a correlation between poor fit and matrix degradation, where the number of cells exhibiting poor fit increases with increasing GM6001 levels (Supplementary Figure S4). These results indicate that EGF-induced matrix proteolysis regulates directional persistence in 3D matrices, particularly in high matrix concentrations. Once a threshold for matrix proteolysis is reached, further changes did not modulate cell speed significantly as shown in Figures 1E and 6C, suggesting that matrix proteolysis does not have a significant effect on the cell-intrinsic increase in cell speed of cells described as motile. These results and the inability of EGF to enhance directional persistence at low matrix concentrations (Figure 1E) suggest that the relative contribution of EGF-induced cell speed and directional persistence

to overall cell dispersion that is mediated by proteolysis is highly dependent on matrix properties.

DISCUSSION

Tumor invasion involves complex interactions between intrinsic cell motility behavior and extrinsic factors in the extracellular matrix environment. In many tumor types, cell motility and invasion behavior are strongly influenced by EGF family ligands (Wells, 1999, 2002), and multiple processes involved in cell locomotion (Lauffenburger and Horwitz, 1996; Ridley *et al.*, 2003) are regulated by EGFR activation, including cell-intrinsic biophysical mechanisms (Maheshwari *et al.*, 1999) as well as proteolytic effects on cell-extrinsic matrix properties (Ellerbroek *et al.*, 1998; Roo-prai *et al.*, 2000; Alper *et al.*, 2001). Because these various processes are regulated by diverse sets of molecular components and pathways (Wells, 2000), it is important to parse the relative contributions of EGF-induced effects on intrinsic cell behavior versus behavior governed by EGF-induced extrinsic matrix modulation.

To obtain cell speed and directional persistence of individual migrating cells driven by EGF-induced motility and matrix proteolysis, we generated real-time 3D movies of glioblastoma cell migration sparsely embedded in a 3D matrix. Detailed analysis of the cell tracks and the application of the persistent random walk model led us to conclude that the stimulatory effects of EGF arose from a concomitant increase in motility, cell speed, and matrix concentration-dependent directional persistence (Figure 1, C–F). Interestingly, translocation speed of moving cells was not significantly influenced by matrix density, whereas the overall motility was substantively affected because fraction of motile cells decreased with increasing matrix density. Although cell speed increased at all matrix concentrations assessed, EGF enhanced directional persistence only at high matrix concentrations. Therefore, although cell speed was the sole contributor of EGF-induced cell dispersion at low matrix concentrations, directional persistence was the predominant contributor at high matrix concentrations. Analogous real-time 2D movies demonstrated that these cells exhibit increased cell speed but decreased directional persistence upon EGF stimulation (Figure 2, B–E), similar to the effect of EGF on fibroblasts (Ware *et al.*, 1998). The modest magnitude of these 2D effects are likely due to a background platelet-derived growth factor autocrine loop present in U87MG cells (Ma *et al.*, 2005). The crucial finding, nevertheless, is that EGF yields a qualitatively opposite effect on persistence in 3D versus 2D.

2D cell migration does not require MMP activity and matrix proteolysis; cell migration was unaffected upon treatment with an MMP inhibitor (Figure 4), whereas 3D cell migration is dependent on matrix proteolysis. Therefore, we hypothesized that, although cell speed is increased as part of the cell-intrinsic, proteolysis-independent programming in both environments, the observed dimensionality-dependent regulation of directional persistence arises from extrinsic sources; when embedded in a 3D proteolysis-dependent environment, EGF induces an integrated cell-intrinsic (to lower persistence) and cell-extrinsic response (to enhance persistence), where the cell-extrinsic response dominates. This idea was supported by highly matrix concentration-dependent cell dispersion and directional persistence in unstimulated conditions (Figures 1, D and F, and 5A). Further detailed analysis of unstimulated cell migration indicates that low persistent movement is governed by matrix confinement (Figure 5), supporting that the cell-extrinsic response arises from EGF-induced matrix proteolysis, whose

role is to clear extracellular steric barriers to enhance cell migration. Quantitative modulation of matrix proteolysis using a broad MMP inhibitor demonstrated a high correlation between matrix proteolysis and directional persistence (Figure 6), confirming that the cell-extrinsic increase in directional persistence is modulated by matrix proteolysis. Translocational speed of motile cells, however, was not affected by modulation of matrix proteolysis once a proteolysis threshold was reached, consistent with the matrix concentration-independent cell speed response measured in Figure 1E. Taken together, we conclude that matrix proteolysis governs directional persistence in high matrix concentration and that overall contribution of matrix proteolysis to cell dispersion mediated through directional persistence, and not cell speed, is dependent on matrix concentration.

