Transforming Growth Factor β Controls the Directional Migration of Hepatocyte Cohorts by Modulating Their Adhesion to Fibronectin

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Transforming growth factor β (TGF- β) has a strong impact on liver development and physiopathology, exercised through its pleiotropic effects on growth, differentiation, survival, and migration. When exposed to TGF- β , the mhAT3F cells, immortalized, highly differentiated hepatocytes, maintained their epithelial morphology and underwent dramatic alterations of adhesion, leading to partial or complete detachment from a culture plate, followed by readhesion and spreading. These alterations of adhesive behavior were caused by sequential changes in expression of the $\alpha 5\beta 1$ integrin and of its ligand, the fibronectin. The altered specificity of anchorage to the extracellular matrix gave rise to changes in cells' collective motility: cohorts adhering to fibronectin maintained a persistent, directional motility, with ezrin-rich pathfinder cells protruding from the tips of the cohorts. The absence of adhesion to fibronectin prevented the appearance of polarized pathfinders and lead to random, oscillatory motility. Our data suggest a novel role for TGF- β in the control of collective migration of epithelial cohorts.

INTRODUCTION

The cytokines of the transforming growth factor β (TGF- β) family have essential and pleiotropic roles in embryonic development, adult homeostasis, and pathology (reviewed in Massague *et al.*, 2000; Bachman and Park, 2005). Although the outcome of the TGF- β signaling is strongly dependent on its cross-talk with other signal transduction pathways, as a general rule it is cytostatic and cytotoxic for epithelia and the immune system, while it stimulates differentiation and migration of endothelial and mesenchymal cells (Siegel and Massague, 2003).

Liver is one of the organs dramatically affected by the TGF- β signaling (reviewed in Bissell *et al.*, 2001). Ectopic expression of the active form of TGF- $\beta 1$ gives rise to liver fibrosis through the differentiation of hepatic stellate cells into myofibroblasts and stimulation of collagen synthesis and deposition (Sanderson *et al.*, 1995). During liver regeneration, TGF- β acts to control the transient stimulation of stellate cells and, through its growth inhibitory action on hepatocytes, the extent of hepatocyte regenerative proliferation (Romero-Gallo *et al.*, 2005). Primary hepatocytes in culture are also sensitive to TGF- β : in this setting the cytokine provokes a strong apoptotic response of both mature and fetal hepatocytes (Fabregat *et al.*, 1996; Gressner *et al.*, 1997), whereas early hepatic precursors survive the same treatment (Clark *et al.*, 2005). The resistance to the apoptotic effects of TGF- β signaling can be achieved by simultaneous activation of MAPK Erk and PI3K survival signaling (Fab-

regat *et al.*, 1996; Janda *et al.*, 2002; Valdes *et al.*, 2004), which likely accounts for continued survival and proliferation of many hepatoma cell lines in the presence of TGF- β (Gressner *et al.*, 1997). Cross-talk with other signal transduction pathways (Janda *et al.*, 2002; Cordenonsi *et al.*, 2003; Kamaraju and Roberts, 2005; Mishra *et al.*, 2005; Katuri *et al.*, 2006), acquisition of mutations invalidating the cytostatic and cytotoxic effects of TGF- β in the course of tumor progression (reviewed by Elliott and Blobel, 2005), as well as the complex effects on the tumor stroma (Bhowmick *et al.*, 2004) and the immune response (Thomas and Massague, 2005), give rise to an apparently paradoxical effects of TGF- β in cancer : acting as a tumor suppressor at the early stages of tumorigenesis, at later times autocrine TGF- β signaling can increase tumor cells aggressiveness and metastasis (reviewed in Massague *et al.*, 2000; Bachman and Park, 2005).

One paradigm through which TGF- β can exercise its oncogenic effects is by provoking the epithelial-to-mesenchymal transition (EMT) and the concomitant acquisition of motility by the tumor cells. EMT, a process in which epithelial cells lose their polarity, scatter, and acquire mesenchymal markers at the expense of the epithelial ones (reviewed in Thiery, 2002; Grunert *et al.*, 2003), occurs during embryonic development and can be easily recapitulated in cell culture, notably by the TGF- β treatment. It is generally believed that the EMT participates in the metastatic progression of tumors (Thiery, 2002; Xue *et al.*, 2003; Yang *et al.*, 2004; Huber *et al.*, 2005). However, because of the extreme difficulty in demonstrating the EMT *in vivo*, the evidence for its physiological importance in carcinogenesis remains circumstantial (Grunert *et al.*, 2003; Thompson *et al.*, 2005).

In culture, many epithelial cell types, including hepatocytes, undergo EMT upon treatment with TGF- β , especially if Ras is concomitantly activated, and the resulting mesenchymal tumors are more aggressive than their epithelial counterparts when injected into animals (Fabregat *et al.*,

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Table 1. Primers and expected sizes of PCR products with each primer pair

Gene	Forward primer	Reverse primer	Size (bp)
E-cadherin	5'-CTGCTGCTCCTACTGTTTC-3'	5'-CTGGCTCAAATCAAAGTCC-3'	141
Snail	5'-ATCTTCCCGGAGCTACCC-3'	5'-ACCAAGAGAGCCAAAGCAGGA-3'	108
Integrin β 1	5'-CTACTGGTCCCACATCATC-3'	5'-GGCATTCAATTTCTCCTTTTC-3'	149
Integrin α 1	5'-CATTTCAGCGATTTTCCATAG-3'	5'-TGAAAGGTGGTGTATGAGG-3'	363
Integrin α 6	5'-TCTCTCGTTCTTCGTTCCAGG-3'	5'-GCAGCAGCGGTGACATCTAT-3'	110
Cutl1	5'-GACCCCTTTCTCCTCCTTTCAG-3'	5'-GCTCCGTTGTCACTCTCGT-3'	275
Integrin α 5	5'-TTACACCCCCCAACTCACAGG-3'	5'-ACACCATTGCTGCCTTCTG-3'	375
Fibronectin 1	5'-CCAGTTCAGAGGAGCATCAG-3'	5'-GGCATTGTCGTTCAAGTGTA-3'	105
GAPDH	5'-GCTCACTGGCATGGCCTTCCG-3'	5'-TGGAAGAGTGGGAGTTGCTGT-3'	200
Hprt1	5'-TTGCTGACCTGCTGGATTAC-3'	5'-TTATGTCCCCCGTTGACTG-3'	120

1996; Janda *et al.*, 2002; Huber *et al.*, 2005; Gotzmann *et al.*, 2006).

