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Corrigendum

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Two-photon laser-generated microtracks in 3D collagen lattices: principles of MMP-dependent and -independent collective cancer cell invasion

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Abstract

Cancer invasion into an extracellular matrix (ECM) results from a biophysical reciprocal interplay between the expanding cancer lesion and tissue barriers imposed by the adjacent microenvironment. *In vivo*, connective tissue provides both densely packed ECM barriers adjacent to channel/track-like spaces and loosely organized zones, both of which may impact cancer invasion mode and efficiency; however little is known about how three-dimensional (3D) spaces and aligned tracks present in interstitial tissue guide cell invasion. We here describe a two-photon laser ablation procedure to generate 3D microtracks in dense 3D collagen matrices that support and guide collective cancer cell invasion. Whereas collective invasion of mammary tumor (MMT) breast cancer cells into randomly organized collagen networks required matrix metalloproteinase (MMP) activity for cell-derived collagen breakdown, re-alignment and track generation, preformed tracks supported MMP-independent collective invasion down to a track caliber of 3 μm. Besides contact guidance along the track of least resistance and initial cell deformation (squeezing), MMP-independent collective cell strands led to secondary track expansion by a pushing mechanism. Thus, two-photon laser ablation is useful to generate barrier-free microtracks in a 3D ECM which guide collective invasion independently of pericellular proteolysis.

Introduction

Cell migration *in vivo* is a complex process defined by determinants of both the cell and the tissue environment on or through which the cell migrates. In interstitial migration through a 3D extracellular matrix (ECM), important physical determinants of cell movement include the degree of ECM alignment, the width of pores present therein, and the ECM stiffness (Erler and Weaver 2009, Wolf *et al* 2009, Friedl and Wolf 2010, Egeblad *et al* 2010b). Thereby, the ECM provides both a physical scaffold for cell adhesion and migratory guidance but also a physical barrier which, if sufficiently dense, hinders or prevents cell movement (Wolf *et al* 2007, Hotary *et al* 2003).

An *in vivo* interstitial ECM is heterogeneous in composition, density and organization. It comprises regions of high density with tightly packed collagen fibers and loose regions composed of random collagen fibers resulting in variable gap diameters and clefts (Wolf *et al* 2009, Provenzano *et al* 2008b, Condeelis and Segall 2003). Both the density

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and degree of alignment of a 3D ECM impact the molecular mechanisms of cell migration, particularly the engagement of integrins and the requirement for pericellular proteolysis (Ruiter *et al* 2002, Friedl and Wolf 2010). The dimensions of ECM gaps and pores determine the physical space available for moving individual cells or cell groups (Friedl and Wolf 2010), as well as the speed and persistence of migration (Ghajar *et al* 2008). *In vivo* loose and randomly organized connective tissue favors single-cell dissemination of amoeboid breast cancer cells (Farina *et al* 1998, Pinner and Sahai 2008, Wyckoff *et al* 2006); conversely regions of highly aligned collagen bundles and myofibers in the deep mouse dermis support cell collective invasion in fibrosarcoma xenografts (Alexander *et al* 2008). *In vitro*, collective invasion into dense collagen lattices requires localized cell-mediated pericellular proteolysis and ECM realignment resulting in elongated gaps and tracks of least resistance (Wolf *et al* 2007). The major cell surface collagenase controlling type I collagen degradation is MT1-MMP/MMP14 (Sabeh *et al* 2004), and consequently membrane-type-1 MMP (MT1-MMP) activity is rate limiting for collective invasion into 3D collagen lattices *in vitro* (Wolf *et al* 2007). However, when and under which ECM conditions pericellular proteolysis is required for cancer cell invasion is controversial, based on divergent results from different labs using different collagen matrix substrates *in vitro* (Hotary *et al* 2003, Provenzano *et al* 2008b, Wolf *et al* 2003) and *in vivo* (Wyckoff *et al* 2006). Thus, the physical determinants of the ECM structure to either support, mechanically impede or arrest cell migration *in vitro* and *in vivo* remain to be determined.

