Matrix Metalloproteinase 2-Integrin $\alpha\beta 3$ Binding Is Required for Mesenchymal Cell Invasive Activity but Not Epithelial Locomotion: A Computational Time-Lapse Study

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Cellular invasive behavior through three-dimensional collagen gels was analyzed using computational time-lapse imaging. A subpopulation of endocardial cells, derived from explanted quail cardiac cushions, undergoes an epithelial-to-mesenchymal transition and invades the substance of the collagen gels when placed in culture. In contrast, other endocardial cells remain epithelial and move over the gel surface. Here, we show that integrin $\alpha\beta 3$ and matrix metalloproteinase (MMP)2 are present and active in cushion mesenchymal tissue. More importantly, functional assays show that mesenchymal invasive behavior is dependent on MMP2 activity and integrin $\alpha\beta 3$ binding. Inhibitors of MMP enzymatic activity and molecules that prevent integrin $\alpha\beta 3$ binding to MMP2, via its hemopexin domain, result in significantly reduced cellular protrusive activity and invasive behavior. Computational analyses show diminished intensity and persistence time of motility in treated invasive mesenchymal cells, but no reduction in motility of the epithelial-like cells moving over the gel surface. Thus, quantitative time-lapse data show that mesenchymal cell invasive behavior, but not epithelial cell locomotion over the gel surface, is partially regulated by the MMP2-integrin interactions.

INTRODUCTION

Cell motility within a three-dimensional (3D) tissue space, often termed invasion, is a dynamic process involved in a host of morphological and pathological events (Harris, 1987). Extracellular matrix (ECM) fibers provide mechanical support for tissue integrity/deformations, act as a scaffold for cell motility, and act as a repository of growth factors and latent enzymes (McCarthy and Turley, 1993; Damsky *et al.*, 1997; Friedl and Brocker, 2000; Davis and Senger, 2005). Cell invasion is often coupled to local ECM degradation (Ellis and Murphy, 2001). In an extreme case, structures termed invadopodia have been observed on the surface of invasive tumor cells. These structures are cellular protrusions that contain surface-bound, externally facing proteases (Chen, 1989; Mueller and Chen, 1991; Monsky *et al.*, 1993, 1994; Kelly *et al.*, 1994).

During normal embryonic development extracellular matrix protease activity is important for successful epithelial-to-mesenchymal transformations (EMT) that accompany neural crest, sclerotome, and renal tubular cell delamination (Cheng and Lovett, 2003; Song *et al.*, 2000; Duong and Erickson, 2004). During epithelial-to-mesenchymal transformations ECM proteolysis may not only remove a physical

barrier, but also expose cell receptor sites and regulatory molecules sequestered in the ECM. Similar proteolytic events are recapitulated during angiogenesis and tubulogenesis in vitro (Bayless and Davis, 2003; Fisher *et al.*, 2006). When deprived of specific proteolytic mechanisms, some cell types change their motility strategy and move through the ECM by means of amoeboid behavior (Wolf *et al.*, 2003).

Degradation of the ECM occurs by a number of secreted proteinases. One family, the matrix metalloproteinases (MMPs), is composed of zinc-dependent enzymes capable of degrading most ECM molecules. The role of MMP2, a secreted MMP, is well established during invasive activity of many cell types (Monsky *et al.*, 1993; Partridge *et al.*, 1997; Koyama, 2004). In particular, the molecular interaction between MMP2 and integrin $\alpha\beta 3$, via the hemopexin C (PEX) domain, was shown to be essential for efficient cell invasion and angiogenesis (Brooks *et al.*, 1996, 1998; Ria *et al.*, 2002; Karadag *et al.*, 2004). An organic molecule, TSRI265, created to selectively block the MMP2-integrin $\alpha\beta 3$ binding acts as a potent antiangiogenic agent, thereby indirectly blocking tumor growth in vivo (Silletti *et al.*, 2001; Leroy-Dudal *et al.*, 2004). By mimicking the PEX domain, TSRI265 competitively blocks MMP2-integrin binding, without disrupting the integrin's affinity for ECM ligands, or suppressing MMP2 enzymatic activity. However, a recent in vitro study found no evidence of a role for MMP2 in angiogenesis. The work also questioned the specificity of the MMP2- $\alpha\beta 3$ binding (Nisato *et al.*, 2005). Motivated by this apparent contradiction, as well as the lack of functional reports on how the PEX-mediated MMP2-integrin interaction modulates cell invasion, we investigated the roles of MMP2 and its

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PEX domain by using an in vitro model of endocardial cushion invasion and dynamic imaging, which faithfully mimics a normal morphogenic process.

Endocardial cushions appear as cell-free swellings between the endocardium and myocardium in the atrioventricular (AV) and outflow tract regions of the embryonic heart shortly after the onset of circulation. The cushions function as primitive valves and are remodeled into mature valve leaflets during subsequent cardiac morphogenesis. The cushions contain a complex ECM sometimes referred to as an expanded myocardial basement membrane, or cardiac jelly (for review, see Eisenberg and Markwald, 1995). Perhaps the cardinal event during cushion morphogenesis is the seeding of the cardiac jelly by motile cells derived from the endocardium through EMT (Mjaatvedt and Markwald, 1989; Potts *et al.*, 1991; Brown *et al.*, 1996, 1999; Nakajima *et al.*, 1998; Ramsdell *et al.*, 1998; Boyer *et al.*, 1999; Nakajima *et al.*, 2000; Boyer and Runyan, 2001; Desgrosellier *et al.*, 2005; Wang *et al.*, 2005). A well-established in vitro model of the cushion seeding consists of transplanting quail AV cushions onto the surface of 3D collagen I gels; the gel is subsequently populated by invasive cells that originated from the endocardium via an epithelial-to-mesenchymal transformation (Runyan and Markwald, 1983). When combined with our dynamic imaging system, this experimental model enables us to record and statistically analyze mesenchymal and epithelial cell motility (Czirok *et al.*, 2002; Zamir *et al.*, 2006).

The time-lapse data demonstrate that agents known to prevent $\alpha_v\beta_3$ -MMP2 binding, via the hemopexin domain, distinctly alter parameters that characterize mesenchymal cell invasive activity through a collagen gel, including the intensity and persistence time of motility, and protrusive activity. The same reagents do not alter concomitant epithelial cell locomotion over a gel surface.

