Cancer Cell
Article

The Inhibition of Tumor Cell Intravasation and Subsequent Metastasis via Regulation of In Vivo Tumor Cell Motility by the Tetraspanin CD151

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DOI 10.1016/j.ccr.2008.01.031

SUMMARY

In vivo tumor cell migration through integrin-dependent pathways is key to the metastatic behavior of malignant cells. Using quantitative in vivo assays and intravital imaging, we assessed the impact of cell migration, regulated by the integrin-associated tetraspanin CD151, on spontaneous human tumor cell metastasis. We demonstrate that promoting immobility through a CD151-specific metastasis blocking mAb prevents tumor cell dissemination by inhibiting intravasation without affecting primary tumor growth, tumor cell arrest, extravasation, or growth at the secondary site. In vivo, this loss of migration is the result of enhanced tumor cell-matrix interactions, promoted by CD151, which prevent dissociation by individual cells and leads to a subsequent inhibition of invasion and intravasation at the site of the primary tumor.

INTRODUCTION

While tumor development at the primary site is cause for the onset and progression of neoplastic disease, the metastatic colonization of secondary tissues is clearly the most fatal aspect of clinical disease (Weiss, 1990; Hanahan and Weinberg, 2000). Tumor cell dissemination involves multiple sequential rate-limiting steps, including tumor cell mobilization, intravasation, and extravasation, which ultimately result in the colonization of a secondary site (Bockhorn et al., 2007; Pantel and Brakenhoff, 2004; Bernards and Weinberg, 2002). Throughout the journey from its original location on route to a distant site, the metastasizing tumor cell interacts with its changing microenvironment. Together with transmembrane signaling receptors, adhesion complexes on the cell surface convey signals from the instructive microenvironment and regulate tumor cell behavior. The ability of the tumor cell to mobilize itself and leave the original tumor site is one of the prerequisite requirements of tumor cell dissemination (Stracke and Liotta, 1992; Fidler, 2003; Cameron et al., 2000; Hynes, 2003). Nevertheless, the role of active migration and invasion in metastasis remains undefined (Bockhorn et al., 2007).

While alternative, passive methods of dissemination have been proposed (Weidner, 2002) the identification of the promigratory factors Rho C and Twist as key contributors to the metastatic phenotype (Clark et al., 2000; Yang et al., 2004) implicates the need for a functional migration machinery. Cellular migration is mediated by the adhesion receptors (integrins), which regulate cellular behavior through cytoplasmic signaling pathways including Rho GTPases and kinases (Felding-Habermann et al., 2001; Guo and Giancotti, 2004). Adhesion receptor function is tightly controlled through regulatory pathways that include integrin-associated transmembrane proteins such as CD98 (Feral et al., 2005), CD47 (Brown and Frazier, 2001), and tetraspanins such as CD82 and CD151 (Bienstock and Barrett, 2001; Hemler, 2005).

SIGNIFICANCE

The most lethal aspect of a malignancy, metastatic dissemination, is thought to involve migration machinery that mobilizes the tumor cell. The aspect of the metastatic cascade that is strictly dependent upon cell motility has, thus far, not been defined clearly. Here, we demonstrate that specific mAb-mediated inhibition of migration, through the integrin-associated tetraspanin, CD151, prevents tumor cells from entering the vasculature and subsequent colonization of secondary organs. The inhibition of migration is due to a dramatic increase in adhesion that prevents cell dissociation and promotes pronounced immobilization of the tumor cell. The described work illustrates the contribution of tumor cell motility to intravasation and defines induced enhancement of malignant cell adhesion within the primary tumor as a potential therapeutic approach.

The integrin-associated protein CD151 is a member of the tetraspanin superfamily characterized by four transmembrane domains and well-conserved cysteine residues in a large extracellular loop (Hasegawa et al., 1998; Sincock et al., 1999; Hemler, 2005). CD151 has been shown to interact directly with the alpha subunit of several integrins through a small domain (QRD$^{194–196}$) within the larger of its two extracellular loops (Kazarov et al., 2002). While associated with several integrins, the strongest CD151-integrin interactions are with the laminin-receptor integrins $\alpha_3\beta_1$ and $\alpha_6\beta_1$ (Serru et al., 1999; Lammerding et al., 2003). Like other tetraspanins, CD151 also interacts with itself, thus forming small microdomains on the cell surface that incorporate integrins together with other integrin-associated proteins (Claas et al., 2001; Charrin et al., 2003), Ig superfamily proteins (Le Naour et al., 2004), and cadherins (Chattopadhyay et al., 2003). CD151 has been shown to mediate integrin function by regulating cytoplasmic signaling. Specifically, CD151 modulates adhesion-dependent activation of RAS (Sawada et al., 2003), and integrin-associated CDC42 signaling (Shigeta et al., 2003). CD151 has been proposed as a cancer marker (Ang et al., 2004); however, while some components of the molecular mechanism of CD151 have been defined in vitro, very little is known about its in vivo functional role in tumor biology. We have previously demonstrated that an antibody directed to CD151 can inhibit spontaneous metastasis (Testa et al., 1999). However, thus far it has been unclear how, or at which step of the metastatic cascade, CD151 contributes to tumor cell dissemination. To address these and more encompassing questions, we have developed quantitative methods of human tumor cell detection based on PCR amplification of *alu* sequences, which allow for an extremely sensitive detection and quantification of each individual step in the metastatic process (Zijlstra et al., 2002). Furthermore, to assess motility of tumor cells in vivo, we have extended this model to now include a useful system for intravital imaging of individual tumor cell motility.

Using these methodologies, we have demonstrated that the immobilization of tumor cells with a unique function blocking anti-CD151 mAb prevents intravasation and subsequent metastasis. The real-time visualization of tumor cell migration in vivo demonstrates that motility at the secondary as well as the primary site is substantially inhibited by the anti-CD151 mAb. Real-time, intravital imaging indicated that the pronounced inhibition of migration was due to an inability of individual tumor cells to detach at the rear of the cell and depart from their original position within the tumor stroma. As a consequence of the inhibition of migration, a dramatic reduction of intravasation at the primary tumor site was observed which accounted completely for the diminished spontaneous metastasis of tumor cells.

RESULTS

Anti-CD151 Antibody, mAb 1A5, Inhibits Spontaneous Metastasis of Human Tumor Cells In Vivo

We have demonstrated previously that human *alu* PCR in conjunction with the chick embryo spontaneous metastasis assay can be used to quantify metastatic behavior of human tumor cells in vivo (Zijlstra et al., 2002). By employing these methodologies in conjunction with a unique metastasis-blocking monoclonal antibody (mAb 1A5), the role of the tetraspanin CD151 in tumor cell dissemination was explored. Animals bearing HEp3 and HT1080 tumors were injected i.v. with control mAb 29-7 or with purified anti-CD151 monoclonal antibody, mAB 1A5. While both antibodies persist in the blood for the duration of the assay and localize to the tumor (Figure S1 available online), only the anti-CD151 antibody inhibits spontaneous metastasis (Figure 1A). This inhibition is target specific because control antibodies, which also bind to the surface of HEp3 cells (29-7, Figure S1), do not interfere with metastasis (Figure 1A). Inhibition of metastasis is not cell lineage specific since spontaneous dissemination of the epidermoid carcinoma HEp3 and the fibrosarcoma HT1080 are inhibited equally by mAb 1A5. In addition, large differences in CD151 expression between HEp3 and HT1080 (inset, Figure 1A) did not affect the ability of mAb 1A5 to inhibit metastasis, nor did the antibody recognize any antigen in normal chick tissue (Figure S1), further emphasizing the importance of tumor CD151 in metastasis. Importantly, the level of inhibition of HEp3 spontaneous metastasis in the SCID mouse by mAb 1A5 (>80%) is similar to that observed in the chick, affirming that the inhibition is not restricted to the chick model (Figure 1B). In both models, the tumor size is unaffected by antibody treatment (Chick: control IgG, 345.7 mg ± 146.7; anti-CD151, ± 352.25 ± 145.6. Mouse: control IgG, 2.87 g ± 1.29; anti-CD151, 2.86 ± 1.39), indicating that the inhibition of metastasis is independent of primary tumor expansion.

Matrix-Mediated Migration of Tumor Cells Is Inhibited by the mAb 1A5 Independent of the Type of Matrix Composition

Previous work has suggested that CD151 influences migration on laminin (Winterwood et al., 2006). To determine if the regulation of migration by CD151 is matrix component specific, the ability of mAb 1A5 to inhibit the matrix-mediated migration of HEp3 cells was assessed using a variety of matrixes. Considering the established association of CD151 with laminin-receptor integrins (Serru et al., 1999) ($\alpha_3\beta_1$ and $\alpha_6\beta_1$), it is not surprising that mAb 1A5 inhibited laminin-mediated migration (Figure 2A). However, mAb 1A5 was also capable of inhibiting migration on collagen type I (Figure 2B), which is mediated through the α_1 and α_2 integrin subunits not known to have strong interactions with CD151. In fact, for each of the tested matrix components (and thus, their corresponding integrin adhesion receptors), antibody treatment reduced the level of migration to that on uncoated filters (Figure 2B). However, mAb 1A5 clearly does not inhibit matrix-independent migration on uncoated filters.