Different *in vitro* collagen-based cell invasion models consisting of 3D collagen lattices after re-assembly of monomeric collagen mimic the structure of connective tissue *in vivo* to varying degree (Wolf *et al* 2009). In most cases, *in vitro* reconstituted fibrillar collagen provides porosity as well as barrier function to moving cells, similar to loose connective tissue *in vivo*, but they lack well-controlled spatial characteristics because smaller and larger pores result from a stochastic collagen fiber polymerization process which fails to recreate defined *in vivo*-like trails (Provenzano *et al* 2008a, Wolf *et al* 2009, Egeblad *et al* 2010a). Consequently, to create spatially defined, non-random models for cell migration through collagen-based 3D environments, random fibrillar matrix polymerization needs to be combined with procedures to generate predefined tracks that mimic particular patterns of connective tissue organization.

We here show how two-photon excited laser microsurgery can be used to generate physical tracks in 3D collagen matrices of defined width and length. The resulting micro- and macropatterned lattices were used to address the conditions for MMP-dependent and -independent collective migration of invasive cancer cells.

Materials and methods

Cell culture and reagents

Dual-color mouse mammary tumor (MMT) cells that express cytoplasmic DsRed2 and nuclear EGFP coupled to histone 2B (Tsuji *et al* 2006) were maintained in RPMI 1640 growth medium (Invitrogen) supplemented with 10% FCS (Sigma), penicillin and streptomycin (each 50 U ml^{-1}) (PromoCell) in a humidified incubator (37 °C, 5% CO_2). The function of MMPs was inhibited with the broad-spectrum inhibitor GM6001 (final concentration 20 μM) (Chemicon). Pericellular collagen degradation was visualized using polyclonal rabbit IgG directed against a collagen cleavage-specific peptide CGP(OH)GPQG (ImmunoGlobe, Germany), using an established neoepitope staining protocol (Wolf *et al* 2007).

3D spheroid culture

Cells from subconfluent cultures were detached with 2 mM EDTA, and multicellular spheroids were generated using the hanging-drop method (Korff and Augustin 1998). In brief, cells were re-suspended in RPMI 1640 growth medium supplemented with 10% methylcellulose, and incubated in 25 μl droplets overnight. After cell aggregation, spheroids were washed in PBS and incorporated into 3D type I collagen lattices consisting of non-pepsinized rat-tail collagen (final concentration: 6 mg ml^{-1}; BD Biosciences) the porosity of which approximates that of dense ECM regions of an *in vivo* interstitial matrix (Wolf *et al* 2009).

Two-photon laser microsurgery

Generation of microtracks ablated from fibrillar collagen within 3D collagen lattices was performed using two-photon excitation at 830 nm with a Ti:sapphire laser (XR, Coherent) on an upright microscope stage (LaVision BioTec) using a 20× NA 0.95 water-immersion objective (Olympus) and a focal power of 400 mW. The regions of interest of 150 μm in length and variable widths and depths were positioned directly adjacent to the edge of multicellular spheroids before the onset of invasion. Following microtrack generation spheroid-containing lattices were maintained at 37 °C and monitored for growth and invasion activity for up to 48 h. At the end-point, cultures were fixed (4% paraformaldehyde, room temperature, 30 min) and reconstructed by confocal microscopy (Olympus FV100), using confocal reflection for visualizing collagen fibers.

Time-lapse microscopy and image analysis

Migration of MMT cells into collagen-ablated microtracks was monitored for 48 h using time-lapse two-photon microscopy. Images were acquired every 15 min at a 830 nm excitation wavelength allowing the simultaneous visualization of DsRed2, EGFP and second harmonic generation (SHG), as described in Friedl *et al* (2007). Z-stacks were obtained with a step-size of 5 μm. Image processing and 3D reconstruction were obtained using the ImageJ (1.40v; W. Rasband, NIH) or ImSpector 3.4 (LaVision BioTec) software.

