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Characterization of cell motility in single heart valve interstitial cells *in vitro*

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Summary. Valve interstitial cells (VIC) are the most prevalent cells in the heart valve, regulating to a large extent the normal biology of the valve and its pathobiological response to disease. In the process of valve tissue repair by VICs, single cell motility is likely to be important, as it is in wound repair by most mesenchymal type cells. We designed *in vitro* experiments using low density monolayer cultures to study the association of morphology and motility in single VICs which expressed alpha-smooth muscle actin. We observed that the morphology of single VICs can be categorized into six types which are reminiscent of the shape of VICs seen in vivo during valve repair. Of these morphologies, round, rhomboid, tailed and spindled shaped VICs were the most common. VICs did change their morphology over time. Rhomboid cells could become tailed or spindle-shaped and vice versa. Using time-lapse imaging and immunofluorescent microscopy, we showed that VIC morphologies reflect differences in cell motility and cell-matrix interactions. Tailed and spindle-shaped VICs were the predominant motile types and were associated with few extracellular fibronectin fibrils and less focal adhesions, as demonstrated by vinculin staining. Round and rhomboid shaped VICs were less motile and were associated with prominent vinculin and extracellular fibronectin fibrils. We found that cell mitosis is an important determinant of VIC migration. Many of the motile VICs were associated with mitosis as the daughter cells separated by migrating as tailed and spindle shaped cells. Thus cell morphology is an important determinant of VIC motility.

Key words: Valve interstitial cells, Cell morphology, Motility, Focal adhesions, Vinculin, Matrix, Fibronectin

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Introduction

Native heart valves are biologically active tissues and although in a hostile hemodynamic/mechanical environment, they function well throughout an individual's life (Chen et al, 2004; Yoganathan et al, 2004). Once disease intervenes resulting in tissue damage, subsequent valve dysfunction may occur leading to serious heart disease and eventually requiring surgical heart valve replacement. Our understanding however of both the biology of heart valve function and the pathobiological response to tissue injury is limited. A new area of investigation, the cell biology of heart valves, transformed the investigation of heart valves from static histopathological studies to dynamic cell biology investigations. This was propelled forward with the development of successful methods to culture valve interstitial cell (VIC) and endocardial endothelial cells (Lester and Gotlieb, 1988a; Paranya et al, 2001).

The concept of "the response to valve injury" was suggested as a general paradigm which would be useful in designing studies to understand diverse valve functions in disease (Mulholland and Gotlieb, 1996; Durbin et al., 2002, 2005; Durbin and Gotlieb, 2002). VICs are the most prevalent cells in the valve and likely regulate, to a large extent, the normal biology of the valve and the pathbiological response to disease. Thus, understanding the fundamental processes that regulate VIC function is critically important. We were the first to extensively characterize in vitro VICs (Lester et al., 1988b). Because of reported heterogeneity in VICs (Lester et al., 1988b, 1993) and differences between VIC and other cell types including fibroblasts and myofibroblasts (Zacks et al., 1991; Taylor et al., 2000), it is expected that regulation of VIC function during wound repair will show important differences from those described in the literature for fibroblasts, smooth muscle cells, endothelial cells, and epithelial cells. We do not know if the response of VICs to disease leads to ultimate chronic dysfunction of the valve because repair was inadequate and/or because repair was excessive leading

to structural disruption of the valve architecture creating chronic dysfunction.

In repair of tissue by mesenchymal-type cells, single cell motility is important and occurs as the primary form of motility. This is in contrast to endothelial and epithelial cells which migrate as sheets of cells or as cells moving in synchrony (Gotlieb and Spector, 1981; Wong and Gotlieb, 1984, 1986; Nikolic et al., 2006). For this reason, we have designed in vitro experiments using low density monolayer cultures to study the association of morphology and motility in single VICs.

Materials and methods

VIC Cultures

Porcine hearts were obtained from a local abattoir, and explants were prepared from the distal third of the anterior leaflet of porcine mitral valves as previously described (Lester et al., 1988b). The atrial and ventricular surfaces of the explants were scraped with a scalpel blade and rinsed with phosphate buffered saline (PBS) pH 7.4 to remove endocardial endothelial cells. The explants were cut into 4x5 mm pieces, placed in 35 mm tissue culture dishes (Falcon; BD Biosciences, San Jose, California), and grown in medium 199 (M-199) supplemented with 10% fetal bovine serum (FBS), and 2% penicillin, streptomycin and Fungizone (PSF; GIBCO BRL, Life Technologies Inc, Rockville, Md) in a humidified 95% air and 5% carbon dioxide atmosphere in an incubator at 37°C. VICs which grew out of the explants were detached with TrypLE Express (Invitrogen Corporation) and subcultured. In all following experiments, cultures of VICs were incubated in 10% FBS M-199 with 2% PSF. VICs between passages 7 to 13 were used.

Morphological observations of VICs

VICs were seeded sparsely on 22x22 mm glass coverslips (Corning Incorporated, Corning, NY) in 35 mm tissue culture dishes at a density of 17,000 cells per coverslip. Following incubation for 24, 48, 72 or 96 hours, VICs were washed three times with PBS, fixed with 4% paraformaldehyde for 15 minutes, and washed three times with PBS at 5-minute intervals. Cell morphologies were observed in PBS and classified into categories under a phase contrast microscope using a 10x eyepiece and a 40x objective (Nikon Canada). For each coverslip, the morphologies of 300-500 VICs were examined by using a method that selected fields randomly and avoided examining the same cell more than once. VICs were seeded in triplicate for each time point. Three sets of experiments were performed. Some coverslips were observed prior to fixation to establish that fixation itself did not alter VIC morphology.