The inhibition of matrix-mediated migration could be due to the disruption of adhesion to the matrix components. However, adhesion to, and spreading on, collagen type I-coated surfaces in vitro was not disrupted (Figure 2C, upper panels). Furthermore, the formation of paxillin containing focal adhesions was enhanced (Figure 2C, lower panels), suggesting that antibody treatment actually enhances matrix interactions rather than disrupting them.

In Vivo Migration Controlled by CD151 Is Critical for Tumor Cell Motility at the Secondary Site, but Not for the Extravasation of Arrested Tumor Cells

The observation that the function-blocking mAb 1A5 implements a broad inhibition of matrix-mediated migration suggests that it

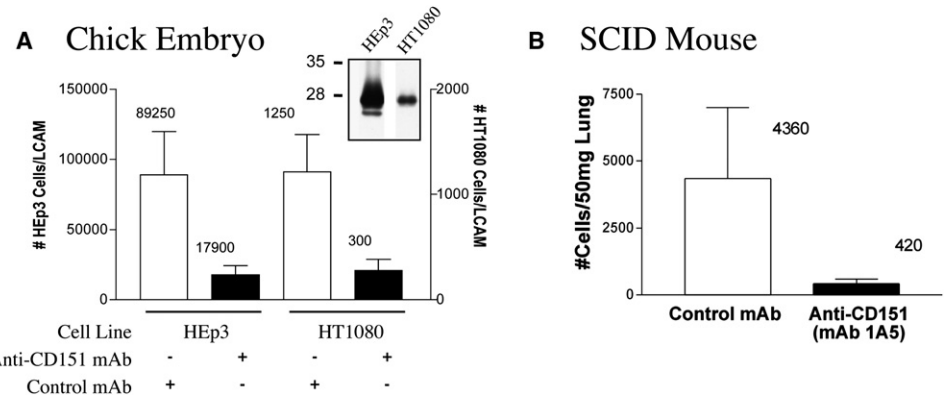


Figure 1. Treatment with Anti-CD151 Antibody, mAb 1A5, Inhibits Spontaneous Metastasis of Human Tumor Cells In Vivo

(A) Control antibody (29-7) or anti-CD151 monoclonal antibody (1A5) was injected i.v. (100 μg) into HEp3 or HT1080 tumor-bearing chick embryos 1 day after applying the tumor cells. The level of metastasis to the chick lower CAM (LCAM) was determined by human *alu* PCR after 7 days as described in the Experimental Procedures.

(B) To confirm that CD151 also impacts metastasis in another in vivo model, human *alu* PCR was used to quantify the level of spontaneous metastasis in SCID mice bearing HEp3 tumors in response to systemic treatment (i.v. injection, 100 μg ×2) with mAb 1A5. Values are represented as mean ± SEM.

might also inhibit migration within a complex matrix substratum such as that found in vivo. To perform an analysis of tumor cell motility in vivo with respect to CD151, we developed the CAM as a biological platform for the visualization of tumor cell arrest, extravasation, and migration. (Figure S2).

To assess if mAb 1A5 could interfere with extravasation and subsequent migration, GFP-expressing tumor cells were injected i.v., and their ability to arrest and disperse within the stroma was assessed by fluorescent microscopy. GFP-expressing HEp3 and HT1080 cells readily arrested in the CAM vasculature in the presence and absence of anti-CD151 mAb (Figure 3A, Day 1). In subsequent days, the arrested tumor cell population in control animals expanded and was capable of disseminating widely throughout the CAM stroma. In contrast, the tumor cell population in mAb 1A5-treated animals proliferated but apparently failed to disseminate as they remained tightly clustered, suggesting these cells cannot migrate (Figure 3A, Day 3 and 5). This inhibition is not Fc-dependent but requires the bivalent nature of the IgG (Figure S5). Both epidermoid carcinoma (HEp3) and fibrosarcoma (HT1080) demonstrate similar inhibition in motility.

To determine if the failure of tumor cells to disseminate in mAb 1A5-treated animals was due to an inability to extravasate, we examined the vascular location of the GFP-expressing tumor cells in the presence and absence of mAb 1A5. At 24 hr postinjection, all of the cells in animals treated either with control antibody or with mAb 1A5 had extravasated (N = 300, Figure 3B, left panels, Z projection). At 5 days postinjection, the extravasated cells had proliferated in both control and mAb 1A5-treated animals. However, in control animals, these cells had migrated throughout the stroma, while in mAb 1A5-treated animals, the extravasated cells proliferated but failed to disseminate and, thus, formed clusters or compact colonies immediately outside the vasculature (Figure 3B, right panels, z-projection). These observations indicate that the inhibition of migration by anti-CD151 mAb 1A5 appears not to be critical for vascular extravasation but prevents active migration of the tumor cells within the stroma after departure from the vasculature.

The Tumor Cell Immobility Mediated by CD151 upon Antibody Ligation Does Not Reduce the Arrest and Growth of Tumor Cells In Vivo

HEp3 cells were injected i.v. in the presence of control mAb or anti-CD151 mAb, and their arrest in both the CAM and lung at 24 hr and the subsequent proliferation of arrested cells 4 days postinjection were determined quantitatively by Alu PCR. A comparable number of arrested cells (~1000 cells in the LCAM and 200 cells in the lung) were detected in treated and control animals (Figure 3C, Day 1). Furthermore, a 5-fold increase in cell number occurs in the LCAM and a 2-fold increase in the lung for both mAb 1A5 and control mAb-treated animals (Figure 3C, Day 4). The tissue-specific lag in tumor cell proliferation seen in the lung was observed previously (Zijlstra et al., 2002). These data confirm that the inhibition of motility by mAb 1A5 does not affect tumor cell arrest or growth in vivo.

Inhibition of Motility by mAb 1A5 Eliminates the Invasive Edge of the Primary Tumor

Although the loss of motility mediated by anti-CD151 prevents migration at the secondary site, it does not limit metastatic colonization by tumor cells injected intravenously (Figure 3). This inability to influence postintravasation metastatic events indicates that the inhibition of migration plays a critical role in the departure from the primary site. To monitor the migratory behavior of cells within the primary tumor, we developed a distinct intravital imaging system (see the Experimental Procedures). GFP-expressing HEp3 cells (~5×10^4) were implanted into the CAM of ex ovo embryos and allowed to form a primary tumor. The animals were treated with a control mAb or mAb 1A5 at 24 hr postimplantation, and the tumors were imaged 7 days later. The hallmark of aggressive metastatic tumors is an irregular invasive front seen at the tumor-stromal interface (Friedl and Wolf, 2003; Hood and Cheresh, 2002; Geho et al., 2005; Liotta and Kohn, 2001). This invasive front is clearly visible in control HEp3 and HT1080 tumors (Figure 4) where tumor cells have invaded the stroma surrounding the primary tumor, thereby generating an irregular

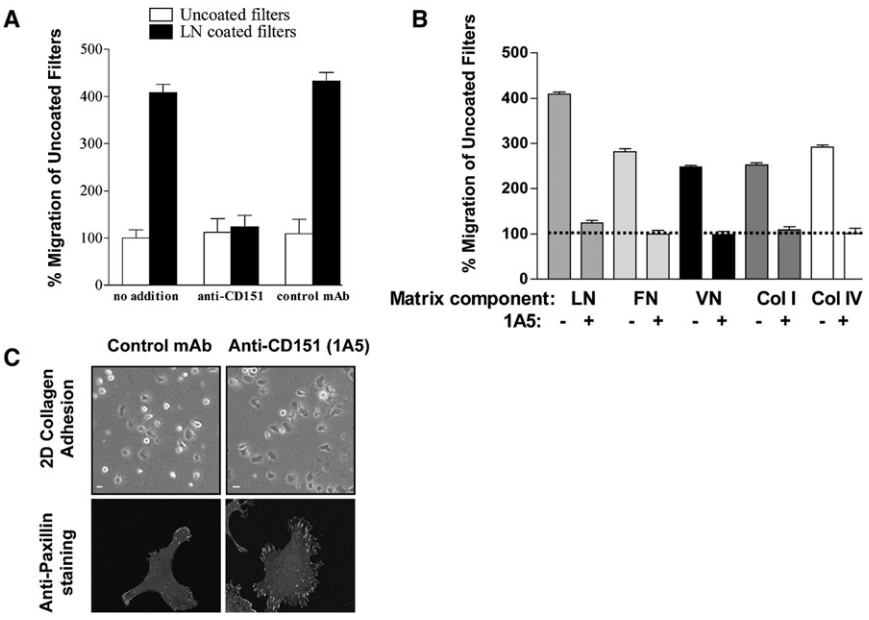


Figure 2. Anti-CD151 Metastasis-Blocking mAb 1A5 Mediates Inhibition of Migration Independent of the Underlying Matrix

Transwell migration of HEp3 cells in response to an underlying matrix was assessed in the presence of mAb 1A5 or an unspecific control mAb. Haptotactic migration of HEp3 in response to laminin (LN, [A]) was assessed during a 16 hr incubation and compared to migration on control (uncoated) filters. (B) Comparative summary of 1A5 inhibition of matrix mediated migration by HEp3 on LN, Fibronectin (FN), Vitronectin (VN), Col I, and Collagen type IV (Col IV). Values in (A) and (B) are represented as mean ± SEM (C) Adhesion of HEp3 cells to collagen type I coated coverslips in the presence of control mAb or mAb 1A5 was visualized using phase contrast microscopy (upper panels). Immunofluorescent staining for Paxillin visualizes the formation of focal adhesion complexes on a collagen type I substrata in the presence of a control mAb or mAb 1A5 (lower panels). A 2- to 3-fold enhancement of paxillin containing focal adhesion complexes was observed in the presence of mAb 1A5. Scale bars, 10 μm.

border. High magnification (100×) imaging demonstrates that individual cells have migrated into the stromal tissue and appear particularly abundant along the stromal vasculature. It is not surprising that tumor cells indeed migrate rapidly along the vasculature (Figure S4 and Movie S8), a phenomenon also reported in a mouse tumor model (Wyckoff et al., 2000). In contrast, mAb 1A5-treated tumors exhibit a very defined border with little or no invasion of the surrounding stroma nor migration along the vasculature for both HEp3 and HT1080 tumors (Figure 4, lower panels).