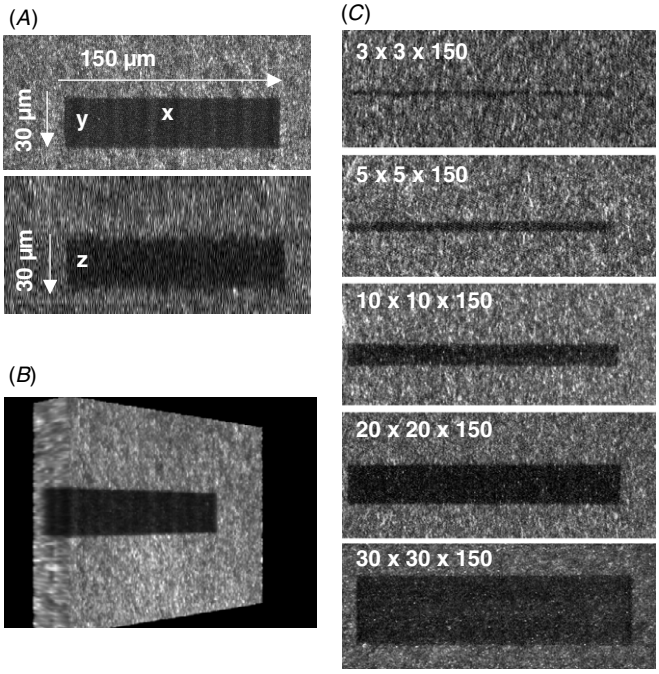

Figure 1. Two-photon laser generation of collagen-ablated microtracks within a 3D matrix. (*A*) *Xy* and *xz* view of a two-photon laser-generated microtrack showing a collagen-cleared lumen. (*B*) 3D reconstruction of a laser-generated microtrack. (*C*) Collagen-ablated microtracks of different calibers. The collagen structure was visualized by confocal reflection microscopy.

Results

Two-photon laser microsurgery to generate microtracks in 3D collagen lattices

To establish an *in vitro* model for naturally occurring clefts and tracks forming trails of least resistance, similar to connective tissue patterns *in vivo* (Wolf *et al* 2009), microtracks within 3D collagen matrices were generated by two-photon laser ablation and used for studying cell migration along barrier-free interfaces. Regions of interest were defined and ablated using a laser power density of 400 mW, resulting in rectangular 3D tracks devoid of collagen structures (figures 1(*A*) and (*B*)). Scanning at higher laser intensities resulted in cell damage and poorly defined edges of the microtracks, consistent with out-of focus energy absorption and overshoot-vaporization. Lower laser power was not sufficient to reliably ablate the collagen fibers and form defined microchannels (data not shown). To vary microtrack geometry, channels were created with constant length (150 μm) but varying widths and depths (3 to 30 μm in both *x* and *z* directions) (figure 1(*C*)). In each case, the channels showed precise medium-collagen interfaces and were largely free of debris inside the lumen (figure 1(*B*)), thus representing a defined model for 3D tracks within a physiological substrate as a border to support cell adhesion and guided migration.

Collective cancer cell migration into collagen-ablated microtracks

The physical structure of an ECM has been shown to influence greatly the efficiency of cancer cell invasion (Harley *et al* 2008) and requirements for pericellular proteolysis (Gaggioli *et al* 2007, Wolf *et al* 2007, Friedl and Wolf 2010). Using dense fibrillar collagen as a physiological substrate, we tested whether collective cell migration into collagen-ablated microtracks requires MMP-dependent pericellular proteolysis. Multicellular spheroids of invasive MMT cells expressing cytoplasmic DsRed2 and nuclear H2B/EGFP (Tsuji *et al* 2006) were embedded within 3D collagen lattices, and subsequently two-photon excited ablation of tracks was performed adjacent to the spheroid–collagen matrix interface (figure 2(*A*)).

MMT cells invaded spontaneously as multicellular strands into the collagen matrix along preformed microtracks (figure 2(*A*), numbers) and further into secondary invasion zones *de novo* generated by the cells themselves (figure 2(*A*), arrowheads). Secondary invasion (figure 2(*A*), white arrowheads) as well as invasion from spheroids into non-dissected collagen (figure 2(*B*)) was accompanied by MMP-dependent pericellular collagen degradation which was detected by collagen degradation neoepitope staining (Col $\frac{3}{4}$) (figure 3), similar to invasive HT-1080 fibrosarcoma cells (Wolf *et al* 2007, Friedl and Wolf 2008). Consistent with MMP-dependent invasion, both collagen degradation and invasion activity were ablated in track-free collagen lattices by the broad-spectrum MMP inhibitor GM6001, whereas spheroid growth remained undiminished (figures 2(*B*), (*C*) and 3). Thus, MMT cells require MMP function for collective penetration into a dense 3D collagen matrix, as described in Friedl and Wolf (2008).

In contrast to non-dissected collagen scaffolds, when microtracks with a track caliber exceeding the single-cell diameter (15–20 μm) were juxtaposed to spheroids, MMT cells maintained collective migration capability despite the presence of GM6001 (figure 2(*B*)). The first tip-like cell migrated individually into the track yet maintained rearward cell–cell interaction with neighbor cells, which subsequently followed while maintaining front–rear as well as lateral cell–cell junctions by moving along the track edge until its lumen was filled completely (figure 2(*D*)). Notably, after microtracks were filled, MMT cells failed to form secondary *de novo* tracks in the presence of GM6001, suggesting that collective invasion into a randomly structured, dense collagen matrix but not barrier-free microtracks requires proteolytic ECM degradation.