However, it has been reported that either the constitutive Erk (Pinkas and Leder, 2002) or TGF- β signaling (Han *et al.*, 2005) alone, in the absence of demonstrable EMT, could increase metastasis in experimentally inoculated tumors. Moreover, epithelial tumor cells are capable of collective migration and intravasation into blood vessels, moving as sheets or cohorts (Friedl *et al.*, 2004; Wicki *et al.*, 2006), suggesting that the EMT is not the sole means by which epithelial cells can become motile and invade local and distant sites.

We have studied the effect of TGF- β treatment on mhAT3F, an immortalized, highly differentiated mouse hepatocyte cell line (Antoine *et al.*, 1992; Haouzi *et al.*, 2005). We show that these cells undergo little, if any, apoptosis and no EMT in response to TGF- β . They are nevertheless sensitive to the TGF- β signaling: a change in the profile of expression of integrins gives rise to spectacular alterations of anchorage. This in turn translates into alterations of collective motility especially in respect to mechanisms enabling a persistent, directional movement of cell cohorts.

MATERIALS AND METHODS

Cell Culture and Treatments

MhAT3F cells were cultured in DMEM supplemented with 5% fetal calf serum, 5 μ g/ml insulin, 1 μ M dexamethasone, 1 μ M triiodothyronine, 250 ng/ml fungizone, 100 ng/ml streptomycin, and 100 U/ml penicillin. AML12 cells were cultured in DMEM:F12 supplemented with 10% fetal calf serum, 5 μ g/ml insulin, 5 μ g/ml transferrin, 5 ng/ml selenium, 0.1 μ M dexamethasone, 100 ng/ml streptomycin, and 100 U/ml penicillin. BMEL (clone C3) cells were isolated from embryonic day (E)14 embryonic mouse livers and cultured on collagen-coated dishes in the RPMI medium containing 10% fetal calf serum (FCS), 50 ng/ml epithelial growth factor, 30 ng/ml insulin-like growth factor II, 10 μ g/ml insulin, 100 ng/ml streptomycin, and 100U/ml penicillin (Strick-Marchand and Weiss, 2002). For the TGF- β treatment cells were grown on dishes coated with collagen (0.15 μ g/cm²). Huh7 cells were cultured in DMEM supplemented with 10% FCS, 100 ng/ml streptomycin, and 100 U/ml penicillin.

The cultures were seeded in six-well plates with 100,000 cells in 2 ml medium for 3 d and then were treated with 1 ml fresh medium containing 5 ng/ml TGF- β 1 (R&D Systems, Minneapolis, MN) or the same volume of TGF- β 1 vehicle (4 mM HCl, 1 mg/ml BSA).

RNA Isolation and Analysis

Total RNA was extracted from cultured cells with the High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) and used for first-strand cDNA synthesis with random hexamers. For semiquantitative RT-PCR, cDNA was amplified with GoTaq Flexi DNA Polymerase (Promega, Madison, WI) according to the manufacturer's instructions and subjected to agarose gel electrophoresis. The mRNA levels were quantified in triplicate by real-time PCR with LC FastStart DNA Master SYBR Green I using a Light-Cycler rapid thermal cycler system (Roche Diagnostics) according to the manufacturer's instructions. PCR cycling conditions were 10 min at 95°C for one cycle followed by 35 cycles at 95°C for 15 s, 60°C (excepted integrin β 1 at

59°C; E-cadherin at 63°C; and Snail, integrin α 5, and glyceraldehyde-3-phosphate dehydrogenase [GAPDH] at 65°C) for 10 s, and 72°C for 20 s. Dissociation curve analysis confirmed that signals corresponded to unique amplicons. Expression levels were normalized by GAPDH mRNA levels for each sample, obtained from parallel assays and analyzed using the relative standard curve method. Normalization with hypoxanthine guanine phosphoribosyl transferase 1 (Hprt1) mRNA gave the same expression patterns (not shown). The primer sequences specific to the genes examined and predicted product sizes are shown in Table 1.

Western Blot Analysis and Pulldown Assay

Cells were homogenized, extracted in Laemmli buffer, and then sonicated and complemented with dithiothreitol (DTT), which was omitted for integrin α 1 analysis. Whole cell lysate (25 μ g) was analyzed by SDS-PAGE and blotted onto nitrocellulose membranes. Blots were incubated 2 h at room temperature with primary antibodies and then incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham, Piscataway, NJ) and activity was visualized by electrochemiluminescence.

Pulldown assay for Rac1-GTPase was performed with PAK-GST protein beads (Cytoskeleton, Denver, CO) according to the manufacturer's instructions and subjected to Western blot analysis.

Sample Preparation and Immunofluorescence

Cells were fixed in 4% paraformaldehyde for 15 min, permeabilized for 2 min in 0.1% Triton X-100 (unless nonpermeabilized cells were used, as indicated), rinsed in phosphate-buffered saline, and blocked with 1 mg/ml BSA. Samples were incubated with primary antibodies followed by the appropriate fluorochrome-labeled secondary antibodies. Actin was visualized by fluorescein isothiocyanate (FITC)-phalloidin or rhodamine-phalloidin labeling (Sigma, St. Louis, MO). Nuclei were counterstained with Hoechst 33258. Golgi apparatus was visualized by α -N-acetyl-galactosamine labeling with lectin *Helix pomatia* agglutinin Alexa Fluor 488 conjugate (Molecular Probes, Eugene, OR). The mounting medium was Mowiol (Calbiochem, La Jolla, CA).

Immunofluorescence confocal images were acquired using Zeiss LSM 510 laser-scanning confocal microscope (Thornwood, NY) equipped with an external argon laser. Images were captured at 0.5 μ m intervals using a Zeiss C-Apo 63 $\times$ objective, or 1 μ m intervals using a Zeiss Achroplan 40 $\times$ objective for moving cohorts analysis and deconvoluted with Huygens Pro and reconstructed in 3D with Imaris (Bitplane, Zurich, Switzerland).

Antibodies

Monoclonal anti-E-cadherin antibody was from Zymed (South San Francisco, CA). Monoclonal anti-ZO-1 antibody was from Chemicon International (Temecula, CA). Monoclonal anti-integrin β 1 antibody (anti-mouse CD29) was from PharMingen (San Diego, CA). Polyclonal anti-integrin α 5 (H-104) and anti-fibronectin (H-300) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti- β -actin antibody was from Sigma. Polyclonal anti-ezrin antibody was from Upstate Biotechnology (Lake Placid, NY). Monoclonal anti-Rac1 antibody was from Cell Biolabs. Fluorochrome-labeled anti-rabbit Alexa Fluor 488, anti-rat Alexa Fluor 594, and anti-rabbit Cy5 were from Invitrogen (Carlsbad, CA).