Time-lapse imaging of VICs

VICs were seeded at low and high densities, 42,500 and 212,500 cells per coverslip respectively on 45 mm diameter round glass coverslips (Fisher Scientific Inc.) in 60 mm tissue culture dishes. Cells attached to the coverslips and at 24 hours, were washed three times with standard fresh media, mounted on a metal adaptor chamber, and transferred into the Nikon incubator apparatus with humidified 95% air and 5% CO_2 at 37°C on top of a TE300 phase contrast microscope. Fifty-six single cells were recorded using a 10x objective in 4, 24-hour videos. The image analysis program used for time-lapse imaging was SimplePCI 6.0 (GTI Microsystems, Tempe, Arizona). An image was recorded every minute over a time period of 24 hours. The finished data sequence was compressed into an .avi file at 15 frames per second.

Immunofluorescent microscopy

VICs were fixed with 4% paraformaldehyde, rinsed in PBS, incubated with 0.2% Triton X-100 in PBS for 5 minutes, and rinsed in three PBS washes at 5-minute intervals. The coverslips were incubated with mouse anti-vinculin (1:50, Jackson Immuno Research, West Grove, Pa), sheep anti-fibronectin (1:100, Serotec, Raleigh, NC), and mouse anti-α-smooth muscle actin (1:100, Sigma Chemical Co., Germany) primary antibodies at room temperature. The coverslips were washed three times with PBS at 5-minute intervals. Secondary antibodies were goat anti-mouse Alexa 568 and donkey anti-sheep Alexa 488 (Jackson Immuno Research, West Grove, Pa). The coverslips were mounted with FluoroGuard reagent (Bio-Rad Laboratories Inc, California). For negative control, equal amounts of protein of mouse immunoglobulin G and sheep immunoglobulin G (Jackson Immuno Research, West Grove, Pa) were used.

Fluorescently labeled coverslips were examined using the 60x objective of a scanning confocal laser imaging system (BioRad MRC 1024, BioRad, Toronto, Ontario, Canada) fitted with an argon-krypton mixed-gas laser with excitation wavelengths of 488, 568, and 647 nm. The confocal system was connected to a Nikon Optiphot microscope (Nikon Canada). Serial optical sections were taken at 0.4-µm intervals for 3.5 to 4.5 µm depending on cell thickness, which allowed for intracellular and extracellular observations, especially for fibronectin localization. Projections of the optical sections were performed with the BioRad software. Two to three hundred cells were examined per coverslip in a random fashion as noted previously. Representative images were photographed. Experiments were repeated in triplicate.

Statistical analysis

For each set of experiments, the percentage of cells exhibiting a certain morphology was compared with those exhibiting other morphologies at each time point and in between the time points using two-way analysis of variance. A value of $p<0.05$ was considered

significant. The Bonferroni method was used to reflect multiple comparisons. These statistical analyses were performed using GraphPad Prism 4 version 4.03 software (GraphPad Software Inc.)

Results

VIC morphologies can be classified into distinct categories

VICs showed six distinct morphologies in low-density culture (Fig. 1). These morphologies all expressed variable amounts of α-smooth muscle actin in a stippled and/or diffuse pattern. Tailed cells showed more prominent α-smooth muscle actin in the leading lamellipodia and in the tail. Some rhomboid cells showed stress fibers (Fig. 2). The round, rhomboid, tailed and spindle shaped morphologies were more common, while half-moon shaped cells and cells with multiple cytoplasmic extensions were infrequent (Fig. 3). The predominance of the rhomboid morphology was generally consistent over the 96-hour time period despite fluctuations in the number of cells classified into the other morphological categories (Fig. 3). The tailed morphology was the second most common morphology, and was also fairly consistent over the 96-hour time period (Fig. 3).

VIC morphologies correlate with time elapsed post-platting

The numbers of VICs exhibiting a particular morphology at a given time following platting change in a time-dependent manner. The change in the numbers of rhomboid and elongated cells at 48 hours compared to 24 hour post-platting may be due to prominent cell proliferation during this time period where nearly every single VIC undergoes mitosis at least once. Daughter VICs at the completion of cytokinesis moved away from each other, often assuming a tailed or spindle shaped morphology (Fig. 4).

During the time period of 48 to 96 hours post-platting, proliferation was less and the number of rhomboid cells increased back up to approximately its original value and those of tailed and spindle-shaped cells decreased. The number of round cells showed a slow decline over the 96 hours. The number of half-moon shaped cells or those with multiple cytoplasmic extensions did not change over time (Fig. 3).