Physical events during MMP-independent migration into narrow microtracks

In vivo invading cancer cells are confronted with the ECM of widely varying patterns and pore sizes (Provenzano *et al* 2008a, Wolf *et al* 2009, Egeblad *et al* 2010a). To test the minimum track dimensions permissive for MMP-independent collective invasion, the dimensions of microtracks were reduced to the technical limit of laser-dissection using a single-line scan. The resulting tracks measured approximately 3 × 3 μm (figure 4(*A*)) which exceeded the lateral point-spread function of the two-photon set-up by factor 4 and the vertical by factor 2 (Andresen *et al* 2009).

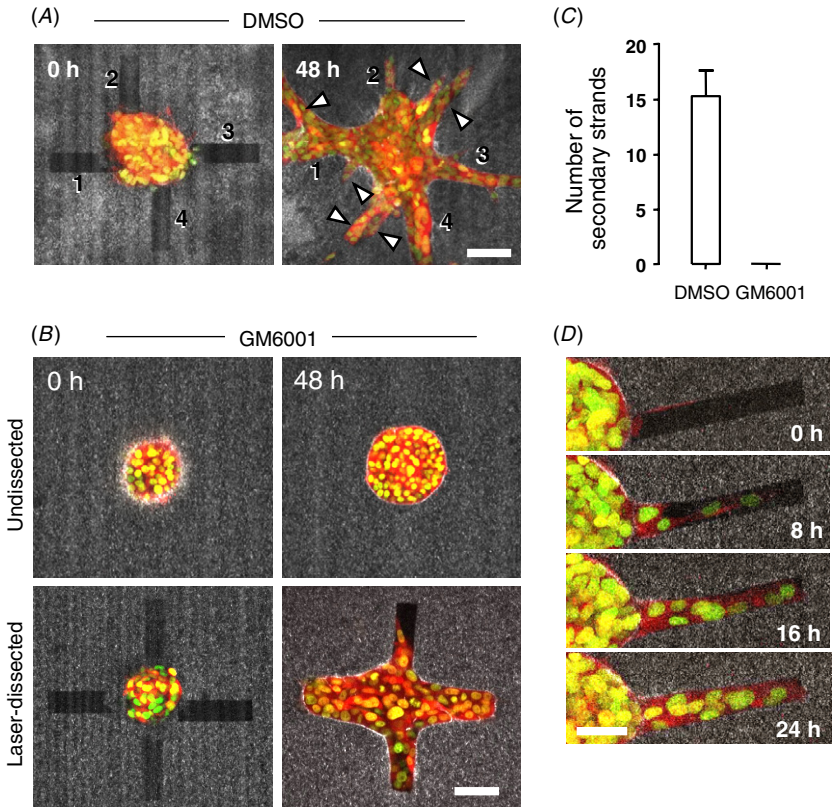

Figure 2. Protease-independent collective migration of MMT cells into collagen-ablated microtracks. (*A*) DMSO-treated control MMT cells develop multiple collective strands invading a native collagen matrix both along laser-ablated microtracks (numbers) and secondary invasion strands into regions of previously unmodified collagen (white arrowheads). (*B*) Blocking of MMP function with a hydroxamate inhibitor GM6001 abrogates invasion of MMT cells into a non-dissected collagen matrix but not into laser-dissected microtracks. Cell proliferation was not affected by GM6001. (*C*) Quantification of secondary invasion strands in DMSO-treated control samples compared to cultures treated with GM6001. (*D*) Time course of MMP-independent collective migration into a laser-ablated microtrack (GM6001-treated culture). Scale bars 20 μm.