Inhibition of Individual Tumor Cell Migration by mAb 1A5 Prevents Invasion of the Adjacent Stroma

To determine if CD151-mediated control of migration was responsible for intratumoral cell motility in vivo, changes in velocity, distance, and directionality of tumor cell motility in HEp3 tumors in response to mAb 1A5 were assessed using intravital microscopy. HEp3 tumor cells were implanted in the CAM, and the motility of individual tumor cells was imaged over a 12.25 hr period in the presence and absence of mAb 1A5 (Figures 5A and 5B). A representative population of motile cells, whose path could be traced during the entire time period, was analyzed by tracking the centroid of individually selected cells. For each individual cell, the total distance migrated and the productive migration (distance from origin) was calculated (Figure 5C). In addition, the velocity, persistent forward motion (persistence), and changes in directionality (angle change) were determined for each cell.

Individual HEp3 cells exhibit a high level of motility (22 μm/hr for a total distance of 288 μm; Movie S1; Figure 5D). This average velocity compares favorably with the high speeds of 20–40 μm/hr recorded in two- and three-dimensional culture in vitro for cancer cells of epithelial origin (Soon et al., 2005; Harms et al., 2005). More important, HEp3 cells exhibit a persistence of 4.39 μm, which facilitates a productive migration of 150 μm in untreated animals (Figures 5C and 5D). Tracking of individual cells clearly demonstrates the ability of HEp3 cells to rapidly penetrate the stroma surrounding the primary tumor (Figure 5A, control tracks 3 and 8). In contrast, the inhibition of motility is readily apparent in mAb 1A5-treated animals (Figure 5B and Movie S2). Tumor cells in antibody-treated animals exhibit an approximate 4-fold inhibition of migration velocity (5.9 μm/hr), which, together with a 3.6-fold inhibition of persistence (1.21 μm), results in a 6.6-fold reduction in productive motility (23 μm, Figures 5C and 5D). The substantial reduction in motility upon treatment with mAb 1A5 is apparent when the traces of individual tumor cells are plotted on a singular origin in a Wind-Rose plot (Figures 5A and 5B, right panels).

In mAb 1A5-treated animals, the tumor cells clearly exhibit some residual movement in the presence of mAb 1A5, and their ability to change direction is unaffected (Figure 5D). Pseudopodial extensions and small forward and backward movements are readily observed within the tumor (Figure 5B, tracks 1 and 2; Movie S2). However, considering an approximate body length of 20 μm, on average, the mAb 1A5-treated tumor cells fail to travel more than one body length from their original position, while in control animals, the average tumor cell travels 5–7 body lengths away from their original start position (Figures 5A, 5B, and 5D).

Inhibition of Motility by Anti-CD151 Is Mediated Mechanistically by the Inability of Tumor Cells to Detach at the Rear of the Cell Body

The migratory behavior of HEp3 cells in vivo was analyzed in greater detail by capturing enhanced images of individual migrating cells in control and mAb 1A5-treated animals (Figure 6). In control tumors, migrating cells can be seen to invade the surrounding stroma as the leading edge of the cell penetrates the mesenchymal tissue (Figure 6A, arrow). When the cell body moves forward in the direction of the invading pseudopodia,

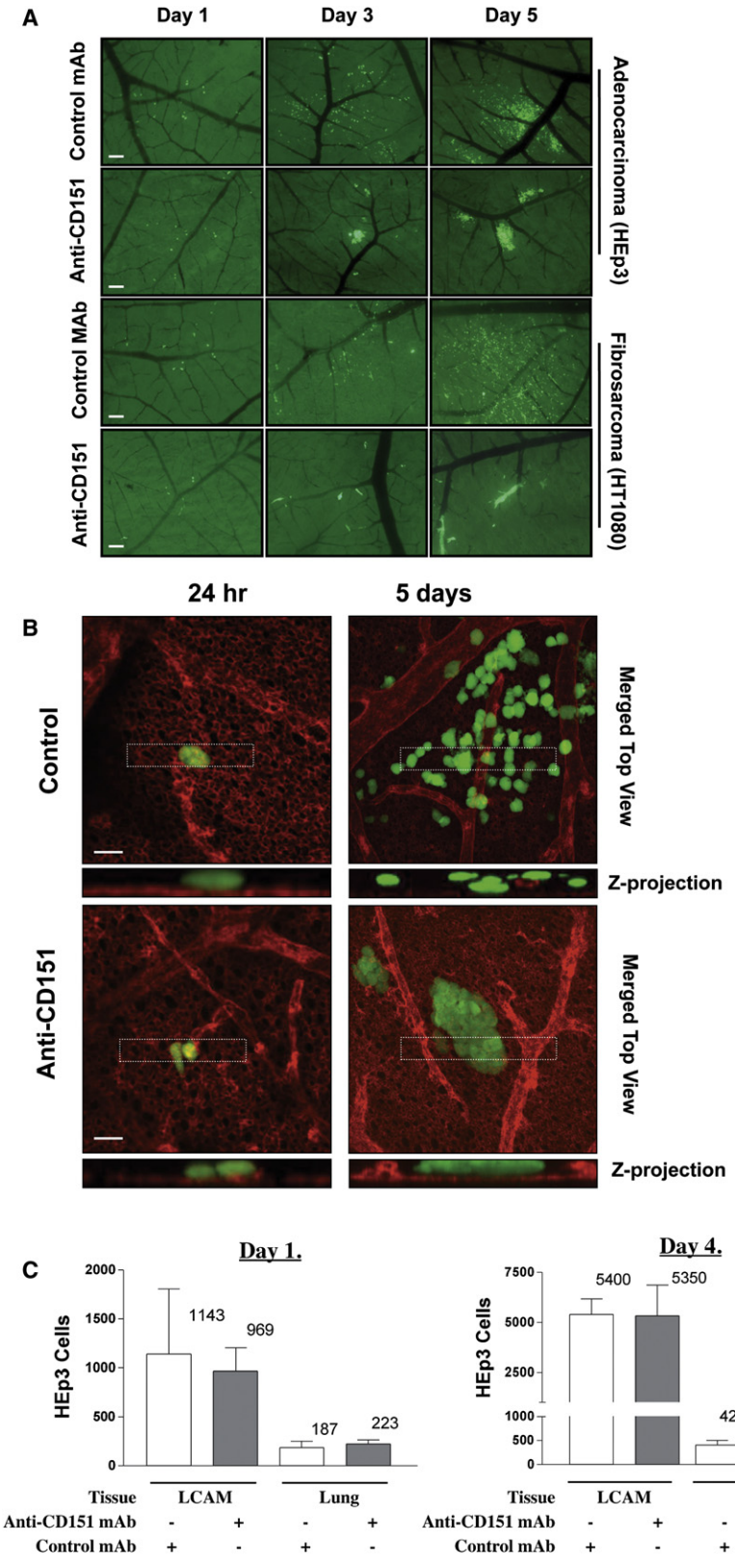


Figure 3. In Vivo, Intrastromal Tumor Cell Migration, but Not the Arrest, Extravasation, or Growth at the Secondary Site of Metastasis Is Affected by Anti-CD151 mAb 1A5

(A) The ability of anti-CD151 to impact in vivo mobility was determined by visualizing the intrastromal mobility of GFP-expressing HEp3 (upper panels) and HT1080 (lower panels) cells in the CAM when coinjected with 100 μg anti-CD151 or a control IgG. Blood-containing vessels are seen in black while GFP-expressing cells are bright green against the dull green glow of the eggshell. Each image is representative of twelve animals imaged in four separate experiments.