MATERIALS AND METHODS

Antibodies and Reagents

Polyclonal antiserum to chicken MMP2 was provided by Dr. Jinq-May Chen, formerly at the Babraham Institute (Cambridge, United Kingdom). The immunoglobulin G (IgG) fraction of the MMP2 antisera and LM609 ascites was purified via protein A and protein G affinity chromatography, respectively, and dialyzed into phosphate-buffered saline (PBS), pH 7.3. Fluorescently labeled (dichlorotriazinylamino fluorescein [DTAF] and Cy5) secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Zinc chelator 44127 was provided by Dr. William Parks (University of Washington, Seattle, WA).

Staging Quail Embryos and Atrioventricular Cushions

Fertile quail eggs (*Coturnix coturnix japonica*; Ozark Egg, Stover, MO) were incubated in a humidified atmosphere at 37°C until the appropriate Hamburger and Hamilton (HH) stage (Hamburger and Hamilton, 1951). Atrioventricular (AV) cushions were designated as "early" stage specimens when the mesenchymal cell population is still sparse within the slightly expanded cardiac jelly space. Such early cushions were removed from approximately HH stage 15 embryos, based on somite number (24–27 somites) and length of incubation (50–55 h). However, maturation of AV cushions exhibits considerable variability calibrated against a staging procedure relying on HH criteria. "Intermediate" stage AV cushions exhibit inferior and superior AV cushions that make contact at their luminal surfaces. Such explants were usually isolated from approximately HH stage 18 embryos based on somite number (30–36 somites) and length of incubation (65–69 h). Cushions from approximately HH stage 23 embryos, based on incubation time (~84 h), typically exhibit a well-defined AV canal; such primordia were considered "late" stage cushions for the purposes of this study.

SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Immunoblot

For immunodetection of MMP2, early, intermediate, and late stage hearts were removed from the quail embryos while immersed in ice-cold embryonic phosphate-buffered saline (EPBS: 137 mM NaCl, 2.69 mM KCl, 8.1 mM Na_2HPO_4 , 1.47 mM KH_2HPO_4 , 0.68 mM CaCl_2 , and 0.49 mM MgCl_2 , pH 7.3).

The AV cushions were dissected free of other heart tissue and held in ice-cold EPBS until all samples were collected. The AV cushions, including the endocardium and a small amount of remaining adherent myocardial cells, were extracted in 50 mM octylglucoside buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM MgCl_2 , 1 mM CaCl_2 , and 2 mM phenylmethylsulfonyl fluoride) on ice. The homogenate was centrifuged for 5 min at 14,000 rpm, and the supernatant was stored at -20°C. Samples were thawed on ice and evaluated for protein concentration using the Bio-Rad (Hercules, CA) DC Protein Assay. Forty micrograms of total protein was loaded per lane on a freshly prepared Tris-glycine 4–12% gradient gel and subjected to electrophoresis using Laemmli electrophoresis buffer (Laemmli, 1970). Prestained SDS-PAGE molecular mass standards (Novex, San Diego, CA) were included for molecular mass estimation. Chicken MMP2, affinity-purified from embryonic fibroblast-conditioned medium (0.5 μg), was loaded as a positive control. Polypeptides were transferred by electroblotting onto nitrocellulose membrane (0.45- μm pore size, Nitropure Nitrocellulose; Micron Separation, Westboro, MA) in Tris-glycine buffer, pH 8.0. Adventitious protein binding was blocked using 5% (wt/vol) whole milk, after which the membranes were probed with rabbit anti-MMP2 serum (IgG fraction) at 0.5 $\mu\text{g}/\text{ml}$ for 1 h at room temperature. The MMP2 antibody was detected with a horseradish peroxidase-conjugated goat anti-rabbit secondary IgG at 0.25 $\mu\text{g}/\text{ml}$, and subsequent immunoreactivity was observed using chemiluminescent techniques (ECL kit; GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom).

Gel Substrate Zymography

Cell extracts were prepared in octylglucoside extraction buffer as described above. Samples for zymographic analysis were diluted twofold using zymogram buffer (0.5 M Tris-HCl, pH 6.8, 10% glycerol, and 0.1% bromophenol blue). Diluted samples were loaded, without boiling, into freshly prepared 10% SDS-polyacrylamide gels, containing 0.3% (wt/vol) gelatin, with a 5% stacking gel. Chicken MMP2, affinity-purified from embryonic fibroblast-conditioned medium, was loaded (0.25 μg) as a positive control. Prestained SDS-PAGE molecular mass standards (Novex) were included for size estimation of proteins exhibiting gelatinolytic activity. After electrophoresis using Laemmli buffer, the gel was dialyzed against 100 ml of 2.5% Triton-X 100 in Tris-buffered saline on a rotary shaker for 1 h at room temperature. Noncontrol lanes were placed in 100 ml of zymogram developing buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 5 mM CaCl_2 , and 0.2% Brij-35, wt/vol) and incubated at 37°C for 24 h. Control lanes were cut from the gel and similarly incubated but in the presence of an enzyme inhibitor, 1,10-phenanthroline (0.3 mM), to demonstrate that subsequent proteolytic clearing was due to metalloproteinase activity. The gels were then stained in 100 ml of 0.25% Coomassie Brilliant Blue 250-R in 45% methanol, 10% acetic acid for 3 h at room temperature on a rotary shaker. Destaining of the gels occurred in three changes of 45% methanol, 10% acetic acid for durations of 15, 30, and 60 min, respectively. The gels were then incubated in 1% glycerol (vol/vol) in H_2O for 1 h at room temperature. Protease activity was evident as unstained clear zones in the gels. Gels were scanned on a flatbed scanner at 1800 dpi.

Whole Mount Immunolabeling

Appropriately staged embryos were removed from the yolk by adherence to filter paper rings, rinsed in EPBS, and then placed ventral side up onto the surface of 5% agar (in PBS) plates. Live embryos were microinjected directly in the AV cushions with an approximate 60-nl single bolus of the IgG-purified polyclonal anti-chicken MMP2 serum using a Medical Systems PL1–100 pico-injector coupled to a Narishige four-channel hydraulic micromanipulator workstation (Drake and Little, 1999; Little and Drake, 2000). Control embryos were microinjected with the IgG fraction of corresponding nonimmune serum. Double-fluorescence labeled embryos were injected in the same manner with a single bolus injection containing both the polyclonal MMP2 IgG and the monoclonal integrin $\alpha_v\beta_3$ (LM609) IgG. Embryos were then cultured as described previously (Drake *et al.*, 1995) for 2 h. Subsequently, the embryos were rinsed in PBS, pH 7.3, and fixed in 3% buffered paraformaldehyde for 30 min. The samples were made permeable in 100% ethanol, rehydrated through a graded dilution of ethanol and water, and then incubated overnight at 4°C in a mixture of Cy5-conjugated goat anti-mouse IgG and DTAF-conjugated donkey anti-rabbit IgG (10 $\mu\text{g}/\text{ml}$ in 3% bovine serum albumin [BSA] in PBS, pH 7.3). After extensive rinsing in PBS, the embryos were embedded in paraffin, sectioned at 7 μm in thickness, and mounted on Superfrost-plus (Thermo Fisher Scientific, Waltham, MA) glass microscope slides. Epifluorescence and differential interference contrast (DIC) images were acquired and processed. Confocal images were generated on an Axioscope (Carl Zeiss, Thornwood, NY) with an MRC-1024 laser scanhead (Bio-Rad, Hercules, CA). Images were processed using NIH Image 1.6 (National Institutes of Health, Bethesda, MD) and Adobe Photoshop (San Jose, CA).