Despite the average cell diameter of 15 to 20 μm and the presence of GM6001, MMT cells migrated collectively into all tested microtracks whereby the dimensions of the microtracks determined whether cells migrated in parallel or were organized in single-cell chains (figures 4(*A*) and (*B*)). After initial immigration, collagen-ablated microtracks were laterally enlarged by invading cell strands, so that resulting tracks eventually matched the diameter of single cells or multicellular strands (figures 4(*A*) and (*B*)). In microtracks that were smaller than the mean nuclear diameter (5–10 μm), the morphology of cells and nuclei adopted a cigar-like shape and cells arranged as chain-like files within 6 h after the invasion onset, consistent with substantial cell deformation and squeezing (figures 4(*A*), insets; (*C*)). In a second phase following initial deformation, the nuclear shape reverted back to roundish morphology (figure 4(*C*)), concomitant to microtrack widening (figures 4(*B*) and (*C*)). Both initial cell deformation and secondary shape reversion with track widening were independent of pericellular collagen degradation in GM6001-treated cultures (figures 3(*A*) and (*B*)), consistent with a physical outward compression that did not require structural collagen remodeling. In conclusion, collective invasion is an adaptive multi-parameter process, the protease requirements of which depend upon the geometry and deformability of both the cell body and the encountered track of least resistance. Thereby, the widening of ECM tracks to accommodate the cell shape during migration occurs by proteolytic remodeling as well as physical outward pushing.

Discussion

Two-photon laser dissection is suited to generate micro- or macro-patterned matrices of defined dimensions to study matrix-guided mechanisms of cell invasion in a physiological substrate, here 3D fibrillar collagen. Established 3D collagen-based models to study mechanisms cell invasion *in vitro* provide cells with a physiological substrate for attachment and migration and recapitulate randomly or partially aligned matrix organization (Wolf *et al* 2009), yet fail to recapitulate aligned clefts and gaps of different calibers present in many connective tissues (Provenzano *et al* 2008a, Alexander *et al* 2008, Friedl and Wolf 2009, Egeblad *et al* 2010b). Substantial recent progress has provided micropatterned surfaces to study directed cell migration along gradients of biomolecules (DeLong *et al* 2005, Lee *et al* 2008, Luo and Shoichet 2004), pre-defined microtracks (Falconnet *et al* 2006, Sarig-Nadir

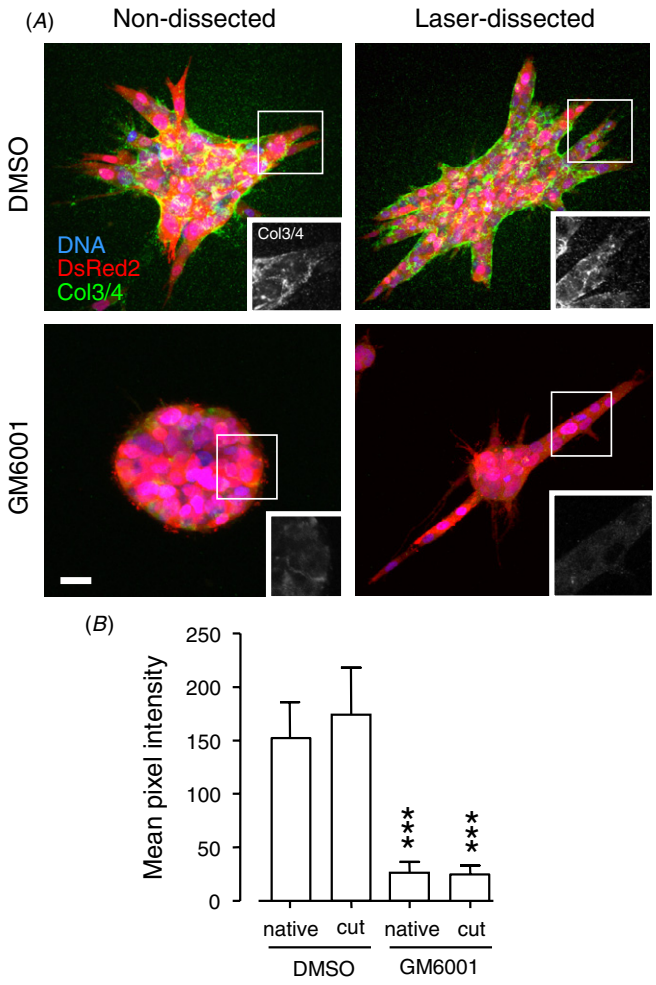

Figure 3. Invasion of MMT cells into a native or a laser-dissected collagen matrix involves pericellular proteolysis. (*A*) Abrogation of pericellular proteolysis of MMT cells in a laser-dissected or an intact collagen matrix after inhibiting MMP activity with GM6001, compared with DMSO-treated control cultures. Scale bar, 20 μm. (*B*) Quantification of collagen neoepitope staining in GM6001-treated and control samples. The remaining cell-associated fluorescence pattern and intensity in GM6001-treated spheroids corresponds to the non-specific fluorescence signal obtained by an isotypic and secondary antibody alone (not shown). Data represent the mean intensity $\pm$ SD of ten pericellular regions after correction for a background signal from cell-free collagen from three independent experiments. Asterisks, $p < 0.0001$, unpaired two-tailed Mann–Whitney test.