RNA Interference

Retroviral vectors (pSIREN-RetroQ, Clontech, Palo Alto, CA) encoding the hairpin shRNA (short hairpin RNA) sequences (Supplemental Figure 1) were cotransfected with a VSVG (vesicular stomatitis virus glycoprotein)-expressing plasmid into 293-T packaging cells. The supernatants were used to infect mhAT3F (200,000 cells) or Huh7 (100,000 cells). Stable populations were selected with 4 μ g/ml puromycin for 3 d.

The shRNA against luciferase, shLuc comes from RNAi-Ready pSIREN-RetroQ Retroviral Vector kit (Clontech).

Time-Lapse Microscopy

The cultures were placed in a 37°C chamber equilibrated with humidified air containing 5% CO₂ throughout the experiment. Time-lapse microscopy was performed with a Leica DMIR 2 microscope (Wetzlar, Germany) using a 20× contrast-phase objective (10× for Figure 3) and a 10× LMC objective for migration assays. Images were taken with a camera (Princeton Instruments, Trenton, NJ) Micromax YHS 1300 (pixel of 6.7 μm) at 15 min intervals for Figure 6 and 30 min intervals for Figures 3 and 7. The movies were created from the time-lapse series using MetaMorph Version 6.2r5 (Universal Imaging, West Chester, PA). Cell occupancy was measured in successive images with 6 h intervals with MRI Cell Image Analyzer (a visual scripting interface for Image J software (<http://rsb.info.nih.gov/ij/>), adapted at the microscope facility Montpellier RIO Imaging by Volker Bäcker and Pierre Travo).

Cell Migration Assays

Growth factor-reduced Matrigel (250 μl, BD Biosciences, San Jose, CA; 6 mg/ml protein content) complemented with human fibronectin (BD Biosciences; 40 ng/ml) diluted in 150 mM HEPES, pH 7, are deposited in a metal ring of 14 mm diameter, previously put in a six-well plate, and incubated at 37°C until Matrigel solidified. Cells ($n = 20,000$) were seeded onto Matrigel, and then 5 ml of medium was added for 3 d. Cells were treated for 2 h with fresh medium containing 5 ng/ml TGF-β1 or the same amount of vehicle, washed, and maintained in medium without TGF-β1. Cell cluster movements, recorded by time-lapse microscopy, were tracked in successive images using MRI Cell Image Analyzer.

Statistical Analysis

Experiments were performed at least three times. Data are presented as mean ± SEM. Comparisons between groups were analyzed by *t* test. Significance was accepted for values where $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.005$ (***).

RESULTS

mhAT3F Cells Undergo neither Apoptosis nor EMT upon TGF-β Stimulation

Similarly to primary cells, immortalized hepatocyte cell lines can undergo apoptosis upon treatment with TGF-β. AML12, an established cell line derived from a mouse transgenic for TGF-α (Wu *et al.*, 1994), was highly sensitive to this treatment (Perlman *et al.*, 2001), with few cells surviving 72 h of culture in the presence of 5 ng/ml TGF-β (Figure 1A, top

Figure 1. Changes in hepatocytes' morphology in response to TGF-β1. Subconfluent cultures of AML12 and mhAT3F cells were treated with TGF-β1 (5 ng/ml) for indicated times and analyzed by low-power phase-contrast microscopy (A and B, top) or fixed and stained for actin (phalloidin-FITC; green) and nuclei (Hoechst 33258; blue) and analyzed by confocal microscopy (B, bottom). Xy and xz planes are shown for the confocal images.

Figure 2. Cell-cell interactions are conserved in mhAT3F after the TGF-β1 treatment. Subconfluent cultures of mhAT3F and AML12 cells were treated with TGF-β1 (5 ng/ml) for indicated times. RNA was assayed by RTqPCR for E-cadherin (A) and Snail (B) expression and normalized by expression of GAPDH. Mean ± SEM from three independent experiments is shown. Statistical significance was calculated relative to the 0 h time point; * $p \leq 0.05$. Protein expression was analyzed in mhAT3F cells by immunoblotting (C) and protein subcellular localization by confocal immunofluorescence analysis before treatment and after 72 h culture in the presence of 5 ng/ml TGF-β1 (D). The nuclei were counterstained in blue with Hoechst 33258 dye. Xy and xz planes are shown for the confocal images.

panels). On the contrary, mhAT3F, a murine cell line derived from a SV40Tag transgenic mouse and selected for a differentiated phenotype (Antoine *et al.*, 1992), was resistant to the apoptotic effects of TGF-β, with <10% of the cells dying after 72 h in the presence of the cytokine (Figure 1A, bottom panels). Before their demise by apoptosis, the TGF-β-treated AML12 cells underwent a change of morphology: they became spindle shaped, lost cohesive intercellular contacts, and developed actin stress fibers (Figure 1B). These changes were reminiscent of an EMT, a conclusion further supported by the observation that the AML12 down-regulated the expression of E-cadherin after treatment with TGF-β (Figure 2A). Contrary to the AML12, the mhAT3F cells did not lose either their epithelial shape, intercellular cohesion, or cortical actin arrangement (Figure 1B). Interestingly, whereas showing no signs of EMT, the mhAT3F cells, flattened under the control conditions, became more cuboidal in response to TGF-β (Figure 1B, bottom panels, z-section). This change of morphology gave rise to an apparently smaller nuclei and more irregular actin staining in the x-axis images (Figure 1B).

Down-regulation of E-cadherin expression is a major characteristic of the EMT (Thiery, 2002). We used RTqPCR to assay the expression of the E-cadherin in cells treated with TGF-β. While the control AML12 cells showed a down-regulation of E-cadherin mRNA, its level remained elevated, and in fact increased slightly, in the mhAT3F cells (Figure 2A). In hepatocytes, E-cadherin transcription is controlled by Snail, a bHLH family transcriptional repressor (Vega *et al.*, 2004). In concordance with the levels of E-cadherin mRNA expression, Snail mRNA was strongly up-regulated by TGF-β in the AML12 cells, whereas it was hardly detectable in mhAT3F cells throughout the experiment (Figure 2B). In addition to the effect on the transcriptional regulation, TGF-β can also regulate the E-cadherin cellular localization and stability (Janda *et al.*, 2006). Accordingly, we used immunoblotting to confirm the maintenance of E-cad-

Figure 3. The mhAT3F undergo a cycle of contraction and respreading under the influence of TGF- β . Cells were grown on plastic dishes, treated with TGF- β 1 (5 ng/ml), and observed by time-lapse microscopy for 96 h. Representative images of a low-power field and threefold enlargement of one cluster (indicated by an arrow in the top panel). Well-spread monolayer cell clusters ($t = 0$ h) contract soon after treatment ($t = 12$ –48 h). A large proportion of clusters resume spreading at later time points ($t = 72$ –96 h).

herin expression in TGF- β -treated mhAT3F cells (Figure 2C). Finally, we tested the subcellular distribution of E-cadherin by immunofluorescent staining of nonpermeabilized cells (Figure 2D). The membrane-bound E-cadherin was clearly detectable before treatment and the labeling intensity increased upon incubation with TGF- β . The accumulation of membrane bound E-cadherin was probably associated with an increase in adherens and/or tight junction formation, because it was accompanied by a strong recruitment of the junctional protein ZO-1 (Figure 2D, bottom panels).