A VIC may exhibit more than one morphology over time

Time-lapse observations show that over time the distinct morphology of an individual VIC may change. Rhomboid VICs arose often from the flattening of small

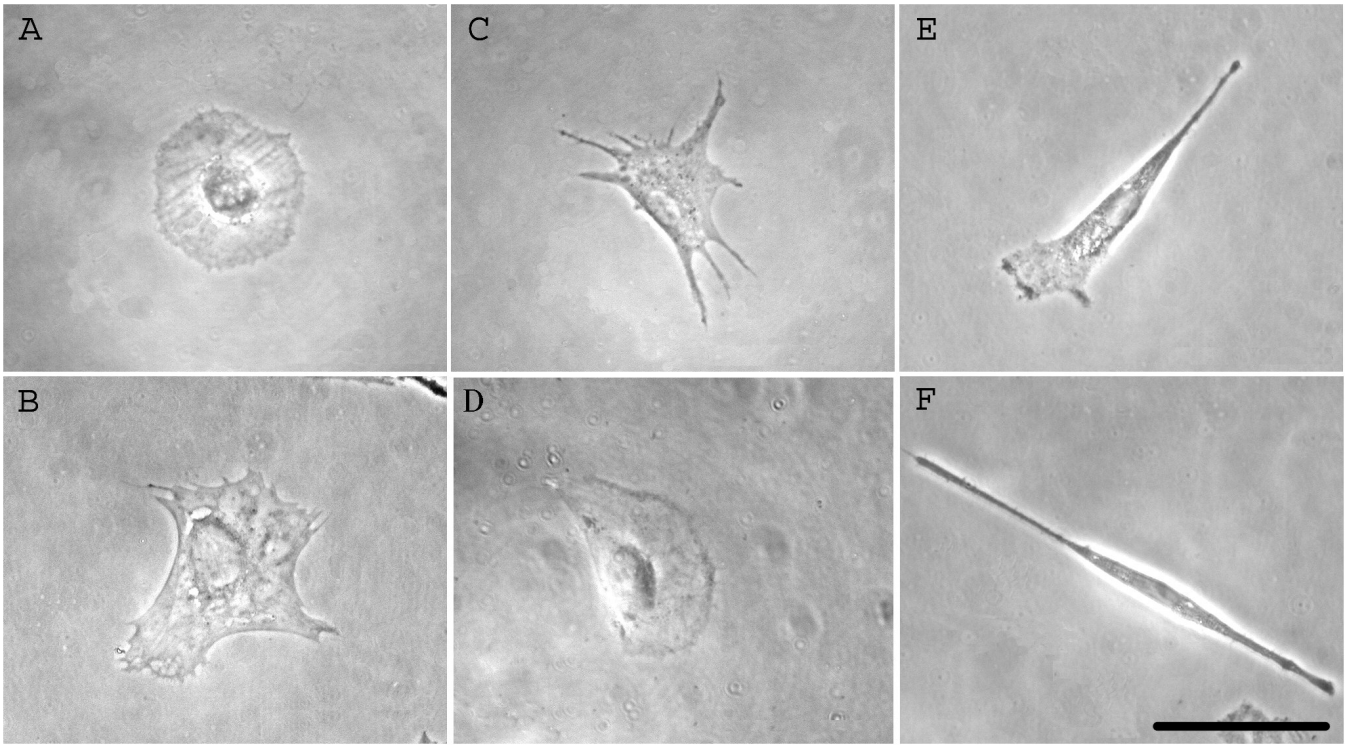

Fig. 1. Phase contrast photomicrographs of single VICs in monolayer culture observed under phase contrast microscopy. At 48 hours post-seeding, six types of morphologies are seen: (**A**) round, (**B**) rhomboid, (**C**) the presence of multiple cytoplasmic extensions, (**D**) half-moon shaped, (**E**) tailed, and (**F**) spindle-shaped. Bar: 10 μm; x 400

round cells during this period. Rhomboid cells sometimes took on the tailed morphology, often abruptly, with the appearance of a frontal lamellipodia (Fig. 5). Moreover, tailed VICs occasionally elongated into a more spindle shape in the process of migration (Fig. 5). It was also observed that a spindle shaped VIC, when migration was slowed, could flatten out into a rhomboid morphology (Fig. 6). There was no specific morphological findings to explain why these changes occurred. These were single cells and cell-cell contact was not a factor in altering the cell shapes which we are describing.

Focal adhesions vary with respect to cell morphology and time elapsed post-platting

Vinculin staining used to identify focal adhesions showed that the round and rhomboid morphologies form considerably more prominent focal adhesions than the tailed and spindle shaped morphologies at both 48 and 72 hours post-platting (Fig. 7). In the round and rhomboid cells, vinculin stained brightly and was dispersed radially in the cell periphery or along the cell perimeter. In the tailed and spindle shaped cells, vinculin stained weakly and was found localized in the front edge of the lamellipodia and along the tail of the tailed shaped VIC, or at the two narrow ends of the spindle-shaped cells. The intensity of vinculin staining in round and rhomboid cells was most prominent at 24 hours (Fig. 7).