et al 2009), or synthetic 3D biopolymers, i.e. polyethylene glycol (PEG) hydrogels as a substrate (Bott *et al* 2010, Kloxin *et al* 2010). Although these studies demonstrate spatial control of cell migration and implicate ECM structures in guiding cell directionality, but they either poorly recapitulate the geometry of tracks and trails that surround the cells three dimensionally, or they represent an artificial substrate with physicochemical properties that differ from native tissue components. Micropatterned collagen gels using microscale cavities (Nelson *et al* 2008, Raghavan *et al* 2010) are suited to construct 3D space with defined geometry into which cells are positioned; yet they do not generate cell-free tracks inside the matrix that the cells can use for individual and/or collective migration. Photosensitive PEG-based hydrogels, which are used for controlled generation of microtracks, provide a 3D scaffold for monitoring cell guidance; however they lack physiological hydrostatic and ligand properties as well as MT1-MMP sensitivity present in native collagens (Kloxin *et al* 2010).

The here developed two-photon laser ablation of fibrillar type I collagen, which is a physiological *in vivo*-like substrate for cell attachment and collagenolytic degradation, is suited to position collagen-free tracks of defined geometry directly adjacent to cells and cell spheroids to be readily used for cell migration and secondary remodeling. The excitation wavelength of 830 nm at 400 mW focal power using a 20 $\times$ 0.95 NA objective was required to evaporate collagen fibers precisely with minimum of remaining debris down to a smallest caliber 3 μm in lateral and vertical directions. As a drawback of two-photon laser ablation, the resulting tracks exceed the technical resolution of the system ($\sim$500 nm lateral and 1.5 μm vertical resolution) which indicates heat-induced destruction of collagen fibers beyond the focal volume by factors 2 to 4. To further reduce the track caliber, an objective lens with a higher numerical aperture combined with an excitation wavelength in the visible range and/or altered laser pulse duration might be useful. The laser ablation approach may be amenable to other 3D fibrillar tissue scaffolds, such as fibrin or PEG lattices and further may be used to generate more complex track structures of varying width, direction and bifurcations for studying tissue-guided biophysics of cell migration.

Several secreted MMPs, including MMP-1, MMP-3 and MMP-13, are effective collagenases; however for invasive cell migration through native fibrillar collagen, MT1-MMP is the rate-limiting effector, the inhibition of which is not compensated by other proteases (Sabeh *et al* 2004, Wolf *et al* 2007). MT1-MMP has a key function in the generation of micro- and macrotracks generated by cancer cells themselves (Wolf *et al* 2007) or stromal cells (Gaggioli *et al* 2007), and the tunnel-like trails are permissive for the formation of collective invasion strands (Friedl and Wolf 2008). Consistent with MMP-dependent collagen remodeling, spontaneous invasion of MMT cells into dense 3D collagen matrices with laser-generated tracks consisted of two processes, (i) the migration into the preformed trails until they were completely filled, and (ii) the secondary penetration of a matrix within cell-derived tracks. Whereas cell-derived track generation was abrogated by broad-spectrum MMP inhibition, migration into preformed microtracks persisted, which provides direct evidence that collective invasion is dependent on sufficient space available in the tissue matrix to accommodate the volume of the cell strand (Friedl and Wolf 2008). *In vivo*, collagen-rich interstitial tissues provide gaps and trails of 3–30 μm diameter, similar to those generated here by laser ablation (Wolf *et al* 2009). MMP-independent collective migration could thus occur in physiological clefts between tightly packed collagen bundles or along pro-invasive structures, such as blood or lymphatic vessels and muscle strands (Alexander *et al* 2008) which may represent preferential tracks of least resistance.