TGF- β Alters the Adhesion of mhAT3F Hepatocytes

Despite their resistance to apoptosis and the maintenance of epithelial morphology in the presence of TGF- β , the mhAT3F are sensitive to this cytokine. We noticed that in the course of the experiment, both individual cells and the entire epithelial islands seemed to condense (Figures 1B and 3). The confocal analysis of Z sections indicated that the apparent condensation was in fact largely due to a change of cell shape from flattened to more cuboidal. Additionally, the well-ordered cell monolayer became more irregular, with the appearance of several cell layers. This process was under way as early as 12 h after addition of TGF- β and became especially marked at 24–36 h of treatment, when some of the

original islets rounded up into compact balls and nearly or completely detached from the culture plate. Quite unexpectedly, these structures, containing cells deprived of anchorage, did not die: within the next hours they reattached and spread on the culture dish (Figure 3 and Supplementary Fig 3videoA). Interestingly, the continuous presence of TGF- β was not required for this response: a 2 h pulse with 5 ng/ml the cytokine was sufficient to elicit the changes of cells' adhesion (not shown).

The reversible loss of anchorage caused by TGF- β was not limited to the specific cell line we studied: murine primary hepatocyte precursors, the BMEL cells (Strick-Marchand and Weiss, 2002), also underwent a cycle of detachment and spreading in response to treatment (Supplementary Fig 3videoB). It should be noted that, in contrast to the mhAT3F cells, the BMELs continued to proliferate in the presence of the cytokine. Thus, the changes of adherence were independent of the cells' proliferative status.

TGF- β Alters the Pattern of Expression of Integrins

We reasoned that for a group of cells to detach from a solid support and round up into a compact cluster, one would predict the maintenance of strong intercellular cohesion and a concomitant loss of adherence to the culture dish, which in our experimental setup would be covered with an endoge-

Figure 4. The mhAT3F change the pattern of integrin gene expression in response to TGF- β . Exponentially growing cells were treated with TGF- β 1 (5 ng/ml). At the times indicated, cells were collected and total RNA was subjected to RTqPCR analysis. The relative abundance of the mRNAs was estimated after normalization with primers specific for GAPDH. ■, TGF- β -treated samples; □, vehicle treated control. Results are presented as mean $\pm$ SEM of three independent experiments. Statistical significance was calculated relative to the 0 h time point; * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.005$.

nous extracellular matrix (ECM), deposited by the cells (Bissell and Maher, 2003). Data presented in Figure 2 argue that the cell-cell interactions were indeed maintained, and possibly strengthened, in response to TGF- β . To investigate the loss of substrate anchorage, we analyzed the profile of expression of integrins, which are the major receptors mediating the ECM attachment. We concentrated on the integrin subunits known to be expressed by hepatocytes under physiological and pathological conditions (Stamatoglou and Hughes, 1994; Scoazec *et al.*, 1996). In agreement with published data (Bhowmick *et al.*, 2001) the RTqPCR analysis of the principal hepatic integrin β mRNA, $\beta 1$, showed a slight increase in response to TGF- β (Figure 4). On the other hand, integrin $\alpha 1$, a preponderant hepatic α chain, did not vary significantly compared with untreated cultures. In agreement with the literature (Nejjari *et al.*, 1999; Bates *et al.*, 2005; Michl *et al.*, 2005), the TGF- β led to a significant increase of the integrin $\alpha 6$ component of the complex, possibly through the upregulation of its transactivator Cutl1 (Figure 4, inset; Michl *et al.*, 2005). However, the most spectacular changes of expression were observed for the integrin $\alpha 5$ subunit, where the TGF- β treatment led to a nearly 10-fold increase of the mRNA level. Thus, similarly to primary hepatocytes, the untreated mhAT3F cells expressed the $\alpha 1\beta 1$ integrin, which is a receptor for collagen. TGF- β induced expression of other α chains, particularly the $\alpha 5$, which, in association with the $\beta 1$ subunit forms a receptor for fibronectin. Interestingly, this change of pattern of integrin expression was accompanied by a transcriptional up-regulation of fibronectin synthesis (Figure 4).

To assess the functional significance of the changes in the mRNA levels, we next asked if the increase of integrin $\alpha 5$ transcription was reflected at the level of protein expression. The minor differences of integrin $\beta 1$ subunit expression revealed by a Western blot analysis were independent of the TGF- β treatment (Figure 5A, middle panel). In sharp contrast, the expression of the $\alpha 5$ subunit, which remained constantly low in the control cells, increased dramatically under the influence of TGF- β (Figure 5, A, top panel, and B).

To function as an adhesion receptor, the $\alpha 5\beta 1$ integrin complex must be expressed at the cell surface. We used confocal microscopy on nonpermeabilized cells to ascertain this point. The surface expression of the $\alpha 5$ subunit was increased in the TGF- β -treated culture compared with the control cells, even at the early time points of the kinetics (Figure 5C, top panels). As seen in the Z-section of the images, the labeling was predominantly at the points of contact with the culture dish. Interestingly, the increased levels of the surface $\alpha 5$ subunit correlated with a strong $\beta 1$ subunit surface labeling (Figure 5C, middle panels), suggesting that the TGF- β treatment resulted in the membrane localization of functional $\alpha 5\beta 1$ heterodimers. The change of repertoire of expressed integrins could account for the alteration of the cells' adhesion. Specifically, in the absence of fibronectin (the $\alpha 5\beta 1$ substrate), the anchorage should be weakened, explaining the detachment of cell clusters observed in our experiments (Figure 3 and Supplementary Fig 3video). We reasoned that the readherence and spreading of cells, observed at later time points, would require a further change of the integrin-ECM interactions. Accordingly, we tested the possibility that the TGF- β affected the pattern of synthesis and secretion of the ECM components and specifically of fibronectin. Little or no endogenous fibronectin could be detected in control cells throughout the experiment or at the early time points in the TGF- β -treated cultures. However, at later time points, the TGF- β stimulation gave rise to a strong level of fibronectin secretion (Figure 5C,