VIC morphologies show differences in fibronectin expression

Cells with round and rhomboid morphologies stained more brightly for fibronectin than the tailed and spindle-shaped VICs, with the former showing more prominent extracellular staining, and the latter more intracellular staining. Intracellular fibronectin was

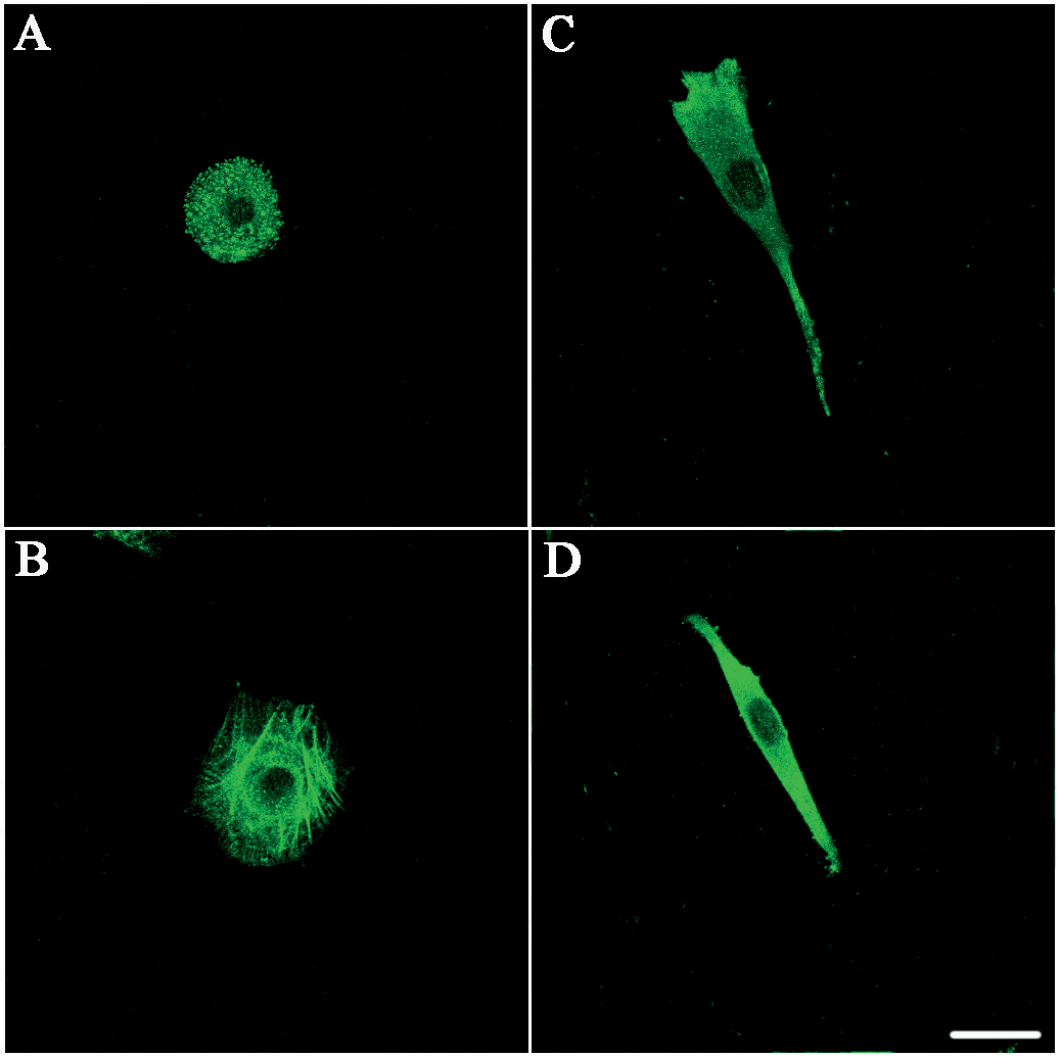

Fig. 2. Confocal immunofluorescent photomicrographs of low-density VICs at 48 hrs post-platting stained for α-smooth muscle actin. Images are taken from projections of z-sections scanned at every 0..4 μm. Single (**A**) round, (**B**) rhomboid, (**C**) tailed, (**D**) spindle-shaped VICs express alpha-smooth muscle actin. Bar: 10 μm; x 600

concentrated around the perinuclear and endoplasmic reticulum region with some general punctate staining. Extracellularly, fibronectin fibrils were present in the matrix underneath the cells. The staining pattern in round and rhomboid cells showed mostly bright extracellular fibrils with some staining in the region of the endoplasmic reticulum. In the tailed and spindle-shaped cells, fibronectin staining was mainly concentrated around the endoplasmic reticulum region with some punctate cytoplasmic staining (Fig. 7). Matrix fibronectin fibrils in the subcellular matrix were only occasionally observed in these morphologies. In tailed