(B) Alterations in the ability of tumor cells to arrest, extravasate, and colonize were assessed by confocal imaging of GFP-expressing HEp3 (green) cells in CAM with Lectin-labeled vasculature (red). Tumor cells coinjected with anti-CD151 or control IgG were imaged at 24 hr and 5 days postinjection. Merged topical view and vertical projection of a resliced Z-stack taken from a selected area (white box) illustrate the location of tumor cells relative to the vasculature. Tumor cells that remain inside the vasculature would be seen as yellow while extravasated cells are only seen in green (for further detail, see Figure S2). Included images are representative of twelve animals from four separate experiments.

(C) *Alu* PCR analysis of experimental metastasis was used to quantify the affect of inhibited tumor cell migration on arrest (Day 1) and growth (Day 4) in the lung and the lower CAM (LCAM). HEp3 tumor cells (50,000) were injected into the allantoic vein of a 10-day-old chick embryo. Tissue was harvested 3 hr after injection to quantify the number of arrested tumor cells or at 4 days after injection to assess the growth of the arrested cells. Similar results were obtained in 3 experiments. n = 5. Values in (C) are represented as mean ± SEM. Scale bars: (A), 200 μm; (B), 20 μm.

the trailing end of the cell (arrowhead) detaches and rapidly contracts toward the cell body (Figure 6A, cells 1 and 2; Movies S3 and S4). These motile control cells were compared to the most motile of the tumor cells in the mAb 1A5-treated animals (such as Figure 5B, track 5, represented as cell 1 in Figure 6B). The enlarged frames in Figure 6B illustrate that the cells in the mAB 1A5-treated animals invade the stroma and appear to be capable of forward movement (see arrow, Figure 6B, cells 1 and 2). However, even though the centroid travels approximately 100 μm from the original position, the rear of the cell fails to follow the movement of the cell body and remains attached close to the original location (see arrowhead, Figure 6B, cells 1 and 2; Movies S5 and S6). A quantification of the migratory behavior of individual cells (n = 250) at the tumor-stroma interface demonstrated that 83.3% of these boundary cells in control tumors were motile (move more than one body length, >20 μm), and only 1.7% of these motile cells failed to detach at the rear of the cell during

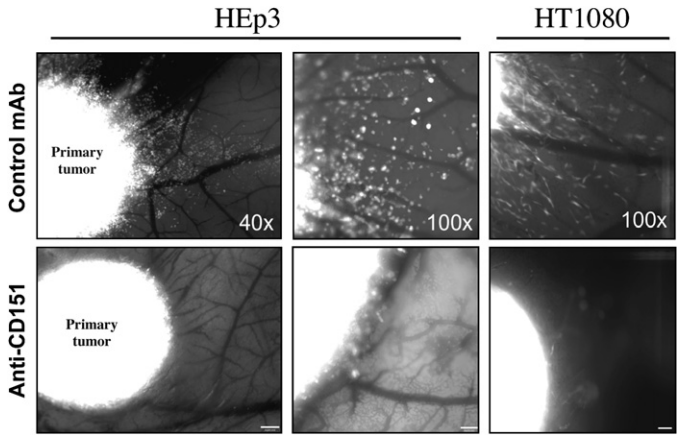


Figure 4. The Inhibition of Migration Prevents Tumor Cell Invasion at the Tumor-Stroma Interface

GFP-expressing HEp3 and HT1080 CAM tumors were imaged intravitally using a newly developed intravital imaging system (see the Experimental Procedures) to visualize the invasive behavior of metastatic tumor cells. Chick embryos were injected i.v. with 100 μg of mAb 1A5 or control mAb 24 hr after CAM implantation of GFP-expressing HEp3 cells. Images were taken 7 days after antibody treatment. The included images are representative of 10 individual tumors generated in two separate experiments with five animals each. Scale bars: (40×), 800 μm; (100×), 100 μm.

the time of observation (12.5 hr). In contrast, in mAb 1A5-treated tumors, 24% of these boundary cells exhibited such motility but 88% of these "motile" cells failed to detach at the rear of the cell. As a consequence, 81.4% of control cells exhibited productive motility compared to less than 3% of the cells in mAb 1A5-treated tumor.

Within a growing tumor, the inability of a proliferating cell to productively depart from its original position should result in a small compact colony of daughter cells. This was indeed true in primary tumors harvested from chick embryos treated with control mAb or mAb 1A5 (Figure S3). In addition, it was shown that in mAb 1A5-treated embryos, daughter cells fail to depart the location of the original parental cell during secondary site colonization (Figure 3B, 3C).

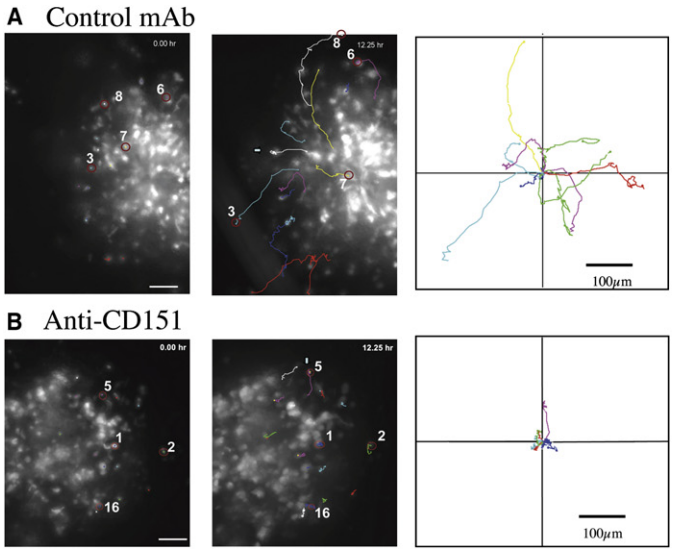
The failure to detach induced by the anti-CD151 mAb is also observed in vitro (Figures 6C and 6D). HEp3 cells treated with a control mAb clearly migrate and exhibit distinct detachment at the rear of the cell. In contrast, treatment with mAb 1A5 prevents detachment at the rear of the cells (white arrows), causing the cell to move back and forth in the same location without achieving productive movement. By quantifying detachment at the rear for individual tumor cells (Figure 6D, n = 30), the failure to detach and migrate productively is apparent (Figure 6D). In a three-dimensional collagen matrix, HEp3 cells which can invade the collagen matrix in the presence of a control mAb (Figure 6E, arrow) are completely immobilized in the presence of mAb 1A5 and form compact colonies (Figure 6E, right panel) as was also seen in the CAM in vivo (Figure 3B).

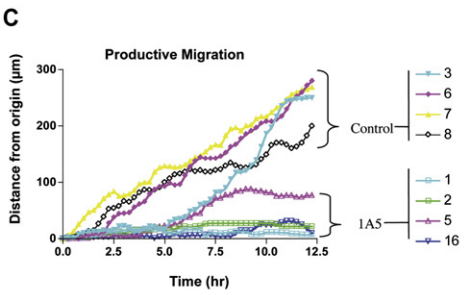
The Loss of CD151 Does Not Significantly Impact In Vivo Motility while Anti-CD151 mAb Treatment Mediates Immobility Only in the Presence of Human CD151

Recent reports of CD151 knockout mice have indicated that the genetic loss of CD151 does not result in dramatic developmental deficiencies (Wright et al., 2004; Sachs et al., 2006; Takeda et al., 2007), suggesting that migration is not critically impaired in these mice. To further explore the immobility mediated by CD151, we compared the in vivo migration of mouse embryonic fibroblasts (MEF), which lacked CD151 expression (MEF$^{CD151-/-}$) with wild-type MEF (MEF-WT) and with MEF$^{CD151-/-}$, which were reconstituted with human CD151 (MEF$^{CD151-/-}$hCD151). As seen in Figures 7E, 7G, and 7I, MEF-WT, MEF$^{CD151-/-}$, and MEF$^{CD151-/-}$hCD151 all migrate into the stroma as individual cells, away from the point of CAM implantation (white arrows, Figures 7E–7J). These observations indicate that the mere absence of CD151 does not significantly impair the in vivo mobility of a migrating cell. Similarly, the downregulation of CD151 expression in HT1080 cells by shRNA (Figure 7K) did not limit tumor migration in vivo (compare Figures 7A and 7C) nor did it inhibit metastasis significantly (Figure 7L). In contrast to the loss of CD151, treatment of HT1080 cells and MEF$^{CD151-/-}$hCD151 with anti-CD151 mAb 1A5 blocks invasion of the stroma (Figures 7B and 7J), and the cells remain tightly clustered at the CAM implantation site. However, cells that lack human CD151 (MEF-WT) or are substantially reduced in CD151 expression (HT1080 shRNA^{CD151}, MEF$^{CD151-/-}$) are unaffected by the antibody (Figures 7D, 7F, and 7H), indicating further that the anti-CD151 mAB is specific and is not targeting other molecules, which might result in off-target inhibition of motility. In addition to these illustrative in vivo results, HT1080 cell migration assays carried out in vitro on collagen coated surfaces also demonstrated that mAb 1A5 inhibits motility only when the CD151 antigen is present at normal levels on the cell surface (Figure S6). These observations demonstrate that cell surface CD151, upon antibody binding, induces pronounced immobility of individual cells.