Figure 5. Changes of the transcriptional profile of integrin expression are reflected at the protein level. Exponentially growing cells were treated with 5 ng/ml TGF- β or the vehicle only as a control for indicated times. (A) Total cell lysate was analyzed by immunoblotting. (B) The Western blots were scanned, and the signal intensity was quantified using the Gene Tools 3.06 software (SynGene, Frederick, MD). Data were normalized with respect to β -actin. □, control culture; ■, TGF- β -treated cells. (C) The surface expression of integrin $\alpha 5$ (top panels), $\beta 1$ (middle panels) integrin subunits and of the extracellular fibronectin (bottom panels) was detected on fixed, nonpermeabilized cells. Nuclei are visualized in blue by the Hoechst 33258 staining. An increase in the surface expression of the $\alpha 5$ is detectable early after the TGF- β treatment, whereas the fibronectin secretion occurs later in the course of response. Orthogonal views of 3D-reconstructed images are also shown.

bottom panels). It would thus appear that the TGF- β treatment of the mhAT3F hepatocytes resulted in the augmented expression of the $\alpha 5\beta 1$ integrin complex, which, in the absence of its appropriate ECM substrate, weakened the cells' anchorage and provoked detachment of cell clusters. At later time points TGF- β signaling gave rise to the synthesis and deposition of fibronectin, the substrate for the $\alpha 5\beta 1$ heterodimer. As a consequence, the cells reattached and spread on the culture dish.

A strong prediction of this hypothesis was that interference with the expression of the $\alpha 5$ integrin subunit should alleviate the loss of anchorage after the TGF- β treatment.

$\alpha 5$ Integrin Chain and Fibronectin Modulate Cell Adhesion after TGF- β Stimulation

Our working model proposed that the loss of anchorage after the TGF- β stimulation was due to a change of integrin

expression pattern. It followed that if the $\alpha 5$ expression was prevented, the $\alpha 1\beta 1$ dimer was likely to remain on the cell surface. In this scenario, the TGF- β would cause no alteration of the major anchorage receptor and would thus not lead to the loss of anchorage. We used RNA interference to inhibit expression of the $\alpha 5$ integrin after the TGF- β treatment. Two different shRNAs tested gave rise to a similar reduction in both mRNA and protein levels in untreated and TGF- β -treated cells (Supplementary Figure 1). As predicted, the interference with the integrin $\alpha 5$ expression allowed the colonies to maintain adhesion to the substrate after the TGF- β treatment (Figure 6A and Supplementary Fig 6video). Because both shRNAs species behaved similarly, only the shRNA A was used in the remaining analyses. To quantify this phenomenon, we took advantage of the fact that upon loss of anchorage the cells compacted into three-dimensional (3D) aggregates. We could therefore measure the changes of the area occupied by cells in three randomly chosen low-power microscope fields during the course of the experiment (portions of which are shown in Figure 6A). As predicted, in the first 48 h of treatment, control cells (shLuc) became highly aggregated and decreased the surface occupancy to ~40% of the initial area (Figure 6B). At later time points the spreading resumed, although it rarely reached the initial surface occupancy (Figure 6, A and B), possibly due to the maintenance of cuboidal cell shape under these conditions. In contrast, cells with diminished expression of the $\alpha 5$ integrin (sh $\alpha 5$) largely retained their spread morphology throughout the entire experiment.

To further investigate the involvement of adhesion through the $\alpha 5\beta 1$ integrin in the changes of cellular morphology after the TGF- β treatment, we next interfered with the expression of the $\alpha 5\beta 1$ ligand, the fibronectin (Supplementary Figure 1). As expected in accordance with the late secretion of fibronectin in the course of response to TGF- β (Figure 5), there was little effect of the fibronectin shRNA on the early loss of anchorage (Figure 6, A and B). However, lowering the availability of fibronectin had an effect on the capacity of the cells to readhere: although the wild-type

cultures resumed spreading at 48 h, the cells expressing the fibronectin shRNA maintained the aggregated phenotype throughout the experiment (Figure 6, A and B). For a quantitative estimation of these data we analyzed five low-power fields, each containing 30–40 cell clusters, from three independent experiments. The rates of shrinking and spreading (Figure 6C) correspond to the slopes of curves in Figure 6B. Lack of the $\alpha 5$ integrin chain significantly inhibited the shrinking behavior ($p \leq 0.0005$), whereas the lack of fibronectin had no significant effect on this process. Conversely, in the absence of fibronectin, the spreading was significantly reduced ($p \leq 0.01$). These data suggest that in response to TGF- β , hepatocytes display a strong preference, and under our experimental conditions, a quasi exclusivity, for adhesion to the ECM via the $\alpha 5\beta 1$ integrin interacting with its ligand, the fibronectin. Importantly, our results cannot be explained by differences in cell proliferation, because the TGF- β treatment gave rise to a G1 arrest in all the populations studied (data not shown).

Changes in Adhesion Correlate with Alterations of Cellular Motility

Changes of adhesion to extracellular matrix, such as those initiated by the TGF- β treatment of the mhAT3F cells, are likely to have a strong impact on cellular motility. The mhAT3F hepatocytes were motile when grown on tissue culture plastic under the standard conditions (not shown). Similarly, seeding cells on a thin layer of Matrigel supplemented with 40 μ g/ml fibronectin, an extracellular matrix preparation whose composition resembles that of the hepatic matrix present in the space of Disse (Bissell and Maher, 2003), permitted spontaneous cell motility. In this case the movement was collective, especially well visible for small cell clusters estimated to contain 10–20 cells (Figure 7A). Contrary to cells grown on plastic, the presence of abundant ECM components allowed the cells to remain attached after the TGF- β stimulation, allowing the analysis of their movement.

Figure 6. Interference with integrin and fibronectin expression modulates the response to TGF- β . Stable populations of mhAT3F cells selected for expression of the control shRNA (shLuc), two independent shRNAs directed against integrin $\alpha 5$ (sh $\alpha 5$ A and sh $\alpha 5$ B) or two independent shRNAs directed against fibronectin (shFnA and shFnB) were grown as exponential cultures, treated with 5 ng/ml TGF- β and observed by time-lapse microscopy for 84 h. (A) Representative images of cell clusters undergoing morphological changes in response to treatment at 0, 42, and 84 h of treatment. (B) Five low-power microscopy fields for each experimental condition, each containing 30–40 cell clusters, were analyzed for changes of surface occupancy by cells, measured every 6 h. The initial coverage, similar for all conditions was arbitrarily set at 100%. The data represent the mean of three experiments $\pm$ SEM. (C) Dynamics of surface occupancy from experiments in B are represented as slopes (calculated by linear regression) of the curves from 0 to 36 h (shrinking) and 48 to 84 h (spreading). Statistical significance was calculated relative to control shRNA (shLuc); ** $p \leq 0.01$ and *** $p \leq 0.005$.