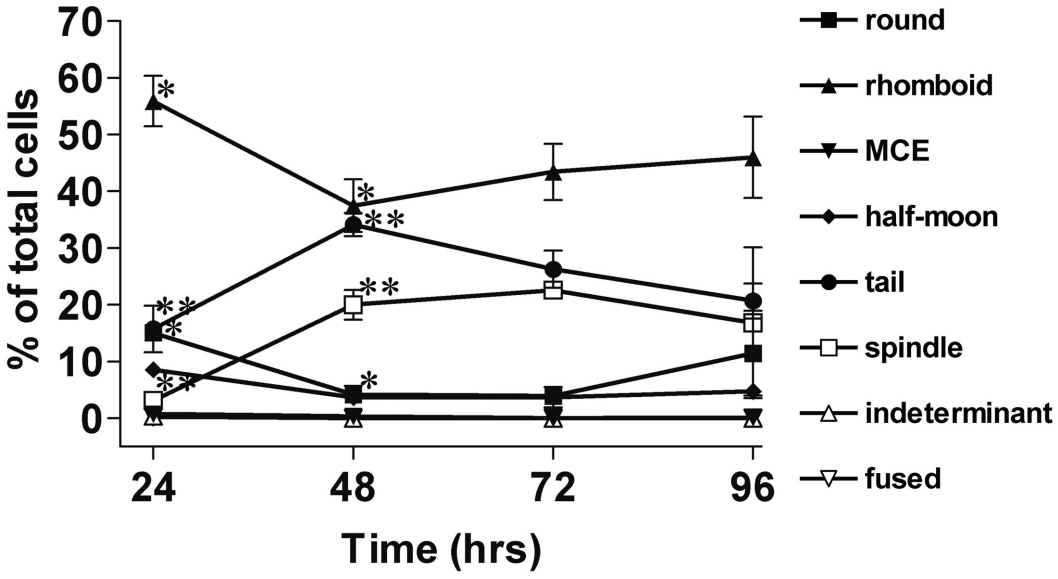

Fig. 3. Morphology of a representative experiment of single BICs in monolayer culture observed under phase contrast microscopy over a 72-hr time period. Three independent experiments,. each in triplicate, were performed. Errors bars denote standard deviation. Note that the percentage of round and rhomboid cells significantly decreased (*) between the time period of 24-48 hours, and the percentage of tailed and spindled cells significantly increased (**) betweeen the same time period. $p<0.05$.

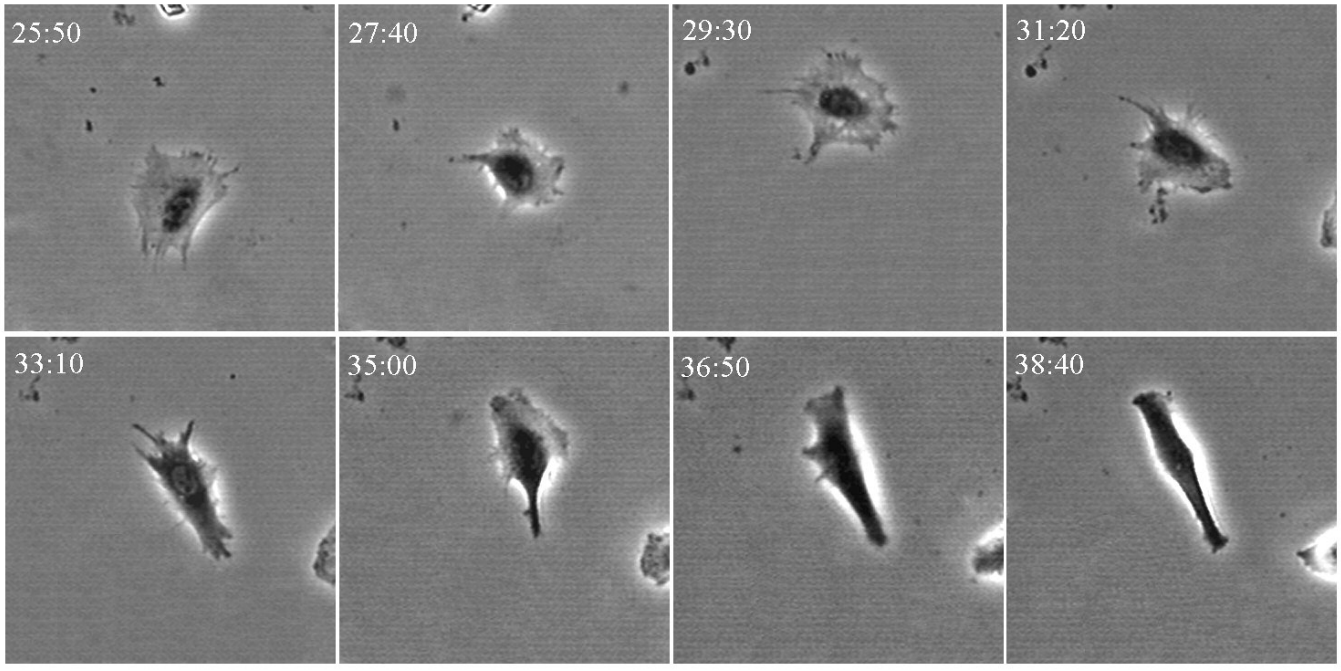

Fig. 4. Time-lapse phase contrast photomicrographs of a dividing VIC giving rise to elongated daughter cells moving away from each other after mitosis. Indicated times denote time-elapsed post-seeding. x 200

cells, matrix fibronectin was sometimes observed on the substratum just distal to the tail (Fig. 8).

Discussion

Our study is the first to describe important aspects of the cell biology of repair in single VICs in monolayer culture. Our time-lapse studies showed that the 6 distinct morphologies of single VICs identified in this study reflect the processes of spreading, proliferation and migration of single VICs, the same processes that occur universally in all forms of mammalian repair. These morphologies are important to understand since they are similar to those seen *in vivo* in histological and ultrastructural studies of normal and injured heart valves (Lester and Gotlieb, 1988a; Lester et al., 1993; Tamura et al., 2000). Our studies show that the morphology of a VIC is associated with the motile activity of the cell, with tailed and spindle-shaped cells showing more active migration than round and rhomboid VICs. These findings are consistent with those in a number of cell types such as lung fibroblasts and kidney epithelial cells (Adams, 2002).