In Vitro Cellular Immunolabeling

Collagen gels were prepared according to Runyan *et al.* (1983). Briefly, rat-tail collagen type I gels (2.0 mg/ml) were made by mixing 4°C collagen type I (BD Biosciences Discovery Labware, Bedford, MA) with a 4°C aqueous solution of PBS and NaOH. To four wells of a 12-well Costar polystyrene culture chamber (Corning, Corning, NY), 400 μl of the collagen solution was aseptically

transferred to coat the bottom of the well. Solutions were allowed to gel at 37°C for 30 min. The gels were incubated for 1 h with 500 μ l of CO₂-independent medium supplemented with 2 mM L-glutamine; 1 $\times$ insulin, transferrin, and selenium (ITS); and 1% Pen/Strep (Invitrogen, Carlsbad, CA). The medium was then aspirated from the wells, and three atrioventricular cushion explants per well were placed onto the gel with a sterile pipette. Explants were allowed to adhere to the gel for 6 h at 37°C before 500 μ l of fresh culture medium was added. Typically, the explants were incubated for an additional 48 h.

To reveal epitopes masked by growth in the three-dimensional collagen gels, the cushion explants were treated with Triton X-100 and trypsin to facilitate antibody binding. Endocardial cushion explants were fixed in PBS-buffered 3% paraformaldehyde for 5 min at room temperature. After two quick washes with PBS, the collagen explanted cushions were treated with 0.3% Triton X-100 (in PBS) for 10 min at room temperature. After another two washes with PBS, the cushions were trypsinized (Sigma-Aldrich, St. Louis, MO) for 2 min while on ice (1.0 mg/ml). The trypsin was removed, and excess was inactivated with 5% goat serum in PBS at room temperature for 15 min. Samples were blocked with 3% BSA overnight at 4°C. Primary antibodies (LM609 at 10 μ g/ml and anti-MMP2 at 5 μ g/ml) were incubated at room temperature for 6 h. The explants were washed overnight in PBS at 4°C. Explants were further washed 5 $\times$ 1 h in PBS. Samples were blocked again in a mixture of 5% goat serum in 3% BSA for 1 h at room temperature. DTAF-conjugated goat anti-mouse IgG and Cy5-conjugated goat anti-rabbit IgG were added at 1:200 dilutions. Secondary antibodies were incubated overnight at 4°C. The collagen explants were washed extensively with PBS and then mounted for imaging.

In Vitro Cellular Outgrowth Assays

Collagen gels were prepared as reported above. Then, 250 μ l of rat tail collagen type I solution was aseptically pipetted into each well of a 24-well cell culture dish and incubated at 37°C for 30 min. The gels were rinsed twice for 15 min with M199 then incubated overnight in defined culture medium: M199 + ITS (Invitrogen). The medium was aspirated from the gels and the explants were placed onto the surface of the gel with a sterile pipette. The explants were allowed to adhere for 4 h at 37°C before defined medium (M199 + ITS), containing inhibitors of MMP activity (EDTA, zinc chelator 44127, rabbit anti-chicken MMP2 antibodies, rhTIMP2, TSRI265 and TSRI359), or corresponding controls, was pipetted onto the gels. The medium was changed every 24 h for a period of 72 h. The explants were fixed in 2% buffered glutaraldehyde, stained with 0.125% Coomassie Blue, and viewed on a Leitz stereomicroscope. Images were recorded using a charge-coupled device camera (DAGE-MTI, Michigan City, IN). The average diameter of cellular outgrowth was measured using Image-ProPlus (Media Cybernetics, Silver Spring, MD). Statistical analysis was performed using SigmaPlot (Systat, San Jose, CA).

In Vitro Time-Lapse Motility Assay

Fertile quail eggs (*C. coturnix japonica*; Ozark Egg) were incubated in a humidified incubator at 37°C until the appropriate stage (HH stage 20, 40–43 somites, and ~72-h incubation). Collagen gels were prepared as described above in 12- or 24-well tissue culture plates. Once the excised cushions attached to the gel surface, fresh culture medium (with inhibitory reagents or corresponding controls) was gently pipetted into the wells. Organic reagents TSRI265 and TSRI359 were used at 10 μ M. Recombinant PEX was used at 10 μ g/ml. Before the recording, the chamber was sealed to maintain a humidified atmosphere within.

Transplanted cushions were imaged by time-lapse scanning ("4D") automated microscope systems (Czirok *et al.*, 2002; Rupp *et al.*, 2003). Briefly, motorized optical microscopes were partially enclosed within an incubator box heated to 37°C. A temperature-regulated airflow within the incubator distributed 38.5°C air across the upper surface of the multiwell plate. The resulting temperature difference prevents water condensation on surfaces within the optical path. An inverted Olympus CK2 microscope, equipped with a 4 $\times$ objective, was used in bright field optical mode to monitor the cell population during the invasion of the collagen gel. In certain studies, a Leica DMR upright microscope with DIC optics and long working distance 10 $\times$ objective was used, which gave better resolution images of individual cells in a smaller field of view. In either case, each cushion was recorded in multiple (10–12) focal planes every 10 min. The depth of field was 25 and 10 μ m for the Olympus 4 $\times$ and Leica 10 $\times$ objectives, respectively.