Anti-CD151-Mediated Inhibition of In Vivo Motility Results in Complete Inhibition of Tumor Cell Intravasation

Since the anti-CD151-mediated inhibition of migration is a consequence of the inability of mAb 1A5-treated cells to detach at the rear of the cell, these tumor cells should not be able to escape from the primary tumor and enter the vasculature (intravasate). The arrival of intravasated human tumor cells in the lower CAM of tumor-bearing chick embryos is readily quantified (Figure 8) using the human *alu* PCR-based assay (Zijlstra et al., 2002). Intravasating tumor cells are first observed in the lower CAM on day 3 and continue to arrive every day thereafter. The intravasated tumor cells which arrive in the CAM proliferate in the ensuing days with an approximate doubling time of 21–24 hr. In order to ascertain the number of intravasating cells arriving in the lower CAM within a single day, we determine the increase in the tumor cell population between day 5 and 6 and compare that with the number predicted solely on the basis of a doubling





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Comparison of Motion Parameters

Parameter	control	mAb 1A5	p value	reduction
Total distance (μm)	288.07 ± 102.78	85.16 ± 36.86	p<0.0001	3.4 fold
Productive distance (μm)	150.83 ± 84.25	23.01 ± 18.94	p<0.0001	6.6 fold
Velocity (μm/hr)	22.96 ±8.31	5.88 ± 2.94	p<0.0001	3.6 fold
Persistence (μm)	4.39 ± 1.43	1.21 ± 0.61	p<0.0001	3.9 fold
Turn angle (degrees)	44.09 ± 7.65	37.61 ± 27.65	0.2736	1.2 fold

Figure 5. Tumor Cell Motility Is Inhibited at the Tumor-Stroma Interface in mAb 1A5-Treated Animals

GFP-expressing HEp3 tumor cells were imaged intravitally for 12.25 hr in order to visualize, record, and quantify the in vivo motility of individual tumor cell (see the Experimental Procedures). Antibody treatment was given i.v. 24 hr after tumor cell implantation and imaging was initiated 48 hr later. Images were captured every 15 min. The motility of tumor cells in a tumor developing on a control animal (A) and a mAb 1A5-treated animal (B) is represented by comparing the first frame (0.00 hr) and the last frame (12.25 hr) of a 12.25 hr movie (see Movie S1 [control] and Movie S2 [mAb 1A5]). The migration of individual cells is visualized as an overlay of dots and lines in each frame (left panels). The tracks of ten representative cells are plotted in Wind-rose plots (right panels) with the initial position of each track superimposed at 0,0 to provide a relative overview of the migration tracks with a single point of origin. The temporal increase in the productive motility of 4 representative tracks from control and treated tumors are plotted in (C). The most motile cell in the mAb 1A5 treated tumor (5) is still far less motile than the average control cells. (D) The motility parameters were determined quantitatively for cells in a tumor from animals treated with either control mAb or mAb 1A5. Motility parameters were determined as an average of 30 cells/tumor with 48 measurements/cell. p values were calculated using Student's t test. A highly significant reduction in forward motility, but no change in the ability to alter direction (turn angle), is apparent. Values are presented as mean ± SEM and representative of 3 distinct experiments. Scale bars in (A) and (B), 100 μm.

of those cells detected on day 5. The number of intravasated cells is the difference between the actual number cells present in the tissue and the number of cells expected due to cell doubling. In control animals, 6050 cells are present in the CAM on day 5 which should grow to ~12,000 cells by day 6 on the basis of their doubling time (dashed line in Figure 8A). However, ~29,000 cells are actually measured on day 6, yielding a calculated value of 17,000 newly arriving cells between day 5 and 6 (area above dashed line, Figure 8A). In contrast, there was no detectable intravasation over the same time period in mAb 1A5-treated animals as the approximately 1000 cells present in the lower CAM on day 5 failed to increase in numbers beyond a doubling (Figure 8A). Tumor growth in all of these experiments (Figures 8A and 8B) was not significantly different between control IgG and anti-CD151 treated animals and increased approximately 50 mg from D5 to D6.

We proposed that the inhibition of motility on any given day would bring about an abrupt inhibition of intravasation. Indeed, when chick embryos bearing HEp3 tumors were injected with mAb 1A5 on day 5, the tumor cell population detected in the CAM on day 5 (2,150) only expands to 4,470 cells by day 6 in close accordance to the cell doubling rate, and no newly intravasating cells are detected (area above the dotted line, Figure 8B). In contrast, control antibody-treated animals yield 11,500 cells on day 6, of which only 4,300 could be predicted from proliferation, thereby indicating a rate of intravasation of ~7,200/24 hr. These quantitative tumor cell measurements are consistent with the in vivo motility and morphological observations and demonstrate that CD151-mediated inhibition of motility subsequently prevents intravasation and thereby ultimately inhibits tumor cell dissemination.

DISCUSSION

The Regulation of Metastasis through Individual Tumor Cell Migration

This work identifies the role of tumor cell migration in metastasis as regulated through the tetraspanin CD151. Using highly sensitive detection of human tumor cells by PCR, along with intravital visualization of tumor cell migration, we determined that CD151 can control deadhesion at the rear of the cell, which thereby promotes immobility of tumor cells and, as a consequence, the successful dissemination of tumor cells from the primary tumor. Contrary to the ablation of CD151 expression, the ligation of CD151 with its cognate mAb 1A5 results in immobilization of CD151-expressing tumor cells. These observations demonstrate that metastasis requires migration as an active component of dissemination and intravasation.

The immobilization mediated by CD151 upon antibody ligation prevents invasion and dissemination at a number of points in the metastatic cascade, including migration within the primary tumor and at the secondary location. However, with regards to the full metastatic ability of human tumor cells, individual cell migration was critical only in the departure from the primary tumor

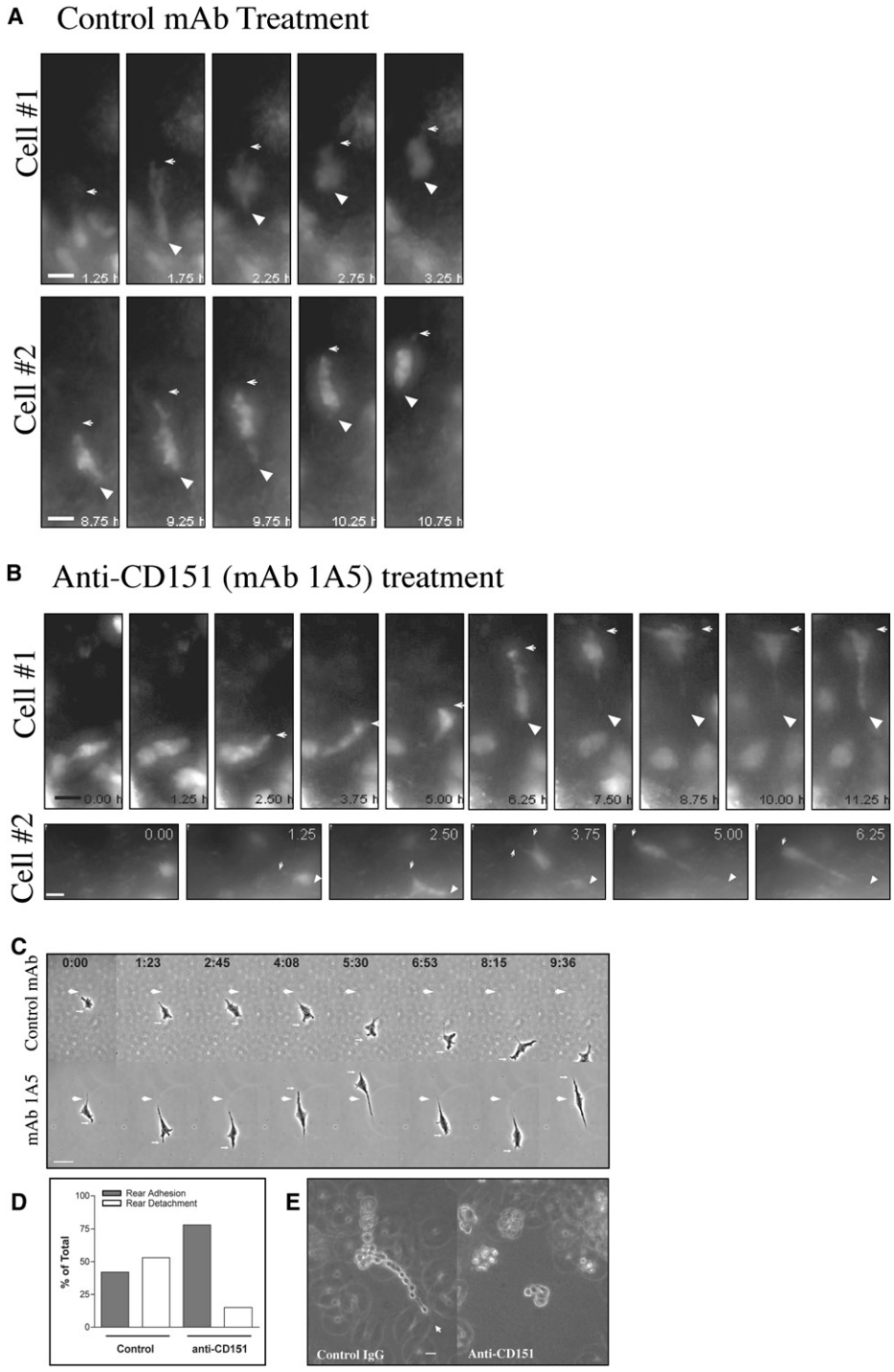

Figure 6. Anti-CD151 Mediated Inhibition of Migration Coincides with Incomplete Detachment at the Rear of the Cells