Our *in vitro* model of studying VICs recently plated in monolayer culture provides a useful window on single VIC migration and proliferation. All the cells studied over the 96 hour time period express α-smooth muscle actin. There were no indication that the motile cells contained more α-smooth muscle actin although rhomboid cells showed more α-smooth muscle actin stress fibers consistent with more substratum adhesion and less motility. The majority of VICs showing round

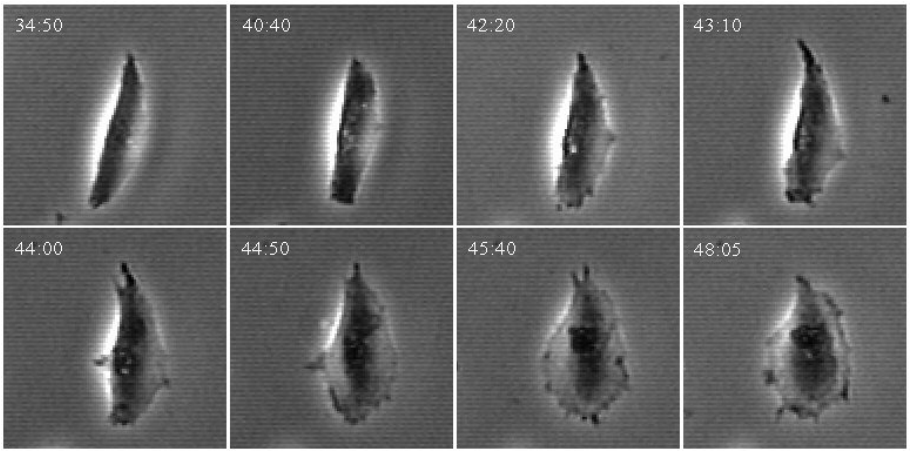

Fig. 5. Time-lapse imaging photomicrographs of a singlle VIC under phase contrast microscopy. Single VICs can sometimes undergo morphological changes. A rhomboid VIC can take on a tailed and a spindle-shaped morphology within a 13-hour time period. Indicates times denote time-elapsed post-seeding. x 200

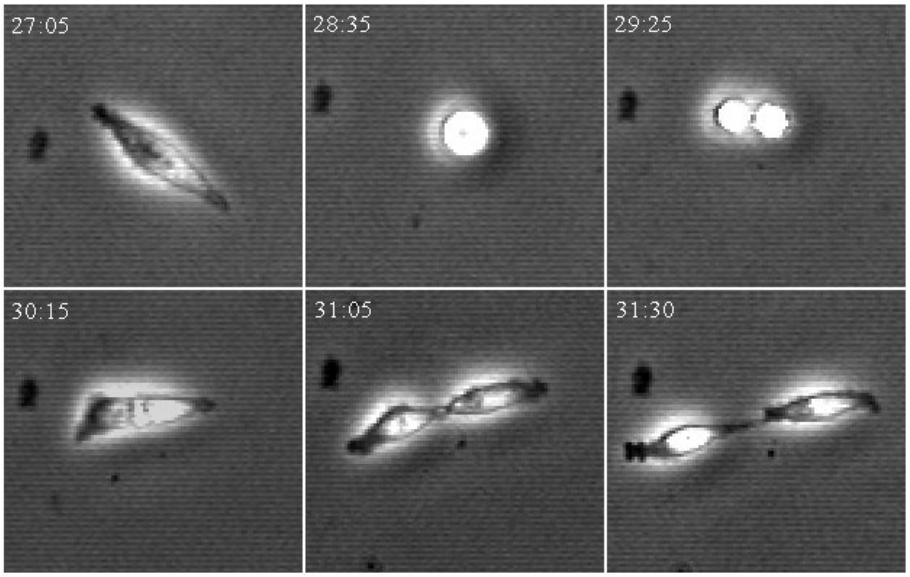

Fig. 6. Time-lapse imaging photomicrographs of a single VIC under phase contrast microscopy. A spindle shaped VIC can take on a rhomboid morphology within a 14-hour time period. Indicated times denote time-elapsed post-seeding. x 200

and rhomboid morphologies at 24 hours post-platting indicates that at this time, most VICs have just attached to the substrate or are beginning to spread, respectively. This interpretation is supported by our time-lapse observations that round cells can flatten out further into rhomboid cells. This change into the rhomboid morphology may be due to extensive Rac activation leading to large lamllipodia extensions, possibly due to such stimuli as the engagement of integrins while round VICs adhered to the substrate (Bialkowska et al., 2000;