Statistical Analysis

The evaluation of cell motility was done using previously established procedures (Mehes *et al.*, 2005; Rupp *et al.*, 2004). Briefly, cells were followed by a manual tracking procedure using custom-written software to browse through the time-lapse z-stacks and to mark the position of selected objects in the consecutive frames. The procedure results in the focal plane (*z*) and the position (*x*, *y*) of the traced cells at a number of consecutive time points (*t*). The focal plane (*z*) information is used to distinguish between cells located at the gel surface, or inside the gel. Within a certain focal plane, the motion of the cells was characterized by their average in-plane (2D) displacement, *d*, during various time intervals of length τ as $d^2(\tau) = \{[x(t + \tau) - x(t)]^2 + [y(t + \tau) - y(t)]^2\}$, where the average $\{\}$ is taken over all possible time points *t*. Thus, we analyze the motion of cells as long as they remain within the optical depth of

Figure 1. Protein extracts from early, intermediate, and late stage AV cushions were subjected to immunoblot analysis and gel substrate zymography. (a) A Western immunoblot prepared using the IgG fraction of a polyclonal MMP2 antiserum. The antibodies detect a dominant band, *M_r* 72,000, at each stage. Three other bands were detected at greatly reduced levels. (b) Gelatin zymography using affinity purified MMP2 as a positive control enzyme. Significant gelatinolytic activity is present in a prominent band, *M_r* 72,000, with two faint bands of substrate clearance corresponding to lower molecular mass forms of MMP. Note that the bands present at each AV cushion stage were also present in the authentic immunoaffinity purified MMP2. A duplicate gel incubated in the presence of 1,10-phenanthroline, a general inhibitor of MMP activity, exhibits no corresponding bands of MMP-specific gelatinolytic activity.

a predetermined focal setting of the microscope, either at the gel surface, or 100 μ m below, inside the gel. The persistence of cell motion was characterized by the diffusion index, α (Mehes *et al.*, 2005), extracted from the functional form of $d(\tau)$ by fitting a power law $d(\tau) \sim \tau^\alpha$ in the $\tau = 3$ - to 10-h range. Cells were classified as persistent or diffusive for $\alpha > 0.75$ or $\alpha < 0.75$, respectively.

RESULTS

Enzymatically Active MMP2 Is Present in Endocardial Cushion Tissue

Immunoblot analysis was conducted on extracts of AV cushions harvested from hearts at early, intermediate, and late invasion stages (Figure 1a). Under nonreducing conditions, a polyclonal antiserum to chicken MMP2 reacts strongly with a polypeptide, *M_r* = 72,000 Da. Based on its apparent molecular mass, this protein band corresponds to the secreted, inactive (prozymogen) form of MMP2. Three faint polypeptide bands are also present. One band of slightly lower mobility, *M_r* = 74,000, and two of slightly higher mobility, *M_r* = 68,000 and *M_r* = 66,000, are present at each stage. Based on band density, the prozymogen, *M_r* = 72,000, seems to be expressed at relatively constant levels, when normalized to total protein per sample. These data suggest that an important means of controlling MMP2 activity, in embryonic tissue, may be by maintaining the protein in a prozymogen form.

Extracts of AV cushions were subjected to nonreducing SDS-PAGE on gelatin zymogram gels to assess the enzyme activity of the various MMP2 polypeptide bands (Figure 1b). Coomas-

Figure 2. MMP2 tissue immunoreactivity at early, intermediate, and late stages of AV cushion morphogenesis. Immunofluorescence microscopy detects MMP2 associated with endocardial cushion mesenchymal cells/tissues. (a) A transverse section through an early stage heart shows mesenchymal cells surrounded by wispy MMP2 immunolabeling (mes). MMP2 antigen is also associated with endocardial cells (arrows), but no immunostaining is detected in the myocardium (myo). The lumen of the AV canal is collapsed (L) in this specimen. A sagittal section through the AV canal of an intermediate stage heart in b shows strong MMP2 immunofluorescence associated with the superior (s) and inferior (i) endocardial cushion tissue. (c) An intermediate stage heart at higher magnification; the MMP2 antigen shows up as fluorescent foci near cells embedded in the cardiac jelly, i.e., cushion mesenchyme. (d) A transverse section through the atrioventricular canal of a late developmental stage heart. Bright punctate MMP2 immunofluorescence is associated with the cushion tissue. Occasionally, there are isolated sites of immunostaining associated with the myocardium (bracket). Insets in a, b, and d provide the anatomical frame of reference for the histological sections.

sie staining revealed gelatin clearance, at all stages, by a protein with an apparent molecular mass of 72,000 Da. Additionally, there are two faint clearance bands with higher electrophoretic mobility, $M_r = 68,000$ and $M_r = 66,000$, which presumably, correspond to an activated intermediate and a fully activated zymogen, respectively. Gel lanes incubated in the presence of 1,10-phenanthroline (an inhibitory divalent cation chelator) did not exhibit clearance of the gelatin substrate.

Clearance of gelatin by multiple molecular mass bands is due to incubation in the detergent-containing zymogram buffer—a process that is known to activate the proenzyme form of MMPs, which under normal conditions is not enzymatically active (Aimes *et al.*, 1993; Brown *et al.*, 1993). Based on Western immunoblot and the zymogram data, the $M_r = 66,000$ protein band is likely the fully processed form of MMP2. The $M_r = 68,000$ band is apparently an intermediate in the MMP2 processing pathway.

MMP2 Is Associated with Endocardium-derived Mesenchymal Cells

To determine the distribution of MMP2 protein, *in vivo*, endocardial cushion tissue in a live embryo was injected

with anti-MMP2 IgG, incubated for 60 min, fixed, sectioned, and subjected to epifluorescence microscopy. Specimens were examined at three progressively older stages of maturation. During early colonization of the cardiac jelly MMP2 immunoreactivity is present as fine wispy-like arrays of fluorescence associated with the motile mesenchymal cushion cell population as well as endocardial cells (Figure 2a). At the intermediate stage of AV cushion development, MMP2 antibodies reveal extensive immunoreactivity, distributed throughout the cardiac jelly/mesenchymal tissue (Figure 2b). When viewed at higher magnification, cushions at intermediate stage of development show that MMP2 staining consists of a punctate staining pattern (Figure 2c). By late stages of AV cushion development, the endocardial cushion tissue is widely immunopositive for MMP2 (Figure 2d). MMP2 protein is also detected in regions of the loose myocardium undergoing trabeculation (Figure 2d, bracket). Thus, during cellular invasion of cardiac jelly ECM, MMP2 is distributed near motile cells that have undergone a phenotypic change from an epithelium (endocardium) to an invasive mesenchyme.