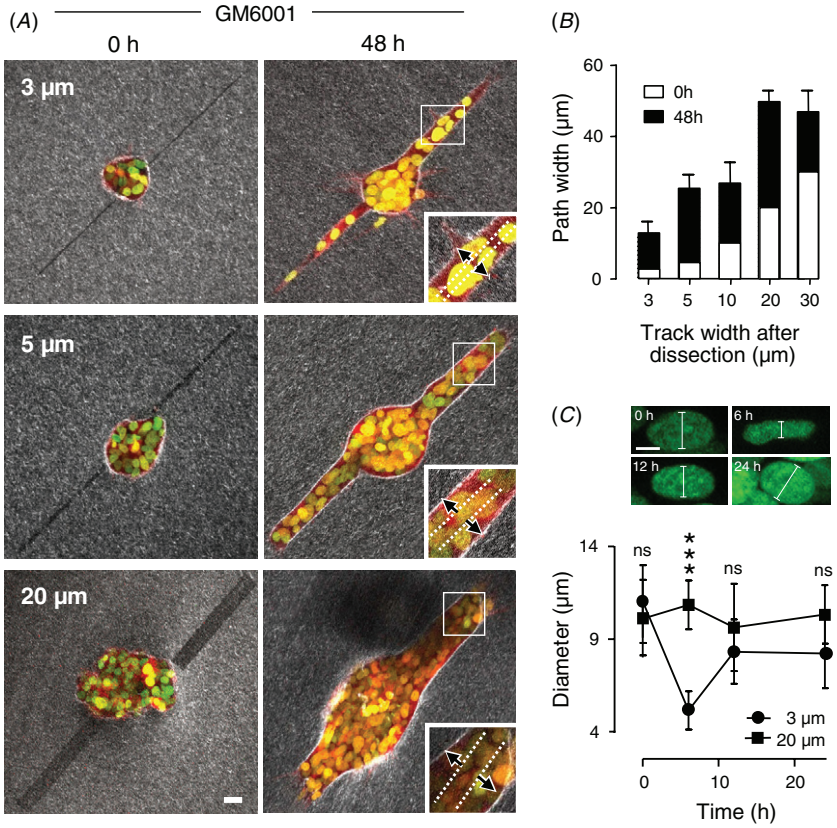

Figure 4. MMP-independent lateral widening of microtracks of different dimensions. (*A*) Geometry of MMT cell strands migrating into microtracks of different dimensions in the presence of GM6001. Scale bar, 20 μm. (*B*) Average microtrack diameter change after colonization by moving cells 48 h after laser ablation. (*C*) Time-dependent deformation of nuclei of MMT cells migrating into microtracks of 3 or 20 μm in the presence of GM6001. Data show the mean nuclear diameter of 30 cells $\pm$ SD from two independent experiments. Asterisks, $p < 0.0001$, unpaired two-tailed Mann–Whitney test.

The mechanisms maintaining MMP-independent migration into laser-ablated tracks comprise two synergistic processes: (i) contact guidance supporting cell alignment and movement along the edge of the track (Condeelis and Segall 2003, Gauvreau and Laroche 2005, Jiang *et al* 2005) and (ii) nonproteolytic displacement of the cell–matrix interface resulting in secondary track widening. Despite inhibition of MMPs and irrespective of the track caliber ranging below or exceeding the respective cell diameter, cell-dependent secondary diameter widening reached by two to threefold enlarged track caliber, suggesting an adaptive pressure-driven process that adjusts the geometry of the ECM as far as matrix elasticity permits. Such cell-derived compression of fibrillar tissue may lead to scaffold condensation, thus locally increasing ECM tension and density. Although the current setting did not permit to directly measure the pressure forces exerted by the cells for secondary track enlargement, cell-induced tissue compression may contribute to increased intro-tumor pressure alongside with other mechanisms, including hydrostatic tissue pressure in tumors caused by defective blood vessels (Vakoc *et al* 2009, Fukumura *et al* 2010, Fukumura and Jain 2007) and increased ECM stiffness caused by proteolytic long-term remodeling and condensation of the ECM structure (Provenzano *et al* 2006, 2008a, 2008b, Egeblad *et al* 2010a), and further act as a diffusion barrier (Davies Cde *et al* 2002). It is unclear to which extent the tissue elasticity *in vivo* allows such cell-pressure-induced tissue deformation; yet the histological sections of concentrically growing primary tumors and metastases suggest a high degree of tumor-induced pressure on the interstitial ECM structure (Pluen *et al* 2001, Netti *et al* 2000). In conclusion, the collagen-based 3D microtrack model is useful to generate defined trails of least resistance and to gain insight into the physical principles of space requirements and pressure-induced tissue deformation by single cell and collective cell migration.

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