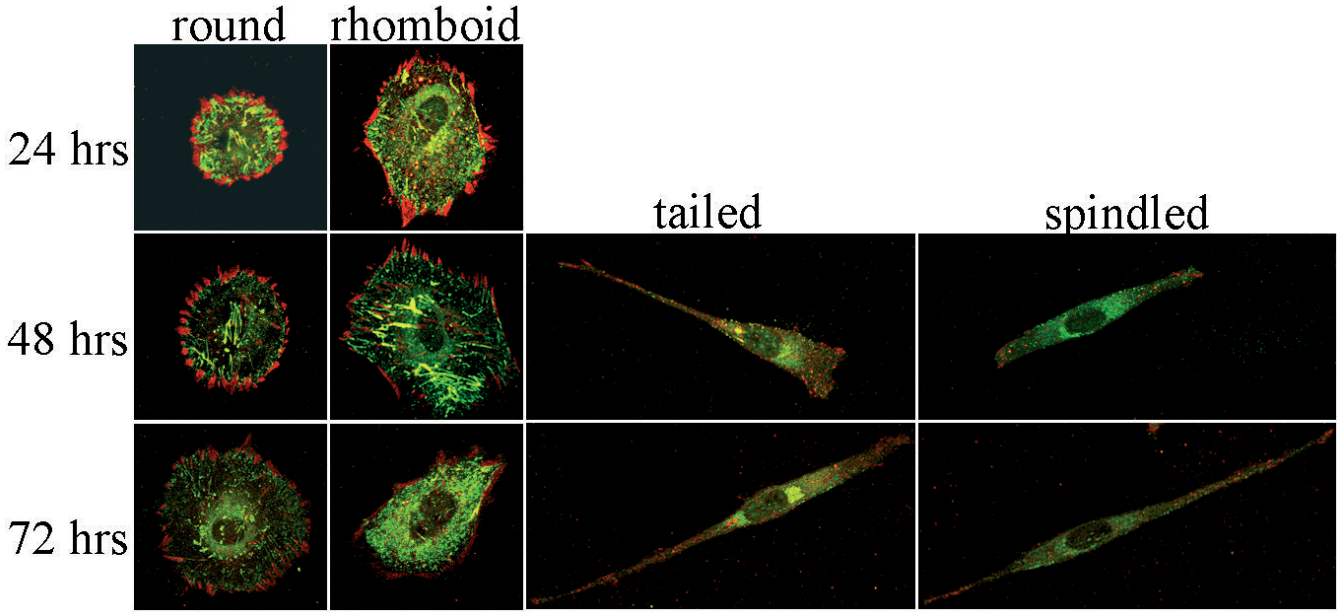

Fig. 7. Confocal photomicrographs of single VICs of different morphologies at 24, 48, and 72 hours post-seeding are double stained for vinculin (red) and fibronectin (green). Images are taken from projections of z-sections scanned at every 0.4 µm. Pattern of focal adhesions are indicated by vinculin. x 600

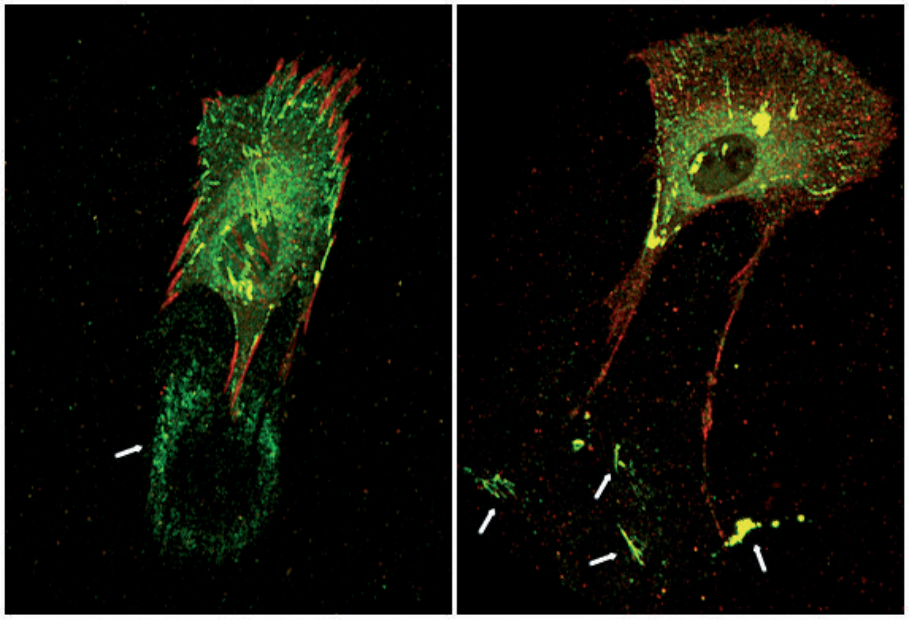

Fig. 8. Confocal photomicrographs of single VICs with tailed moprhology double stained for vinculin (red) and fibronectin (green). Images are taken from projections of z-sectiins scanned at every 0.4 µm. Fibronectin is left just behind the tailed morphology as the cell migrates (arrows). Vinculin staining identifies focal adhesions. x 600

del Pozo et al., 2000). Moreover, the distribution of ruffling lamellipodia all around the periphery of rhomboid cells and the lack of polarization explain the low motility of this type of morphology (Allen et al., 1998). After VICs have spread out into a rhomboid shape, they may be sensing their environment for external cues such as growth factors, cytokines and extracellular matrix components while remaining stationary or moving around slightly. Such sensing activities may be accomplished by their occasional protrusions of long filopodia which is controlled by Cdc42, the activation of which has been linked also to integrin engagement (Etienne-Manneville and Hall, 2001). VICs may also begin to migrate in a definite direction, likely in response to some unknown environmental cues which results in actin assembly and polarization of the cell. Several likely candidates for establishing such polarization in migration include basic fibroblast growth factor-2 (bFGF-2) (Ettenson and Gotlieb, 1995) which we have shown promotes VIC migration (Gotlieb et al., 2002) and the activation of cellular Rac1 and Cdc42 signaling pathways resulting in the interactions of adenomatous polyposis coli (APC), IQGAP1 and CLIP-170 to form a complex that regulates actin cytoskeleton and microtubule dynamics (Etienne-Manneville and Hall, 2001; Watanabe et al., 2004).