Selected fields from real-time movies were captured, enhanced, and processed to reveal the details of migration in the three-dimensional stroma of tumor-bearing animals treated with control mAb (A) or anti-CD151 mAb 1A5 (B). Also, see Movies S3, S4, S5, and S6. A white arrow indicates the leading edge of the migrating cell and a white arrowhead indicates the trailing end of the same cell. The scale bar is 20 μm and the time is marked on each frame. Note that, due to the differential in migration rates, the time line for the control cells is 0.5 hr/frame while the 1A5-treated cells are shown at 1.25 hr/frame. Anti-CD151-induced loss of detachment was confirmed in vitro by visualization (C) and quantitation (D) of HEp3 cell migration on a collagen monolayer (200×, large arrow indicates start position of the rear and small arrow indicates the protrusive edge. Also see Movie S9). The impact on invasion was assessed in vitro using HEp3 cultivated in 3-dimensional collagen (E) where the loss of invasion (white arrow, left panel) and the formation of compact circular colonies was evident (right panel). Scale bars: (A), (B), and (C), 20μm; (E), 10 μm.

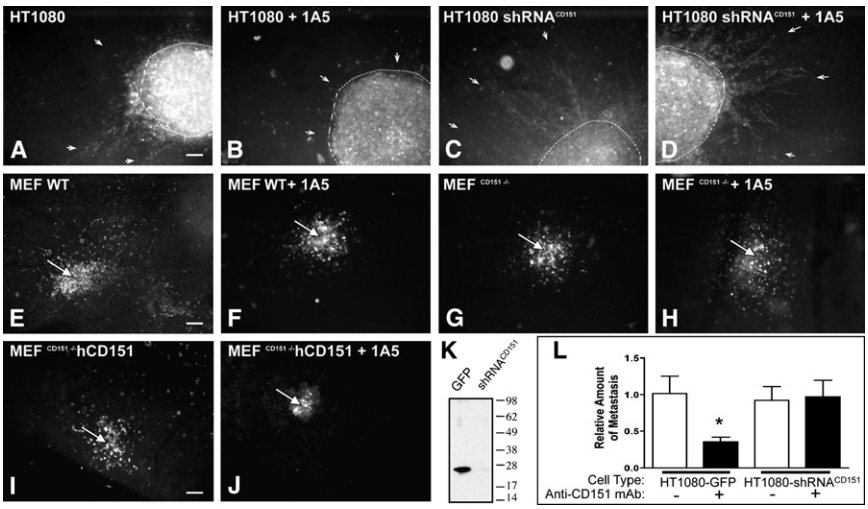

Figure 7. Anti-CD151 mAb Ligation Promotes Immobility while the Loss of CD151 Fails to Impact In Vivo Motility

The necessity of CD151 expression for in vivo motility of tumor cells and MEF was assessed by implanting fluorescently labeled cells with reduced or ablated CD151 expression into the CAM and determining their ability to depart the implantation site. The ability of HT1080 cells stably transduced with an expression vector containing GFP (HT1080) or CD151-specific shRNA (HT1080 shRNA^{CD151}) to migrate out as individual cells was assessed in the presence of control antibody (A) and (C) or anti-CD151 mAb 1A5 (B) and (D) at 4 days after tumor cell implantation. The white dotted line demarks the border of the primary tumor while arrows indicate the stromal invasion of tumor cells. Likewise, the inability of anti-CD151 mAb 1A5 to prevent individual wild-type MEF ([E] and [F], MEF wt) and CD151 knockout MEF ([G] and [H], MEF$^{CD151-/-}$) from mobilizing, departing the implantation site, and entering the stroma was illustrated by fluorescent microscopy 4 days after implantation. Similarly, the mobility of MEF$^{CD151-/-}$ reconstituted with hCD151 ([I], MEF$^{CD151-/-}$ hCD151) was assessed in the CAM. To illustrate the ability of anti-CD151 to actively promote immobility, the ability of MEF$^{CD151-/-}$hCD151 to disseminate from the implantation site was determined in the presence of mAb 1A5 (J). Each image is representative of 3 individual experiments with 3 animals each. A white arrow (E–J) indicates the injection site. Downregulation of endogenous CD151 in HT1080 shRNA^{CD151} is confirmed by western blotting of whole-cell lysates with anti-CD151 antibody (K). The impact of ablated CD151 expression on metastasis and the specificity of anti-CD151 treatment was further investigated by quantifying metastasis of HT1080 GFP or shRNA^{CD151} in the presence or absence of anti-CD151 mAb 1A5 (L). Values represent the mean ± SEM where n = 8. Scale bars, 200 μm.

and entry into the vasculature (intravasation). In contrast, arrest, extravasation, and proliferation at the secondary location appeared unaffected by the lack of productive motility. Tumor cells introduced directly into the circulation in the presence of anti-CD151 during experimental metastasis were able to arrest, extravasate, and proliferate within the surrounding stroma.

The Impact of Tumor Cell Immobilization by Anti-CD151 on the Progression through Individual Stages of Metastasis

It is of interest that extravasation, a process that involves active motility by the tumor cells (Luzzi et al., 1998; Chambers et al., 1992) (Movie S7), is not prevented by the inhibition of CD151-mediated migration. However, since extravasation requires arrested tumor cells to pass through a thin layer of endothelial cells and basement membrane without dissociating from any previously formed matrix contacts, this process can be achieved in a single body length. Individual cells in mAb 1A5-treated animals exhibit the ability to extend one body length (~15–20 μm) even though they fail to dissociate from their original location (Figure 6). This distance would be sufficient to pass through the vascular wall and enter the surrounding stroma. Since in vivo growth is not affected by mAb treatment, no diminishment of secondary site colonization occurs during experimental metastasis.

In contrast to experimental metastasis, spontaneous metastasis is inhibited >80%, thereby emphasizing the importance of CD151 in the departure of tumor cells from the primary tumor. Cells within the primary tumor exhibit a continuous mobility, illustrating that no tumor cell is "static" or entirely immotile. While much of the movement appears random, in fact a portion of the cells exhibit a persistent forward movement that facilitates invasion of the stroma (Figure 5A). This forward mobility is not always outwards but is also directed into the tumor mass (track 7 in Figure 5A), indicating that the migratory clues are not a simple, unidirectional chemotactic stimulus from the stroma. When plotting the productive migration of tumor cells, we observe that many migratory cells move forward in bursts of activity preceded or followed by a period of nonproductive movement. This migration appears to be guided at least in part by the vasculature (Figure S4 and Movie S8).

The 3- to 4-fold reduction in overall (total) tumor cell motility would not account for the near absence of invasion and the >80% reduction in spontaneous metastasis. However, a quantitative analysis of the motion parameters demonstrates that both the velocity and the persistence of movement are reduced 3- to 4-fold. Together, they are responsible for a 6- to 7-fold reduction in productive movement away from the point of origin (Figure 5D). Furthermore, because the mAb 1A5-treated tumor cell fails to depart its original position, even as it possesses some minimal movement, no significant productive departure from the primary tumor can be achieved, and thus, little or no metastasis can occur.

The inhibition of migration, it should be emphasized, does not result in the disruption of adhesive interactions but rather in the prevention of deadhesion. Our direct in vivo and in vitro observations of this behavior are corroborated by the quantitative measurements of migration. A disruption of adhesive capacity would have resulted in a complete loss of any movement, thus yielding a "0" value for all migration parameters. Conversely, loss of chemotactic or directional movement would lead to a reduction in persistence and a subsequent reduction in productive motility without changes in velocity or even total migration. In contrast, the joint decrease in velocity and persistence observed in mAb 1A5-treated animals corresponds with the reduced ability to deadhere. Rear detachment of migrating cells has previously been suggested to be the rate-limiting step in 2D migration in

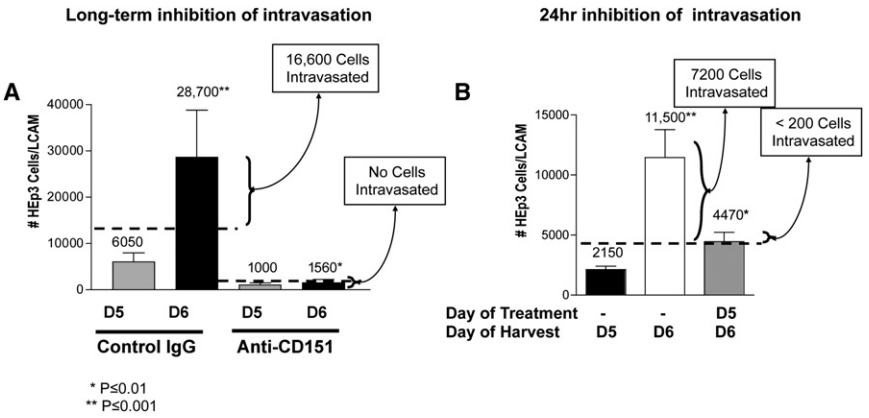

Figure 8. Function-Blocking Anti-CD151 Antibody, mAb 1A5, Inhibits Tumor Cell Intravasation

(A) Intravasation in animals treated i.v. on day 1 with mAb 1A5 or control mAb (mAb 29-7) as determined by *alu* PCR. The dashed line indicates the number of cells expected on day 6 based upon the growth (24 hr doubling time) of cells detected on day 5. The bar volume above the dashed line represent the number of intravasated cells. Similar results were obtained in 3 experiments. n = 6.