MMP2 and $\alpha_v\beta_3$ Integrin Colocalized on Motile, Invasive Cushion Cells

To test whether MMP2 and $\alpha_v\beta_3$ integrin distribution patterns are similar (Bello-Reuss *et al.*, 2001; Brooks *et al.*, 1996; Karadag *et al.*, 2004), endocardial cushion tissues at intermediate stage of development were subjected to double immunofluorescence microscopy using the *in vivo* labeling protocol described above. Figure 3c shows the simultaneous double-immunofluorescence staining pattern of $\alpha_v\beta_3$ inte-

grin (Figure 3a) and MMP2 (Figure 3b). The distribution patterns for the two antigens were similar, with a substantial degree of colocalization (Figure 3c). Less intense MMP2 immunoreactivity was observed in association with the myocardium (Figure 3b, brackets). The micrographs show that, at the tissue level of resolution the physical distribution of MMP2 antigen and the $\alpha_v\beta_3$ integrin antigen are virtually superimposable and occur as fluorescence strands extending throughout the cushion tissue.

Figure 3. Simultaneous MMP2 and $\alpha_v\beta_3$ integrin double immunofluorescence microscopy. (a–c) Intermediate stage endocardial cushions. (d–f) Single whole-mounted mesenchymal cell within a 3D collagen gel. At the tissue level, $\alpha_v\beta_3$ (a) and MMP2 (b) antigens show considerable overlap (c). The images in a and b were compiled from z-axis projections of 12 1- μ m optical sections by using a laser scanning confocal microscope (LSCM). (d) $\alpha_v\beta_3$ immunoreactivity outlining the surface of a cushion mesenchymal cell fixed while moving through a 3D collagen gel. (e) MMP2 immunoreactivity. (f) Superimposition of the two images. Note the abundant yellow fluorescence that indicates a considerable degree of MMP2- $\alpha_v\beta_3$ colocalization (f), including the distal tips of filapodia (arrows). d–f images were acquired near the resolution limit of light optics using a 100 $\times$ objective lens (LSCM). To detect cryptic $\alpha_v\beta_3$ antibody binding sites, in this whole-mounted specimen, it was necessary to unmask the epitopes using detergent (Triton X-100) and mild trypsin treatment (see *Materials and Methods* for details).

The two antigens, $\alpha\beta 3$ integrin and MMP2, were also examined near the resolution limit of conventional light optics (Figure 3, d–f). To accomplish high-resolution imaging, endocardial cushion explants were used. This whole-mounted preparation allowed excellent optical access to the motile mesenchymal cells within the 3D collagen gel. Initial attempts to detect immunoreactive $\alpha\beta 3$ on the surface of mesenchymal cells residing within the substance of the collagen gel were unsuccessful; whereas the MMP2 antigen was readily detected.

After a series of empirical trials it was found that limited trypsin-treatment combined with a nonionic detergent enabled the monoclonal antibody LM609 to bind its cognate cell surface $\alpha\beta 3$ epitope (see *Materials and Methods* for details). Thus, the $\alpha\beta 3$ integrin epitope was not accessible for antibody recognition when present on whole-mounted mesenchymal cells that were moving through hydrated collagen gels at the time of fixation. However, extraction with Triton X-100 detergent combined with controlled trypsinization unmasked the LM609 epitope, on or near the surface of the previously motile whole-mounted cells (Figure 3d). The epifluorescence images show that both integrin $\alpha\beta 3$ (Figure 3d) and MMP2 (Figure 3e) are distributed over a considerable portion of the mesenchymal cell surface, including the distal tips of filopodial protrusions (arrow)—indeed, at most positions on the cell surface, the two fluorescent images are superimposable (Figure 3f).

MMP2 and $\alpha\beta 3$ Integrin Association Is Required for the Normal Locomotion of Endocardial Mesenchyme

To investigate the functional role of MMP2–integrin $\alpha\beta 3$ association during endocardial mesenchyme motility, cushions at early/intermediate stages of development were excised and explanted onto hydrated 3D native rat-tail collagen gels with concentrations of either 1.7 or 2.0 mg/ml (also see Runyan and Markwald, 1983). As revealed in Supplemental Figure 1, irrespective of the gel concentration, two cell populations emigrated from the AV cushion explants—spindle-shaped mesenchymal cells that invaded the gel and endocardial-like cells that moved across the gel–medium interface. The behavior of both cell populations was recorded at multiple distinct focal planes by using automated digital time-lapse microscopy.

For functional analysis the physical association between MMP2 and $\alpha\beta 3$ integrin was disrupted using two experimental reagents. The first reagent is a recombinant form of the human hemopexin polypeptide (PEX), which is the MMP2 domain that binds to integrin $\alpha\beta 3$. Western immunoblots of quail endocardial cushion extracts demonstrated a reactive band, M_r 29,000 Da, which corresponds to the migration position of human recombinant PEX polypeptide (data not shown). Administration of the recombinant human (rh)PEX polypeptide significantly reduces mesenchymal cell motility *through* the 3D collagen gel matrix (Figure 4, a–f, and Supplemental Movie 1), compared with untreated cells (Figure 4, a'–f'). Endocardial-like cellular motility over the surface of the 3D gel, however, is not affected by the PEX polypeptide (Figure 4g).

The physical association of MMP2 and integrin $\alpha\beta 3$ on the surface of mesenchymal cells is required for persistent motility, i.e., MMP2– $\alpha\beta 3$ binding promotes directionally persistent mesenchymal cell motion. Compared with untreated cells (Figure 4, a'–f'), when MMP–integrin binding is blocked with PEX, cellular protrusions collapse frequently (Figure 4, a–f) and new protrusions often form in altered directions. Moreover, the treated mesenchymal cells tend to exhibit a less persistent, more diffusive, motile behavior. The

Figure 4. Time-lapse DIC microscopy of motile cells within and on collagen gels (see Supplemental Movie 1). Each image is an individual frame from time-lapse movies of cultures either treated with rhPEX (a–f) or left untreated (a'–f'). In the absence of a perturbing reagent, spindle-shaped cells move through the 3D collagen gels by extending slender filopodia (e.g., e'). Normally, their motility is highly persistent with infrequent changes of direction (a'–f'). Conversely, mesenchymal cell motility is significantly reduced in cultures exposed to rhPEX domain; in addition to impaired motility, the rhPEX-treated cells are amorphous and poorly extended (a–f). Contrary to expectations, cushion-derived endocardial-like cells, moving across the surface of the 3D collagen gels, are not affected by rhPEX (g). The data strongly suggest that PEX-mediated MMP2–integrin $\alpha\beta 3$ binding is required for persistent mesenchymal cell motility but not for endocardial-like cell locomotion across a free collagen gel surface.

PEX polypeptide does not affect endocardial-like cell velocity, persistence of motility, or protrusive activity.