Elongated tailed or spindle-shaped VICs are derived from either rhomboid VICs that develop polarized lamellipodia promoting directed migration, or as daughter cells which separate after cell division by pulling away from each other and often moving off in opposite directions. These observations show that under the conditions of our experiments, a significant amount of observed motility in single VICs can be attributed to events following cell division. It is likely that if cell division is inhibited or reduced, VICs would show less directional motility. This may suggest a therapeutic target to inhibit or reduce VIC proliferation by targeting cell-cycle proteins that regulate cell proliferation. This type of approach has been successful in preventing restenosis using drug-eluting stents that inhibit smooth muscle cell proliferation (Blagosklonny et al., 2006; Parry et al., 2006). Furthermore, recent studies of endothelial daughter cells hold similarities with our observations where it was noted that hemodynamic shear stress promoted alignment of endothelial daughter cells following mitosis, both in vivo and in vitro, in the axis parallel to flow following proliferation (McCue et al., 2006).

Focal adhesions are large dynamic cell contacts located near the cell periphery which anchor stress fibers and mediate strong focal attachment of cells to the substrate. They are composed of a large complex of structural and signaling proteins which link actin microfilament bundles (stress fibers) to integrins, such as $\alpha_v\beta_3$ and $\alpha_5\beta_1$, which in turn bind to the extracellular matrix. These proteins include focal adhesion kinase (FAK), cytoskeletal molecules, vinculin, paxillin, talin, and α-actinin (Pelham and Wong, 1997; Katz et al., 2000; Zamir and Geiger, 2001; Geiger et al., 2001; Adams, 2002). The pattern of focal adhesion distribution in each different morphology likely defines their motile characteristics. As adhesion increases, migration slows, and vice versa. In mammalian cell movement, there is a critical strength of adhesion to provide optimum conditions for migration. These optimum conditions have not been studied in VICs in both 2D and 3D matrices. The radial distribution of focal adhesions along the perimeter of round and rhomboid cells allow the entire VIC to be flattened and anchored to the substrate. The presence of fewer focal adhesion anchoring points, in the front edge of the lamellipodia and along the tail of tailed cells or at the two ends of spindle shaped cells, allow these cells to move more easily and freely. At 48 hours post-platting, the numbers of VICs exhibiting the more motile tailed and spindle shaped morphologies are significantly increased, likely due to morphological changes and due to cell division. It is thus expected that at this time, cells with elongated morphologies show less prominent vinculin staining than round and rhomboid cells, and they do indeed show this. The decrease in vinculin staining from 24 to 72 hours post-platting may reflect the tendency of some VICs that have already attached to the substrate to become more motile. The decreased staining over time may also signify the gradual reduction in focal adhesions in round or rhomboid cells which may go on to become elongated.

VICs are known to extensively synthesize extracellular matrices *in vivo* (Rabkin et al., 2001; Fayet et al., 2007). Extracellular matrix is well known to regulate cell functions related to repair including proliferation and migration (Chen et al, 2004). Fibronectin is synthesized and secreted in a soluble form through the cellular secretory pathway after which it is assembled into extracellular fibrils in the cell substratum (Yang and Hynes, 1996; Danen et al., 2002; Sottile and Hocking, 2002). Intracellular fibronectin staining in the endoplasmic reticulum region of VICs arises from the former, and extracellular fibrils underneath the VICs arise from the latter.

In round and rhomboid VICs, the bright staining of extracellular fibronectin fibrils up to 72 hours post-platting show that these cells lay down a fair amount of fibronectin fibrils in their matrix during this time period. Despite the scarce or absent fibronectin fibrils, the endoplasmic reticulum fibronectin staining in tailed and spindle shaped cells indicate that these cells also synthesize and secrete fibronectin. The different staining patterns of fibronectin between the less motile round and rhomboid cells and the more motile tailed and spindle shaped cells are explainable by the observed behaviour of these cells. Due to the more stationary nature of the round and rhomboid cells, having stayed in a constant area for a longer period of time, they have more opportunity to synthesize and assemble the observed fibrils underneath them. The more motile morphologies may not stay in the same area long enough to assemble detectable amounts of extracellular fibrils, although

intracellular expression of fibronectin is still detectable. Furthermore, the occasional observation of fibronectin fibrils distal to the tail of tailed cells may suggest that extracellular fibronectin remains on the substratum after the cells have moved forward. This can occur if a rhomboid cell, having synthesized fibronectin fibrils extensively in its substratum, takes on the tailed morphology as described previously and moves away from its original position.

Our study demonstrates that the morphological heterogeneity of single VICs observed in wound repair *in vitro* and *in vivo* corresponds at least *in vitro* with their behavioural characteristics in cell spreading, division, proliferation, and migration. The differences in expression patterns of focal adhesions and fibronectin are consistent with the differences observed in motile activity among the different morphologies of VICs. Notably, cell division is an important factor in establishing morphologies that, at least for the short term, result in active VIC motility.

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