Interestingly, neither 2D nor 3D cell speed exhibited a significant dependence on matrix properties despite a variation of 1000-fold ligand density or 20-fold matrix stiffness (Supplementary Figure S1). Such variation has been demonstrated to strongly influence epithelial and fibroblast-like cell migration (Palecek *et al.*, 1997; Gup-ton and Waterman-Storer, 2006; Zaman *et al.*, 2006). We hypothesize here that U87MG cells may not follow such migratory patterns, or more likely, that the matrix property variations were not sufficient to display a change in cell speed. Nevertheless, independence of cell speed on matrix ligand density or stiffness facilitated our ability to parse the effects of matrix steric hindrance and proteolytic degradation on directional persistence.

We focused our study on a human glioblastoma line due to its highly invasive and migratory properties in *in vitro* collagen matrices and physiological responsiveness to EGF; amplification and overexpression of EGFR are observed in 50% of glioblastoma patients and correlate with poor prognosis (Shinojima *et al.*, 2003). Surgical resection of high-grade glioblastomas is ineffective as they display a diffuse, single-cell invasion pattern, which was modeled by our *in vitro* migration assay. Our assays illustrated that MMP activity is critically important for invasion, consistent with the prominent portrayal of MMPs in the literature as therapeutic targets for glioblastoma treatment. Furthermore, parsing of cell-intrinsic and proteolysis-mediated cell-extrinsic effects may be facilitated by our results, suggesting that U87MG cells do not appear to undergo mesenchymal-amoeboid transition in presence of high levels of MMP inhibitors as described previously in fibrosarcomas and breast carcinomas (Wolf *et al.*, 2003). This result, however, could be due to higher matrix densities and smaller pore sizes used in our study here compared with previous work. Although the bulk matrix elastic moduli involved in these studies were comparable to those of the brain (Georges *et al.*, 2006), the native brain environment lacks high concentrations of fibrillar collagens (Nakada *et al.*, 2007). Nonetheless, the glioblastoma invasion environment, including the perivascular space, is reported to contain laminin, fibronectin, and collagens (Gladson, 1999), much of which is secreted by the tumor cells themselves. In our experiments, U87MG cells were seeded in the collagen matrix for a total of 24 h before imaging, making it likely that the cells had conditioned their environment by secreting matrix proteins. In addition, glioblastomas are reported to express $\alpha_2\beta_1$, fibrillar collagen binding integrins, and the β_1 subunit plays an important role in glioblastoma biology (Paulus *et al.*, 1996). U87MG cells also expressed collagen I specific proteinases (Figure 3, A and B) and MMP-2 (data not shown).

The scope of this study was to identify the net effect of EGF stimulation in regulating motility and proteolysis-me-

diated migratory behavior. Thus, questions, such as what the detailed molecular mechanism of the signaling pathways downstream of EGF-inducing protease release are, as well as how this cell-intrinsic and extrinsic regulation of persistence convoluted to give rise to an increase in persistence, were left as interesting avenues for future research. Many downstream components of the EGF receptor have been shown to induce expression and activation of MMPs and other proteinases while activating the intrinsic motility machinery. They include downstream effectors such as PI3K, Rac1, and MAPK, and transcription factors like AP-1 (Zhuge and Xu, 2001; Mamoune *et al.*, 2004; Van Meter *et al.*, 2004; Kojanne *et al.*, 2007). High spatial and temporal resolution imaging studies of cell protrusion and matrix degradation in presence of molecular manipulations could be powerful in parsing the mechanisms of directional persistence regulation. Most interestingly, it has been suggested that activated MMP-1 acts on collagen fibrils by moving along the fiber in a biased diffusion while acting as a molecular ratchet tethered to the cell surface (Saffarian *et al.*, 2004). This directed proteolysis of molecular scale might significantly contribute to polarized cell protrusions in presence of the 3D extracellular matrix, effectively governing 3D migration persistence. EGF-induced increase in MMP-1 release could intensify this effect, which contributes to the increase in directional persistence in high matrix concentration environment.

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