The second inhibitory reagent TSRI265 is an organic compound designed to mimic the hemopexin polypeptide and to thus prevent MMP2–integrin $\alpha\beta 3$ binding (Brooks *et al.*, 1996; Silletti *et al.*, 2001). When endocardial cushions were incubated in the presence of compound TSRI265, mesenchymal cell motility (Figure 5b') is significantly reduced compared with a control organic compound, TSRI359 (Figure 5b). In contrast, endocardial-like cells moving across the upper surface of the collagen gel are unaffected by either organic compound (Figure 5, a and a' and Supplemental Movie 2). Thus, cultures treated with either the recombinant human PEX or the TSRI265 inhibitor show significantly reduced mesenchymal cell motility; whereas endocardial-like cell locomotion across the surface of the collagen gel is unaffected.

Figure 5. Trajectories of cells emigrating from AV cushion explants. Colored lines represent cell trajectories. The red color designates the beginning of the path, which changes to blue at the end of the path. A' and b' show cultures incubated with a biologically active hemopexin-domain mimetic compound TSRI265, whereas a and b depict cultures treated with TSRI359, a control organic compound. a and a' are images taken at the surface of the collagen gel, whereas b and b' depict a focal plane 100 μm below the collagen gel surface. Cells in control cultures (a and b) display normal motility, as do TSRI265-treated cells moving across the collagen gel surface (a'). In distinct contrast, the active TSRI265 compound inhibits mesenchymal cell locomotion through the collagen gel (b'). Note the significantly shorter, more tortuous, trajectory plots. These data are entirely consistent with the rhPEX polypeptide data. For clarity, the explanted cushion tissue is shown, but the surrounding outgrowth of motile cells was erased using Adobe PhotoShop software.

Control and treated cultures (number of analyzed explants: 8 untreated, 3 TSRI359-treated, 4 TSRI265-treated, and 6 rhPEX-treated) were statistically evaluated to quantify cellular dynamics. For each explanted endocardial cushions, 20–40 cells were traced throughout the time-lapse image sequences (e.g., Figure 4). The cells were picked randomly in two predetermined focal planes, one at the collagen gel surface (resulting in a total of 180 untreated, 40 TSRI359-treated, 156 TSRI265-treated, and 74 rhPEX-treated cells) and the other 100 μm below the surface, well within the substance of the 3D collagen matrix (resulting in a total of 114 untreated, 96 TSRI359-treated, 119 TSRI265-treated, and 47 rhPEX-treated cells).

The motility of tracked cells was characterized by $d(t)$, the average displacement, d , as a function of elapsed time, t (Figure 6a). Persistence of cellular motility can be characterized by the diffusion index, α , a derivative of $d(t)$ (e.g., Mehes *et al.*, 2005). The functional form of $d(t)$ depends on the type of motion, and for a number of idealistic scenarios $d \sim t^\alpha$ holds. In the case of persistent, linear, consistent motion, $\alpha = 1$. For a mathematical random walk where each step is an independent random variable with the same distribution, $\alpha = 1/2$. A motion bounded within a certain domain yields $\alpha = 0$ for a large enough t . As Figure 6a demonstrates, the motion of the untreated (Figure 4, a'–f') and PEX-treated cells (Figure 4, a–f) can be approximately characterized with $\alpha = 1$ (green) and $\alpha = 1/2$ (red), respectively. Thus, cells were classified as diffusive or persistent on the basis of the calculated persistence index. Although, the majority (85%) of cells is persistent under nonperturbed conditions, both PEX and TSRI265 treatment substantially reduce the proportion of persistent cells, by up to 50% (Figure 6b). The statistical analysis reveals that *mesenchymal* cells within PEX- or TSRI265-treated cultures moved 60 and 33% less compared with untreated/negative-control cultures, respectively (Figure 6c). The observed reduction in cell motility is limited only to cells within the collagen gel—no effect is seen on the motility of endocardial-like cells moving across the gel surface (Figure 6d).

Inhibition of MMP Enzymatic Activity Suppresses Mesenchymal Cell Motility

To confirm that MMP2-driven proteolytic activity has a significant impact on general cell emigration from the cushion explants, chemical inhibitors of enzymatic activity were tested, i.e., the divalent cation chelator EDTA, and the zinc ion chelator 44463. Both inhibitors decreased general cellular outgrowth from explanted cushions. EDTA decreased the diameter of cell outgrowth by 39%, whereas the zinc chelator 44463 diminished outgrowth by 35% (Figure 7). The IgG fraction of a MMP2 polyclonal antiserum elicited an average 27% decrease in cellular outgrowth compared with explants cultured in the presence of nonimmune rabbit IgG at the same concentration. Tissue inhibitor of metalloproteinase (TIMP) 2 was the most effective of all the reagents used to inhibit overall cellular outgrowth. Explants cultured in the presence of 10 μM of recombinant human TIMP2 exhibited a 44% reduction in overall outgrowth (Figure 7). Higher concentrations of compound 44463 were tested with no significant decrease in motility.

We cultured intermediate stage AV cushions on collagen gels infused with DQ-gelatin to investigate how the PEX domain-mediated binding of MMP2 and integrin $\alpha_v\beta_3$ modulates MMP2 enzymatic activity. This commercial reagent displays induced fluorescence when subjected to enzymatic proteolysis. After a short incubation (6 h), explants were treated with either 1,10-phenanthroline, TSRI265, or TSRI359 reagents. Some explants were kept as untreated controls. Twenty-four hours after treatment, the signal of unquenched fluorescence was imaged using wide-field epifluorescence and confocal microscopy. As Figure 8 reveals, fluorescence signal indicating cleaved gelatin is most strongly localized around cell surfaces. However, fainter tracks through the collagen are also visible. Although TSRI265 reduces the number of cells within the gel, there is no substantial reduction in the emitted fluorescence signal per cell when compared with controls. In contrast, treatment with 1,10-phenanthroline effectively blocks the cleavage of DQ-gelatin.