Figure 7. $\alpha 5$ integrin subunit is necessary for the maintenance of directional migration in response to TGF- β . Exponentially growing mhAT3F cells, stably expressing the indicated shRNAs, were seeded on a growth factor-reduced matrigel complemented with 40 ng/ml fibronectin, treated with TGF- $\beta 1$, and analyzed by time-lapse microscopy using the LMC optics. (A) Spontaneous migration of two cell clusters, in the absence of TGF- $\beta 1$, showing the position of cells at 0, 36, and 72 h and the paths traveled. (B) mhAT3F expressing either firefly luciferase shRNA (shLuc) or integrin $\alpha 5$ shRNA (sh $\alpha 5$) were grown on fibronectin-supplemented matrigel and treated with 5 ng/ml TGF- $\beta 1$ for 2 h, rinsed, and maintained in culture for an additional 72 h. RNA extracted at indicated times was assayed by RTPCR for changes of expression of integrin $\alpha 5$ and fibronectin. Expression of GAPDH served as a control. Velocity (C) and persistence (D) of cell clusters locomotion was analyzed in microscope fields containing ~300 cell cohorts. Data are presented as the mean velocity (C) or the mean ratio (D) of distance to path over three periods of 24 h. Means $\pm$ SEM of four microscope fields for every condition are shown. (E) Representative tracings of paths traveled over 24 h by control (shLuc) or $\alpha 5$ shRNA expressing cells at day 3 after treatment with TGF- β or its vehicle as a control. (F) Data from D are represented as a measure of movement directionality (D/P; see text) for each individual cell cluster. Migration was measured during 24 h and starting 24 h after the TGF- β treatment.

Similarly to the behavior of cells grown on plastic, a short pulse of TGF- β elicited the changes of expression of the $\alpha 5\beta 1$ integrin and its substrate, the fibronectin (Figure 7B). The treatment had no effect on the velocity of clusters' locomotion measured up to 3 d later (Figure 7C). Diminishing the level of the $\alpha 5$ integrin subunit by ectopic expression of an appropriate shRNA (Figure 7B) had no effect on the spontaneous cell motility either, whereas the stimulation with TGF- β decreased slightly the motility of these cells (Figure 7C).

In addition to velocity, another aspect of cell motility is the persistence, or directionality, of movement. It can be represented as a ratio of the distance (D) between the start and the end point over the total path (P) traveled. We traced the paths and measured the D and P values over a 3 d period for at least 250 cell clusters for each experimental condition studied. An example of two tracings is shown in Figure 7A. The clusters of spontaneously moving mhAT3F cells had a mean D/P ratio of 0.25 (Figure 7D), with a nonnegligible proportion of cohorts moving in a highly persistent, directional manner (Figure 7E). The TGF- β treatment had a marginal effect on the directionality of movement, whereas bringing down the level of the $\alpha 5\beta 1$ receptor did not affect the parameters of locomotion at all (Figure 7, D and E). However, the cells with low $\alpha 5\beta 1$ integrin expression had a drastically altered response to the TGF- β : they switched their mode of locomotion from quite persistent to nearly random oscillations with a mean D/P ratio < 0.1 at $t = 72$ h (Figure 7, D–F, and Supplementary Fig 7 video).

TGF- β Activates Rac1 and Increases Membrane Activity in Migrating Cohorts

Rho family GTPases are intimately involved in the control of cell movement (Sahai and Marshall, 2002) and its directionality, both for individual cells (Danen *et al.*, 2005; Pankov *et*

al., 2005) and for cell cohorts (Friedl and Wolf, 2003; Omelchenko *et al.*, 2003; Farooqui and Fenteany, 2005). In our experimental system, a short pulse of TGF- β gave rise to a long-lasting activation of Rac1, as measured by a pulldown assay performed 48 h after treatment (Figure 8A). This effect, observed both with and without $\alpha 5\beta 1$ signaling, translated into an increased membrane activity polarized to a subset of cells in the cohorts. The membrane activity was visualized by recruitment of ezrin, an ERM family protein that links Rac1 activation to the changes of actin cytoskeleton, necessary for cellular migration (reviewed in Bretscher *et al.*, 2002). Although the total amount of ezrin was the same in the cell cohorts under all experimental conditions studied (Figure 8B), the TGF- β -treated cultures consistently showed stronger labeling of cells in the upper part of the cohorts, which were partially embedded in the matrix (see Figure 8, C, xz plane, and F, for a schematic representation of a cohort's morphology). Under the control conditions (i.e., during spontaneous migration), usually one, but occasionally two or three of the ezrin-labeled cells protruded from the main body of the cohort. The protruding cells, but not all ezrin-labeled cells, had characteristic pseudopod-like extensions (Figure 8C, a–p). The protrusions of cells containing membrane-localized ezrin were also observed in TGF- β -treated cultures (Figure 8C, e, f, m, and n) and in cells with diminished $\alpha 5$ expression (Figure 8C, c, d, k, and l). However, no protruding cells were seen in cohorts composed of cells with low $\alpha 5$ expression that were treated with TGF- β (Figure 8C, g, h, o, and p). Nevertheless, the ezrin labeling was still limited to cells positioned at the upper edge of the cohort. In other words, the presence of protrusions composed of cells with strong membrane activity was correlated with the maintenance of directional movement of cell cohorts.