(B) To assess the immediate impact of mAb 1A5-arrested intravasation on metastasis, HEp3 tumor-bearing animals were injected 24 hr prior to harvesting the LCAM. The number of intravastated tumor cells in CAM tissue harvested on day 6 after a mAb treatment on day 5 was compared with the number of cells found in day 6 CAM from untreated animals. Values are represented as mean ± SEM. *$p \leq 0.01$, **$p \leq 0.001$. Tumor sizes in (A): control: D5, 143.5 ± 61; D6, 216 ± 100. mAb 1A5: D5 167 ± 72, D6 200 ± 85. Tumor sizes in (B): D5, 115 ± 40; control: D6, 200 ± 66; mAb 1A5: D6, 191 ± 71.

vitro (Gallant et al., 2005; Palecek et al., 1998). We present an observation that specifically demonstrates that an integrin-associated tetraspanin can control the cell's adhesive machinery such that it fails to disengage and, thus, prevents productive motility of a tumor cell in vivo. That this can occur within a complex ECM such as the tumor stroma with multiple adhesion ligands, indicates the importance of deadhesion in tumor cell motility.

While the exact mechanism by which CD151 controls migration is currently unknown, the direct interaction of CD151 with the laminin-receptor integrins ($\alpha3\beta1$ and $\alpha6\beta1$) suggests that the tetraspanin might regulate migration through direct control of integrin function. Indeed the expression of CD151 correlates positively with migration on laminin (Winterwood et al., 2006; Sterk et al., 2002) and CD151 has been shown to potentiate ligand binding activity of $\alpha3\beta1$ and $\alpha6\beta1$ (Nishiuchi et al., 2005; Lammerding et al., 2003). Most importantly, CD151 can facilitate adhesion strengthening (Nishiuchi et al., 2005; Lammerding et al., 2003), a process that can reduce migration by inhibiting detachment of the cell (DiMilla et al., 1993; Palecek et al., 1997). Our in vitro and in vivo observations suggest that CD151 can promote immobility on a variety of matrixes in addition to laminin. Furthermore, the mere expression of CD151 does not appear to be sufficient for regulating migration in vivo (Figure 7), an observation which is supported by the normal embryonic development of three independently generated CD151 knockout mice (Wright et al., 2004; Sachs et al., 2006; Takeda et al., 2007). Human patients with CD151 deficiencies suffer principally from regional skin blistering, sensorineural deafness, and kidney failure. The latter is also found in knockout mice. However, neither human nor mouse fails to form organs or heal skin wounds in the absence of CD151 suggesting that, at a cellular level, cells which lack CD151 retain a functional migration machinery. The migration of $MEF^{CD151-/-}$ in CAM stroma (Figure 7) confirms this hypothesis. Conversely, the near complete failure of CD151-positive human tumor cells and $MEF^{CD151-/-}$-expressing human CD151 to detach in response to anti-CD151 mAb demonstrates that antibody-bound cell surface CD151 can promote tumor cell immobility through a dominant-negative effect on migration. This action is likely to be mediated through an associated effector molecule because tetraspanins act primarily as scaffolding proteins in the membrane.

While direct interaction between integrins and CD151 is evident, several nonintegrin membrane proteins, which can regulate motility, are also associated with tetraspanin-enriched microdomains (Claas et al., 2001; Hemler, 2005). These membrane proteins include growth factor receptors (Klosek et al., 2005), Ig superfamily proteins and cell adhesion molecules (Le Naour et al., 2006), and E-Cadherin (Chattopadhyay et al., 2003), each of which is capable of impacting migration indirectly. The continued ability of CD151-negative cells to migrate in vitro and in vivo indicates that CD151 is neither sufficient nor necessary for migration. In contrast, the inhibition of migration in CD151 expressing tumor cells with the anti-CD151 mAb 1A5 indicates that the tetraspanin can promote immobility. This immobilization does not appear to depend on steric hindrance because monomeric Fab fragment of 1A5 binds to CD151 on tumor cells but does not promote immobility (Figure S5). In the absence of catalytic domains in CD151, it is likely that CD151 recruits elements that promote immobility or sequesters elements needed for deadhesion upon ligation with the cognate antibody. The identification of the partner(s) that associate with CD151 and mediate control over cellular (de)adhesion should reveal the molecular mechanism responsible for controlling deadhesion. Such identification studies are now ongoing.

Preventing Metastatic Dissemination through the Inhibition of Migration

The microenvironment to which a metastasizing tumor cell is exposed en route to a secondary site is both an architectural as well as a signaling framework that is continually instructive with regards to cellular behavior (Bissell and Labarge, 2005). The primary approach to intervening with the invasive nature of cancer has been focused on the identification and disruption of extracellular signals that promote migration (Wyckoff et al., 2004; Clark et al., 2000), the disruption of adhesive properties (Murphy-Ullrich, 2001; Czekay et al., 2003), or the disruption of migration mediated signaling downstream of the cell surface sensors (Guo and Giancotti, 2004). Since adhesive interactions with these highly instructive surroundings are in large part responsible for migration, it may seem intuitive that disruption of such adhesions would prevent tumor cell dissemination. However, our data present a case study in which the contrary, the inhibition of

deadhesion, can be an equal or even more powerful approach to preventing metastasis.

The disruption of a single adhesive function through blocking antibodies or peptides directed toward the ligand or its receptor (integrin) can readily disrupt in vitro adhesion and migration (Ruoslahti, 1996) or alter the survival (Stupack et al., 2006) and morphology (Kenny and Bissell, 2003) of tumor cells within the matrix. However, within the complexity of the in vivo stroma, it is difficult to disrupt the multitude of adhesive interactions effectively. In fact, disabling or blocking a single proteolytic or adhesive event often causes a tumor cell to alter their mechanism of migration without the loss of productive motility (Friedl and Wolf, 2003). In contrast, a single interfering step that prevents dissociation can immobilize the target cell without having to out-compete newly available adhesion sites, continuously renewing chemotactic stimuli or matrix modifying enzymatic activities. This is apparent in our ability to promote immobility on multiple matrixes to which the tumor cells adhere (Figure 2). The immobilization that occurs does not prevent localized protrusion and forward movement of the cells. In contrast, immobilized cells can be seen protruding into the immediate surroundings and adhering to the newly invaded space. However, as the rear of the cell fails to dissociate, the cell halts upon extending one body length and remains immotile or returns to its original location (Figure 6). Ultimately, the consequence of this inability to dissociate blocks the invasive behavior of tumor cells and prevents intravasation and, thereby, the metastasis of primary tumor cells.

The role of migration as an active contributor to metastasis is a central question at the forefront of metastasis research (Bockhorn et al., 2007). The immobility mediated by CD151 upon antibody ligation is in vivo proof of principle that migration is an active mechanism that contributes to metastasis. Furthermore, it is to our knowledge the first direct evidence demonstrating that regulation of deadhesion can function as a powerful antimetastasis target. Improving the stabilizing interactions of tumor cells with the microenvironment has already proven successful in reverting the malignant phenotype independent of its genetic instructions (Weaver et al., 1997). Negative regulators of migration, which enhance adhesion of tumor cells to their surroundings, could be highly effective in preventing the invasive behavior of tumors as well as inhibiting metastasis. The CD151-mediated inhibition of migration clearly demonstrates the role of migration in tumor cell departure from the primary tumor and the role of CD151 and cell deadhesion in tumor cell motility, intravasation, and subsequent metastasis.

EXPERIMENTAL PROCEDURES

Cells, Antibodies, and Reagents

The epidermoid carcinoma cells (HEp3) and fibrosarcoma cells (HT1080) were maintained as described previously (Zijlstra et al., 2002). CD151−/− mouse embryonic fibroblasts ($MEF^{CD151-/-}$) were kindly provided by the laboratory of Dr. Sonnenberg (Netherlands Cancer Institute). Wild-type MEF and $MEF^{CD151-/-}$ were immortalized using pBRSV (ATCC) encoding SV40 Large T antige, and human CD151-GFP expression was reconstituted in $MEF^{CD151-/-}$ ($MEF^{CD151-/-}$hCD151) by transduction with PLXN containing CD151-GFP. Transduction with shRNA containing the target sequence GCCTCAAGTACCTGCTGTTTAC was used to downregulate CD151 expression in HT1080 cells ($HT1080shRNA^{CD151}$). Positive and negative cell sorting was used to collect $MEF^{CD151-/-}$hCD151 and $HT1080shRNA^{CD151}$, respectively. The anti-CD151 monoclonal antibody 1A5 (mAb 1A5) and control antibodies directed to the surface of HEp3 cells were obtained by subtractive immunization (Zijlstra et al., 2003; Testa et al., 1999) and purified endotoxin free by protein-G sepharose affinity purification. To generate cell lines expressing green fluorescent protein (GFP), cultured cells were infected with PLXN-GFP and selected by neomycin selection without clonal propagation. Selected cells were passaged in vivo to maintain the metastatic phenotype.