Figure 6. Statistical analysis of digital time-lapse data. (a) Average displacement (micrometers) versus elapsed time (hours) in a double logarithmic plot, which was calculated for the two cells shown in Figure 4 and Supplemental Movie 1. In the case of a control cell that moves persistently (green symbols), the displacement is proportional to the elapsed time (green line). For a PEX-treated cell, which seems to move randomly, the displacement is proportional to the square root of time (red line). Such analysis of displacement versus time curves permits the classification of cell motility as persistent or diffusive as described in *Materials and Methods*. (b) The graph depicts the proportion of mesenchymal cells that display highly persistent motility through 3D collagen gels under various treatment conditions. Agents designed to interfere with MMP2- $\alpha\text{v}\beta 3$ association reduce the percentage of persistently moving cells. (c) Displacements of control and inhibitor-treated mesenchymal cells moving within the 3D collagen gel. Compared with control cultures, the PEX domain and its mimetic analog inhibited the motility of mesenchymal cells moving through the substance of the collagen gels. In contrast, d shows the displacements of endocardial-like (epithelial) cells moving across the scaffold at the gel/medium interface. Unlike mesenchymal cells, the motility of the endocardial-like cells was unaffected by the two inhibitors; indeed, endocardial cell motility was statistically undistinguishable from control cultures (d). PEX, a recombinant human hemopexin polypeptide; TSI265, a synthetic organic inhibitor of MMP2- $\alpha\text{v}\beta 3$ integrin binding; TSI359, a control compound for the active organic inhibitor TSI265.

Figure 7. Endocardial cushion cell outgrowth in the presence and absence of MMP proteolysis inhibitors. General inhibitors of MMP enzyme activity decrease cell outgrowth from explanted endocardial cushions. The divalent cation chelator, EDTA (10 μM), decreased outgrowth by 39%, whereas a zinc chelator (44463; 25/50 μg/ml) diminished up to 35%. Specific inhibition of MMP2 activity also decreased outgrowth of endocardial cushion cells. Antibodies to MMP2 decreased outgrowth up to 33% (20/50 μg/ml), whereas TIMP2 (10 μg/ml) reduced diameter of outgrowth by 44%. These data connect MMP2-mediated proteolysis to cell motility. All values were statistically different ($p < 0.001$) based on Student's t test.

DISCUSSION

MMP2 Is Active during Endocardial Mesenchyme Invasion

MMP2 is an enzyme expressed by a broad variety of invasive cell populations both in the normal and pathological state. The enzyme catabolizes a number of cardiac jelly constituents, including collagen I, fibronectin, and basement membrane collagen IV. Further MMP2 substrates include collagens V, VII, and X, as well as gelatin. Our immunolocalization data established that MMP2 is abundant in AV and outflow tract cushions at the earliest stages of cushion mesenchyme motility; MMP2 continues to be present in cushion tissue throughout the invasion process. Thus, MMP2 clearly has the potential to modulate an important stage of cardiac morphogenesis—the interval when endocardial endothelial cells transform and invade the cardiac jelly ECM (Song *et al.*, 2000). In fact, murine endocardial epithelial-to-mesenchymal transformation is positively correlated with levels of MMP2 expression (Enciso *et al.*, 2003). The expression patterns in Figures 2 and 3 suggest that MMP2 is not required for the transformation event itself, but rather, for mesenchymal cell invasion.

The immunofluorescence microscopy data are supported by Western immunoblot analysis and gel electrophoresis zymography. Both of which confirm that the MMP2 prozymogen is expressed during the cardiogenic stages characterized by endocardial mesenchyme invasion.

MMP2 and Integrin $\alpha\text{v}\beta 3$ Binding

MMP activity is tightly regulated at several levels, from transcription, to proenzyme activation and finally by inhi-

Figure 8. Endocardial cushion explants grown on collagen gels mixed with DQ-gelatin, a reagent that displays induced fluorescence when enzymatically cleaved. (a) An untreated (control) endocardial cushion incubated for 24 h. The colors have been inverted for better contrast such that fluorescence signal is represented by dark color. The strongest signal is associated with the cells (black punctate dots). Fainter tracks of fluorescence can be observed in the collagen. Treatment with the control organic reagent (b) displays similar results. Explants incubated in the presence of 1,10-phenanthroline (c), a general metalloproteinase inhibitor, display minimal cell migration, with the majority having migrated before exposure to the reagent. (d) An endocardial cushion exposed to the TSRI265 organic reagent. Migrating cells are present but in fewer numbers. Additionally, fluorescence tracks are apparent but are lighter in intensity. The insets in a, b, and d are single sections from a z-series through the collagen gels collected using a laser scanning confocal microscope with a 10 $\times$ objective. The images in a–d were collected with a 5 $\times$ objective. The fields of view are 1700 μ m $\times$ 1360 μ m, and the inserts are 165 $\times$ 165 μ m.

bition with TIMPs. Most MMPs are secreted into the ECM in a proenzyme form that is later activated by cleavage of the amino-terminal 80 amino acids. This processing to an active form is accomplished by one of the five membrane-type (MT) MMPs residing on the cell surface, by activated protein C, or by the plasminogen activator-plasminogen cascade (Nguyen *et al.*, 2000; Morrison *et al.*, 2001; Zahradka *et al.*, 2004). A final step in regulating active MMPs is inhibition by small inhibitory proteins called TIMPs. MMP2 is secreted in association with TIMP2, which mediates cell surface binding of the latent complex (Corcoran *et al.*, 1996). Several proteases, MMP2, MT1-MMP, TIMP2, and integrin α v β 3 were shown to colocalize in caveolae in human endothelial cells, (Puyraimond *et al.*, 2001), and the MT1-MMP:TIMP2:MMP2: α v β 3 complex was shown to promote maturation of MMP2 in carcinoma cells (Deryugina *et al.*, 2001). Our double immunofluorescence microscopy reveals that there is a high spatial correlation between MMP2 and the α v β 3 integrin, at the tissue level of organization (Figure 3, a–c). These immunoreactivity profiles in embryonic hearts are further supported by the codistribution analysis using cultured whole-mounted cushion tissue: The surface of invasive cells displayed extensive codistribution of α v β 3 and MMP2 antigen, specifically on cells that were fixed while moving through collagen gels (Figure 3f).

Cellular Motility

Our data demonstrate that MMP2 is actively involved in the invasion and motility of endocardial mesenchymal cells into and through collagen I gels, and suggest that similar mechanisms could operate in vivo. The work also strongly suggests that while MMP2-integrin α v β 3 binding is necessary for mesenchymal cell motility, such activity is not required for endothelial cell spreading on the gel surface. Furthermore, functional studies focusing on the MMP2 hemopexin domain suggest that the α v β 3 integrin is an active participant in MMP2 activation, or in localizing active MMP2 to the surface of motile mesenchymal cells.