Figure 8. Protrusion of pathfinder cells with strong membrane activity correlates with the directional movement of cell cohorts. MhAT3F cells expressing either the control (shLuc) or the integrin $\alpha 5$ directed (sh $\alpha 5$) shRNA were grown on Matrigel supplemented with fibronectin. The cells were treated with TGF- β for indicated times. (A) Rac1 activation was assayed by a GTPase pulldown assay using PAK1-GST as a bait. (B) The level of ezrin expression was assayed by immunoblotting and normalized by GAPDH expression. (C) Cells in panels a–p were fixed, permeabilized, and stained with an anti-ezrin antibody (green). F-actin was visualized by phalloidin staining (red) and nuclei by Hoechst 33258 (blue). Cells in panels q–t were fixed and labeled with anti-fibronectin antibody (purple), then permeabilized, and stained with phalloidin (red) and Hoechst 33258 (blue) to visualize the nuclei. The cloud of fibronectin partially envelops the cohorts, leaving the protruding cells free of fibronectin staining. The arrows indicate the direction of movement deduced from the position of the protruding cells. Insets, glycoproteins (black), strongly enriched in the Golgi apparatus (arrowheads) were revealed with Alexa Fluor 488–conjugated lectin HPA. Two regions of the cohort (identified by squares) are shown. (D) Ezrin (green) and fibronectin (purple) labeling indicate distinct localization of the markers. Nonpermeabilized cells were first labeled with an anti-fibronectin primary antibody, followed by Cy5-conjugated anti-rabbit secondary antibody. The cells were then permeabilized and labeled with an anti-ezrin antibody and revealed with an anti-rabbit antibody coupled to the FITC fluorochrome. This staining protocol accounts for a high background of green labeling of the extracellular fibronectin. However, the membrane-associated ezrin labeling is fully specific of the anti-ezrin antibody (not shown). (E) A 3D reconstruction (top panel) and a section through a middle of a cohort (bottom panel), showing a distinct ezrin localization (green) in the top part as well as in the protruding cell. Actin is visualized in red and nuclei in blue. (F) A schematic representation of a 3D cohort shown in E. The section (indicated by brackets) reveals the cortical organization of actin, position of the Golgi apparatus, accumulation of ezrin in a subset of cells and the cloud of fibronectin in the rear of the cohort. Note the swelling of the matrix behind the cohort; it can be visualized in the Supplementary Fig 7 video and could be due either to a deformation of the matrix, as depicted in the drawing, or to the tail of fibronectin dragged behind the cohort.

Protrusions Point in the Direction of Movement

To investigate if the protrusions corresponded to pathfinder cells that defined the direction of the cohort's movement, we looked at the orientation of the Golgi apparatus relative to the nucleus (Ridley, 2001). Interestingly, whereas the Golgi was always found in front of the nucleus in the protruding cells, its position appeared random in the remaining cells in the cohorts (Figure 8C, bottom panels).

An additional asymmetry of the cohorts became apparent upon staining with an anti-fibronectin antibody (Figure 8, C, bottom panels, and D). The cohorts were extensively decorated with fibronectin; however, the cloud of fibronectin enveloped the sides and the rear of the cohorts and was absent from the front, composed of polarized cells with membrane-mobilized ezrin (Figure 8D). Interestingly, the TGF- β -treated cohorts, in which the adherence to fibronectin was prevented by knocking down the $\alpha 5$ integrin subunit and whose motility was random, were also polarized in respect to ezrin and the pericellular fibronectin (Figure 8Ct). Thus, polarized localizations of the activated ezrin and the fibronectin were not sufficient to sustain a persistent directionality of the cohort's migration. In contrast, ezrin-rich protrusions are characteristic of persistently migrating cohorts. A 3D reconstruction (top panel) and a section (bottom panel) of a control cohort are shown in Figure 8E. A schematic drawing of the same cohort is represented in Figure 8F.

DISCUSSION

Because of its highly pleiotropic action, both tumor promoter and tumor suppressor activities have been ascribed to TGF- β (reviewed in Massague *et al.*, 2000; Bachman and Park, 2005). It is generally believed that the growth suppressive activities, which predominate at the onset of tumorigenesis, are alleviated in the course of tumor progression, allowing full manifestation of the cytokine's tumor promoting activities, in particular by facilitating invasiveness and metastasis as well as stromal effects (Siegel and Massague, 2003; Thomas and Massague, 2005). In addition to its effect on the epithelial to mesenchymal transition (EMT), leading to resistance to apoptosis and increased motility and metastasis (reviewed in Huber *et al.*, 2005), TGF- β can promote metastasis in the absence of EMT (Han *et al.*, 2005), presumably by stimulating collective cell migration (Nabeshima *et al.*, 1995; Friedl *et al.*, 2004; Condeelis *et al.*, 2005).

The most studied mode of cell locomotion, both individual and collective, relies on dynamic interactions of surface receptors of the integrin family with their ligands, the components of the extracellular matrix. One of the well-known effects of TGF- β signaling is a dramatic change of the integrin expression profile and as a result, altered cell adhesiveness and migration (Heino *et al.*, 1989; Stamatoglou *et al.*, 1992; Zambruno *et al.*, 1995; Nejari *et al.*, 1999; Bates *et al.*, 2005).

In the liver, the TGF- β signaling, which is central to the control of embryonic development and in response to injury, is also involved in major hepatic pathologies, such as fibrosis and hepatocellular carcinoma (HCC; reviewed in Bissell *et al.*, 2001). The cytokine is cytostatic and cytotoxic for both mature and fetal hepatocytes (Fabregat *et al.*, 1996; Gressner *et al.*, 1997; Perlman *et al.*, 2001), although a significant fraction of cells escape these effects and undergo EMT (Vega *et al.*, 2004). However, high levels of active TGF- β can be found in well-differentiated HCC, with no signs of EMT, suggesting that acquisition of the mesenchymal phenotype is not the

sole mechanism of escape from the antiproliferative and proapoptotic TGF- β signaling (Idobe *et al.*, 2003). Similarly, we show that the mhAT3F hepatocytes, which retain a high level of differentiation (Antoine *et al.*, 1992; Haouzi *et al.*, 2005), are resistant to apoptotic effects of TGF- β without undergoing EMT. They are nevertheless sensitive to this cytokine, because even a short pulse of TGF- β initiates long-lasting changes in the expression of integrins and their ligands.

Healthy hepatocytes have a restricted repertoire of integrins expressed on their surface: $\alpha 1\beta 1$, $\alpha 5\beta 1$, and $\alpha 9\beta 1$ account for the quasi totality of this class of receptors (Couvelard *et al.*, 1998). The pattern becomes somewhat more complex in hepatocellular carcinoma where, in addition to an increase in the $\alpha 5$ subunit, the $\alpha 3$ and $\alpha 6$ chains are also abundantly expressed (Scoazec *et al.*, 1996). We found that TGF- β induced high level expression of $\alpha 5$ and $\alpha 6$ in the mhAT3F cells, whereas the $\alpha 3$ expression remained constantly low (data not shown). This altered integrin expression was accompanied by the loss of anchorage. The reattachment and spreading that occurred later was linked to the synthesis and the deposition of fibronectin, the $\alpha 5\beta 1$ ligand. Although the alterations of the pattern of integrin expression by the TGF- β have been previously described, to our knowledge, the ensuing dramatic loss of adhesion independent of cell death that we report here had not so far been documented. Importantly, the changes of cells' adhesive behavior were truly due to the TGF- β signaling, because a knockdown of its cognate receptor T β RI by an shRNA fully alleviated this response (data not shown).