The Chick Embryo Metastasis Model

The quantitative spontaneous and experimental metastasis assays were performed as described previously (Zijlstra et al., 2002). In spontaneous metastasis assays, the tumor cells are placed directly on the CAM where a primary tumor forms over a period of 7 days, while in experimental metastasis, the tumor cells are injected i.v. and allowed to colonize organs for 7 days. At the end of indicated time periods, tumors are harvested, weighed, and processed for histology. Metastasis is determined by harvesting lower CAM (LCAM), lung, and liver and processing the tissue for the detection of human DNA by *alu* PCR. For i.v. delivery of antibodies, the injections were performed the day after tumor cell application unless indicated otherwise. To analyze arrest, extravasation, growth, and colonization, 5×10^4 cells were injected i.v. into each egg at developmental day 12. Human tumor cell metastasis in the ex ovo model was developed on the basis of our previously established CAM angiogenesis model (Seandel et al., 2001; Lewis et al., 2006). Human tumor cell behavior was analyzed in shell-less embryos as described below after i.v. injection using a glass capillary needle or during primary tumor formation after topical application of tumor cells directly onto the CAM.

Mouse SCID Spontaneous Metastasis

To assess the metastasis of HEp3 cells in mice, 3×10^6 cells were injected subcutaneously and tumors were allowed to form for 17 days. Control mAb or mAb 1A5 was administered i.v. at 5 days and 13 days after tumor cell injection. The lungs of tumor-bearing animals were harvested at the end of the experiment, and 50 mg of lung tissue was processed as described for chick lungs (Zijlstra et al., 2002). All animals (mice and chick embryos) were housed, maintained, and treated by procedures approved by our Institutional Animal Core and Use Committee (IACUC) and state and federal guidelines for the humane treatment of laboratory animals.

Detection of Human Tumor Cell Arrest, Growth, Intravasation, and Metastasis

The detection of human tumor cell metastasis in chick tissue was based upon the quantitative detection of human *alu* sequences present in chick tissue DNA extracts (Zijlstra et al., 2002). To approximate the actual number of tumor cells present in each tissue sample the *alu* signal from experimental samples was interpolated with a standard curve. A separate standard curve was generated for mice and chicken. The actual number of tumor cells/50 mg lung tissue could be determined over a range of 50 to 100,000 cells/50 mg tissue. Tumor cell arrest was determined by quantifying the number of cells present in a tissue within 24 hr after i.v. injection. In vivo tumor cell growth was quantified by determining the expansion of arrested cells from day 1 to day 4. Intravasation was determined by comparing the number of cells that appeared in lung, liver, or lower CAM of tumor-bearing animals on day 5 versus day 6. The increase in cell number that could not be accounted for by growth of the cells present on day 5 is considered the number of intravasated cells.

Human Tumor Cell Motility

In Vitro Migration Assays

Cellular migration in vitro was analyzed using a Boyden chamber system as previously described (Degryse et al., 1999). Pore filters (8 μm) were coated with indicated matrix components, and matrix-mediated migration was analyzed 16 hr after 50,000 cells were applied to the top chamber. Antibodies were applied at 10 μg/ml unless stated otherwise. Migrating cells were quantified in ten random fields of three replicate wells.

End Point In Vivo Extravasation and Motility Assays

The ability of tumor cells to extravasate was analyzed during experimental metastasis by imaging the location of GFP-expressing tumor cells with respect to the vasculature by which they arrived. To achieve this, 1×10^5 GFP-expressing HEp3 and HT1080 cells were i.v. injected and allowed to arrest and colonize the CAM for 24 hr to 5 days at which time the CAM was harvested and

mounted under a glass coverslip in standard IF mounting medium. Immediately prior to harvesting, 50 μg of Rhodamine-conjugated *Lens Culinaris* Agglutinin (LCA) (Vector Laboratories Inc., Burlingame CA) was injected i.v. to label the chick vasculature (Jilani et al., 2003). Tumor cells were considered to have extravasated when the GFP signal was no longer inside the Rhodamine labeled vasculature, as determined by confocal imaging (MRC1024 laser scanning confocal microscope) (Biorad, Hercules CA).

The motility of human tumor cells during experimental metastasis in vivo was assessed by imaging tumor cells in the CAM after i.v. injection. 1×10^5 GFP-expressing tumor cells were injected i.v. on embryonic day 12 and colonization of the CAM as well as tumor cell dissemination within the CAM stroma was assessed by harvesting CAM from 3 animals at indicated time points and imaging the tumor cell population by epifluorescent microscopy (Olympus BX60, Melville, NY). Digital images were acquired using the C-view imaging software (DVC, Austin, TX) and further processed using Photoshop (Adobe, San Jose, CA).

As the arrested tumor cell population grows, the expanding tumor cell population disseminates throughout the CAM stroma. Failure of the proliferating tumor cells to migrate results in compact colony formation.

Intravital Real-Time Imaging of Tumor Cell Motility

To achieve real-time imaging of tumor cells migrating within the tissue stroma and the primary tumor itself, a chick-embryo-imaging unit was constructed. This unit was designed for the purpose of maintaining the chick embryo under proper temperature and humidity as well as immobilizing the tissue to be imaged while providing a self-contained unit that prevents exposure of the microscope electronics and objectives to humidity and heat. This chick-embryo-imaging unit was built around the shell-less (ex ovo) system developed previously (Seandel et al., 2001) in which chick embryos are maintained without the shell in a plastic dish. The CAM, which is normally located immediately underneath the eggshell in ovo, is now exposed as a free-floating organ atop the developing embryo. Migration of cells within the primary tumor was monitored by imaging a tumor generated within the CAM underneath a coverslip. This was achieved by implanting a mixture of GFP-expressing and non-GFP tumor cells directly into the CAM mesenchyme using a capillary needle at developmental day 10 and covering the area with a sterile coverslip. The animal was subsequently returned to the incubator and imaged at predetermined time points by immobilizing the coverslip in the chick-embryo-imaging unit.

Image Capture and Processing

Real-time imaging of tumor cell migration was achieved by capturing a four-dimensional image series of the entire tumor within the CAM. A 150 μm image stack was captured with a 15 μm step size every 15 min for 12.5 hr (50 frames) using an upright epifluorescent microscope with a motorized Z stage (Carl Zeiss, Thornwood, NY) controlled by Openlab (Improvision, Lexington, MA). For each time point, a 60 μm stack containing the in focus images of the tumor was selected and projected as an "extended focus" image using Volocity (Improvision, Lexington, MA). Image drift and rotation was correct by using the Stack_Reg plugin (Biomedical Imaging Group, http://bigwww.epfl.ch/) running in the open-source software ImageJ (NIH). Cell tracking and motility quantitation was completed by visually determining the centroid (center of the cell body) at every 15 min interval and tracing it from one frame to the next using the Manual_Tracking plugin running in ImageJ. These traces were used for migration analysis of the motion parameters velocity, turn angle, and persistence as described previously (Mandeville et al., 1995). Total migration was defined as the total distance migrated, while productive migration was defined as the absolute distance from the point of origin. The ability of a cell to mobilize itself (motility) is defined as centroid movement in excess of one body length (>20 μm).

Histological Analysis of Chick Tissues

For fluorescent staining, each tissue was frozen in Tissue-Tec mounting media, and 6 μm sections were fixed in Zinc-Formalin and processed for immunological detection of tumor antigens. To detect antigen expression, tumor tissue was incubated with tumor-specific mouse monoclonal antibodies, and primary antibody binding was visualized with fluorescently labeled secondary (anti-mouse) antibody. To visualize in vivo tumor localization of tumor specific antibodies, tumor tissue and control tissues from antibody-injected animals were incubated with fluorescently labeled secondary anti-mouse antibodies only.

SUPPLEMENTAL DATA

The Supplemental Data include six supplemental figures and nine supplemental movies and can be found with this article online at http://www.cancercell.org/cgi/content/full/13/3/221/DC1/.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants R01 HL068648 (to H.S.), R01 CA105412 (to J.P.Q.), NSERC PDF-313420 (to J.L.) and American Heart Fellowship 0225103y (to A.Z.) We thank Dr. Neil Dilley for assessing motility parameters, Chenxing Li for tissue processing, Rebecca Mellor for technical assistance, and Alicia Palestini for administrative assistance. TSRI manuscript no. 17769-CB.

Received: October 10, 2005
Revised: October 12, 2007
Accepted: January 30, 2008
Published: March 10, 2008

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