Our results are in accord with work using other model systems in which MMP2- α v β 3 binding is required for proper MMP2 function. The α v β 3 integrin has been shown to be

necessary for vascular cell survival, proliferation, and invasion during angiogenesis both in vivo and in vitro models (Bayless *et al.*, 2000; Davis, 1992; Stromblad and Cheresh, 1996). Furthermore, Brooks *et al.* (1996) showed that the α v β 3 integrin and MMP2 form a complex on motile endothelial cells; and that MMP2 is proteolytically active. Related studies demonstrate that a specifically-truncated, noncatalytic fragment of MMP2 (the hemopexin-like domain) prevents binding of MMP2 to integrin α v β 3, and thereby disrupts angiogenesis (Brooks *et al.*, 1998).

Similarly, an organic reagent (TSRI265) that prevents binding of MMP2 to the α v β 3 integrin, in vitro, inhibits tumor angiogenesis (Silletti *et al.*, 2001). Pfeifer and colleagues demonstrated that viral delivery of PEX suppressed neovascularization by specifically blocking MMP2 activation (Pfeifer *et al.*, 2000). Although our data do not directly demonstrate that α v β 3 integrin and MMP2 are physically bound in a macromolecular complex; the time-lapse functional assays do show that a recombinant hemopexin domain, rhPEX, and TSRI265, markedly inhibit three physical parameters of motility: displacement per unit time, persistence time of motility, and protrusive behavior (Figures 4–6).

MMP2 Function Stabilizes Cell Protrusions and Increases Persistence Motility during Invasion

Perhaps the most interesting functional data regarding rhPEX polypeptide/TSRI265-induced experimental perturbations are the studies on cell protrusive behavior. Mesenchymal cells in control collagen gel cultures, like all mesenchymal cells, extend filopodia in the direction of locomotion; however, rhPEX-treated cells retract their filopodia and exhibit a random walk-like motility, sometimes returning toward the explant (Supplemental Movie 1). Work by others on cell invasion, is consistent with our results. For example, it was recently reported that during cancer metastasis the coordinated expression and physical association of the α v β 3 integrin and MMP2 is required for the invasive activity of transformed cells (Felding-Habermann *et al.*, 2002; Leroy-Dudal *et al.*, 2004). Interestingly, treatment with α v β 3 integrin-blocking antibodies (LM609) did not markedly affect cushion mesenchymal cell invasive activity in our assay

(data not shown). Based on our *in vitro* labeling studies, which required enzymatic unmasking of the LM609 epitope, we believe that the inaccessibility of ligated antigen to the LM609 antibody explains the apparent lack of effect of LM609 on cushion mesenchymal cell invasion. Alternatively, LM609 may not influence MMP2 localization at the cell surface, and the blocked $\alpha v\beta 3$:ECM binding is compensated for by other integrins, such as $\alpha 5\beta 1$.

The observation that mesenchymal cells and endocardial-like cells responded differently to the presence of recombinant PEX or a PEX mimetic was unexpected. Computational analyses of the digital time-lapse data demonstrate that the endocardial-like cells, whether in control explants or in experimentally treated explants, exhibit highly persistent trajectories that radiate from the explanted cushion; irrespective of whether PEX was present or not. In short, endocardial cell motility across a surface does not seem to involve $\alpha v\beta 3$ integrin-MMP2 binding. To the best of our knowledge these are the first data showing that integrin-MMP binding may be a mechanism that distinguishes mesenchymal cell motility through a matrix from epithelial cell locomotion over an ECM scaffold/surface. Migration across a “free” surface, a liquid/scaffold interface for example, is physically distinct from moving through the substance of a 3D fibrillar extracellular matrix, and consequently might involve a different motility mechanism—one that does not require PEX-derived MMP2 binding capability. However, proteolytic MMP activity seems to play a role in cellular locomotion both in the gel as well as across the surface. A variety of general MMP inhibitors (Figure 7) decrease the overall cellular outgrowth of all cells, mesenchymal and endocardial alike, from explanted endocardial cushions.

PEX and TSRI265 Reagents Prove Specific Response

Nisato and colleagues recently described studies in which the pharmacological effects of recombinant PEX polypeptide were lost when contaminating lipopolysaccharide was neutralized: “The PEX polypeptide neither affected angiogenesis nor bound integrin $\alpha v\beta 3$. Moreover, no specific binding of pro-MMP-2 to integrin $\alpha v\beta 3$ was found” (Nisato *et al.*, 2005).

We consider unlikely the possibility that our results using PEX are a consequence of contaminants for a number of reasons: 1) in our assay rhPEX, and its organically synthesized mimetic, TSRI265, elicited the same cell biological responses, namely, that mesenchymal cell persistence of motility and protrusive rates were all inhibited using both reagents. Because there is no possibility that TSRI265 could be contaminated with microbial by-products (lipopolysaccharide) our contention that inhibiting $\alpha v\beta 3$ -to-MMP2 binding reduces motility, persistence, and protrusive rates remains valid; 2) mesenchymal cells, but not endocardial-like cells, were affected by PEX and TSRI265; thus, the effects are not a trivial response to a contaminant that affects all cell types; 3) Figures 7 and 8 show that MMP2 enzyme activity is required for endocardial cushion cellular outgrowth into 3D collagen gels, which directly links cell invasive behavior to MMP2-mediated proteolysis, whereas our TSRI265 data link normal invasive cell behavior with MMP2-to- $\alpha v\beta 3$ binding. Thus, our data establish a logical connection between mesenchyme invasive behavior and MMP2-to- $\alpha v\beta 3$ binding, irrespective of PEX polypeptide-based data.

CONCLUSIONS

The data herein strongly suggest that MMP2-hemopexin domains are part of a mechanism necessary for efficient mesenchymal cell motility through a 3D collagen scaffold.

By extension, the data suggest that active MMP2, specifically, is required for the invasive behavior of endocardial-derived mesenchymal cells *in vivo*. This study provides insight into the cellular mechanisms by which cells, which have undergone an epithelial-to-mesenchymal transition, invade an extracellular matrix. The work demonstrates a fundamental difference between mesenchymal invasive motility versus epithelial (endocardial) cell motility across the surface of an ECM scaffold.

This study is a vivid example of the importance of dynamic computational analysis, because the differences between experimental and control cell motility were not readily apparent using fixed culture assays (e.g., persistent locomotion vs. random locomotion). These data may also be useful in the broader context of understanding epithelial conversion to a motile phenotype, because some cushion endocardial cells display epithelial motility, whereas other cells engage in mesenchymal motility. Our computational approach provides a model to analyze cell motile behavior, cell adhesion and ECM proteolysis by a clinically relevant cell population. The work may also shed light on cellular behaviors that contribute to diseases such as pathological angiogenesis and tumor cell metastasis.

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