Although spectacular, the changes observed in cells grown on plastic are likely to represent a deformation of a physiological response. In contrast, the same cells grown on a support reminiscent of the loose hepatic extracellular matrix present in the space of Disse (Bissell and Maher, 2003) provide a more convincing model of physiologically relevant conditions. In consequence, we have cultured hepatocytes on Matrigel supplemented with fibronectin. In this setting, the changes of adhesion no longer led to detachment, but rather to alterations of the cells' migratory behavior. TGF- β generally decreases the motility of nontransformed epithelial cells (Siegel and Massague, 2003; Tian and Phillips, 2003), although the opposite effects have also been reported (Zicha *et al.*, 1999). Under our experimental conditions there was little inhibition of the overall hepatocyte motility. This could be due to a short duration of treatment in our experiments, because the continuous presence of the cytokine did slow down the movement of the cells (data not shown). Additionally, it could reflect cell type differences relative to signaling through the Alk1 and Alk5 receptors, described as mediators of contradictory effects of TGF- β on migration of endothelial cells (Goumans *et al.*, 2002). Finally, the effect of TGF- β on collective migration reported here could differ from that observed for single cells, despite the fact that the two modes of locomotion share several control mechanisms (reviewed in Friedl and Wolf, 2003).

Despite the maintenance of cellular motility, TGF- β had a strong impact on the type of the hepatocyte locomotor activity. First, although in the absence of TGF- β the spontaneous motility was not sensitive to the loss of the integrin $\alpha 5$ expression, after treatment with the cytokine the cells slowed down when the $\alpha 5$ chain was depleted. Interestingly, adhesion to fibronectin via the $\alpha 5\beta 1$ integrin is also essential for the synergistic effect of TGF- β and hepatocyte growth factor (HGF) on the collective motility of colorectal carcinoma cells (Shimao *et al.*, 1999).

Second, and more dramatically, the TGF- β treatment rendered the directionality of the movement strongly dependent on the $\alpha 5$ integrin subunit. Importantly, this behavior was not a particularity of the mhAT3F cells, because cohorts of Huh7, a human hepatoma cell line, responded similarly to TGF- β (data not shown). This is in contrast with a reported change from a directional to a random locomotion in isolated cells that was correlated with the adhesion through the $\alpha 5 \beta 1$ fibronectin receptor (Danen *et al.*, 2005).

The directionality of single-cell movement has also been reported to be governed by Rho family GTPases and in particular by Rac1 or the balance of RhoA and Rac activities (Danen *et al.*, 2005; Pankov *et al.*, 2005), two downstream targets of TGF- β signaling (Massague *et al.*, 2000). We confirmed that TGF- β treatment led to a long-lasting activation of Rac1, further strengthened in the presence of a functional $\alpha 5$ integrin subunit, in accordance with the reported specific effect of the $\alpha 5 \beta 1$ engagement by fibronectin on Rac1 signaling (Mettouchi *et al.*, 2001). However, even the strongest level of Rac1 activation in our system was not inhibitory of directional migration: indeed, the loss of directionality was observed in cells that displayed the intermediate levels of active Rac1. Clearly, a global estimate of Rac1 activation does not measure its activity in a subset of cells determining the motility of a cohort. However, overall Rac1 activity correlated with the membrane recruitment of ezrin in a subset of cells, suggesting that the activation of Rac1 was coupled to the mobilization of the actin cytoskeleton and membrane activity (Ridley, 2001) of a few cells located at a tip of each cohort. These cells appeared to lead the movement and therefore determine its directionality: the protrusions consisting of one to three cells always originated from these ezrin-rich tips. Importantly, the protrusions pointed in the general direction of the cohorts' movement, as witnessed by the polarization of the Golgi apparatus in the protruding pathfinder cells. In contrast, but similarly to rows of cells behind the front of migration in wound closure assays (Kupfer *et al.*, 1982), other cells in the cohort did not reorient their Golgi apparatus in the direction of the movement. It would thus appear that the pathfinder, protruding cells determine the direction of the movement and drag the rest of the cells behind them.

The hepatocyte cohorts described by us, while not fully enclosed in the matrix, are largely embedded in it and are themselves organized in 3D (Figure 8, C, z-sections, and F). Their motility appears to be a mixture of the fibroblastoid and of the amoeboid type (Friedl and Wolf, 2003; Sahai and Marshall, 2003): the pathfinder cells protrude from the cohort and extend actin-rich pseudopod-like processes, they are polarized in the direction of the movement, but are also enriched in active membrane bound ezrin. The remaining cells of the cohort are not polarized, have no visible pseudopod-like extensions, but are capable of movement in the direction defined by the pathfinders. This situation resembles collective movement of *Drosophila* border cells, where differences in the strength of signaling within constituents of a cell cluster defines leading cells, characterized by actin-rich protrusions, which communicate the direction of migration to the rest of the cohort (Bastock and Strutt, 2007; Bianco *et al.*, 2007).

What determines the persistence of collective migration in the hepatocyte cohorts? It cannot be the cohorts' polarization per se, because randomly moving cohorts (TGF- β -treated cells with low integrin $\alpha 5$ expression) had polarized membrane activity, visualized by activated ezrin, presumably reflecting a polarized Rac1 activity. However, these cell clusters did not extend polarized protrusions. Taking into ac-

count our data on changes of the cells' adhesive properties in response to TGF- β , we propose that the protrusions that sprouted from the ezrin-rich tips required stabilization by binding to matrix components. This interpretation is consistent with the observation that, in contrast to the behavior of migrating border cells (Bianco *et al.*, 2007), the identity of the leading cell was maintained for long periods of time (Supplementary Fig 7 video). When occasionally a different leading cell emerged from a cohort, it caused an abrupt change of the direction of the collective movement (see for example the TGF- β -treated cells in Supplementary Fig 7 video). Remarkably, after the TGF- β treatment, fibronectin became the obligatory adhesive substrate for the protruding cells. Thus, although the migratory behavior under the unstimulated conditions was independent of adhesion to fibronectin, an exposure to TGF- β initiated a long-lasting response of a strong requirement for $\alpha 5$ integrin to maintain persistent motility along fibronectin fibers, which are the most abundant and ubiquitously distributed components of the extracellular matrix in the liver (Martinez-Hernandez and Amenta, 1993). When the anchorage to fibronectin was abolished, the cell clusters engaged in random oscillations, reminiscent of an exploratory behavior, described in neoplasia (Vasiliev, 2004).

Our results suggest that the TGF- β signaling, essential in liver embryonic development and frequently augmented in hepatocellular carcinoma (Bissell *et al.*, 2001), might improve the capacity of hepatocytes to travel across long distance.

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