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# In wound repair vimentin mediates the transition of mesenchymal leader cells to a myofibroblast phenotype

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**ABSTRACT** Following injury, mesenchymal repair cells are activated to function as leader cells that modulate wound healing. These cells have the potential to differentiate to myofibroblasts, resulting in fibrosis and scarring. The signals underlying these differing pathways are complex and incompletely understood. The *ex vivo* mock cataract surgery cultures are an attractive model with which to address this question. With this model we study, concurrently, the mechanisms that control mesenchymal leader cell function in injury repair within their native microenvironment and the signals that induce this same cell population to acquire a myofibroblast phenotype when these cells encounter the environment of the adjacent tissue culture platform. Here we show that on injury, the cytoskeletal protein vimentin is released into the extracellular space, binds to the cell surface of the mesenchymal leader cells located at the wound edge in the native matrix environment, and supports wound closure. In profibrotic environments, the extracellular vimentin pool also links specifically to the mesenchymal leader cells and has an essential role in signaling their fate change to a myofibroblast. These findings suggest a novel role for extracellular, cell-surface-associated vimentin in mediating repair-cell function in wound repair and in transitioning these cells to a myofibroblast phenotype.

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## INTRODUCTION

In the normal repair process, mesenchymal cell populations restore tissue function in response to tissue insult or injury; however, their susceptibility to becoming myofibroblasts results in a response that promotes and sustains the fibrotic disease process. Fibrosis is a devastating progressive disease, its pathology characterized by the excessive production of extracellular matrix proteins like collagen I, with myofibroblasts being a major producer of this fibrosis-causing matrix. Fibrosis affects almost every organ of the body, causing irreparable damage wherever it occurs (Carver and Goldsmith, 2013).

As fibrosis is an outcome of so many distinct disease states, it is considered a leading cause of death (Tsou *et al.*, 2014).

Factors currently known to induce specific aspects of the fibrotic response to injury repair or pathogenesis include mechanotransduction-signaling events that involve integrin receptors, the actin cytoskeleton, TGF $\beta$ , and collagen I (Schwartz, 2010; Huang *et al.*, 2012; Wong *et al.*, 2012; Duscher *et al.*, 2014; Hinz, 2015). However, it remains unclear how cells normally tasked with directing wound repair are signaled to alter their fate and differentiate to myofibroblasts, the cell type associated with fibrosis. Key features of myofibroblast differentiation include 1) high levels of expression of the intermediate filament protein vimentin, a mediator of cell migration and wound repair; 2) expression of  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) and its organization into the stress fibers that mediate myofibroblast contractile function; and 3) production of extracellular matrix proteins such as collagen I that are secreted into the extracellular milieu. These myofibroblast properties create an aberrant microenvironment that leads to loss of tissue function.

Vimentin has emerged as a critical regulator of the wound-healing process in many distinct tissues and cell types. In studies of skin wound-repair, this protein has been demonstrated to function as a signaling integrator in tissue regeneration and healing

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Abbreviations used:  $\alpha$ SMA,  $\alpha$ -smooth muscle actin; ECM, extracellular matrix; ECZ, extracapsular zone; PBS, phosphate-buffered saline; PTM, posttranslational modification; TN-C, tenascin-C.

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(Cheng *et al.*, 2016). Our own studies with an *ex vivo*, mock-cataract-surgery (MCS), epithelial wound–healing model have shown that vimentin is a defining characteristic of a resident mesenchymal “repair” cell population (Walker *et al.*, 2010) that is located in niches among the cells of the lens equatorial epithelium. This mesenchymal cell population is activated on wounding to immediately migrate to and populate the leading edge of the injured epithelium (Walker *et al.*, 2010; Menko *et al.*, 2014a). Vimentin is required for the function of this mesenchymal leader cell population in directing the collective migration of the injured epithelium to close the wound (Walker *et al.*, 2010; Menko *et al.*, 2014a). Furthermore, we have found that when these cells are activated by wounding, they are also conferred with the ability to invade, a phenotype that is also dependent on vimentin (Bleaken *et al.*, 2016). This finding is consistent with other studies showing that vimentin is required for an invasive-cell phenotype (Satelli and Li, 2011).

Here we investigate whether the properties of vimentin that confer leader cells with the ability to modulate wound repair and direct invasion also make these cells vulnerable to environmental factors that can alter their differentiation pathway to that of a myofibroblast. Other studies have linked vimentin intermediate filaments to the development of fibrosis. Mice lacking vimentin have exhibited both reduced scarring following corneal injury (Bargagna-Mohan *et al.*, 2012) and a diminished fibrotic response to bleomycin-induced injury of the lung (dos Santos *et al.*, 2015). However, the role of vimentin in inducing the fibrotic response and the specific features of vimentin that are associated with the fibrotic phenotype are not yet clear.

While long understood for their role as a highly stable, stress-resistant cytoskeleton, vimentin intermediate filaments are now appreciated as highly dynamic structures that exist in distinct assembly states and subcellular distributions that define their functions (Lowery *et al.*, 2015; Herrmann and Aebi, 2016). The different states of vimentin organization range from nonfilamentous precursors to unit filaments to a highly organized cytoskeletal network, each with distinct cellular tasks (Herrmann *et al.*, 2009; Lowery *et al.*, 2015; Herrmann and Aebi, 2016). Soluble forms of vimentin are associated with signaling (Perlson *et al.*, 2005), while an organized vimentin cytoskeletal network is critical to processes such as resisting stress and providing cell shape (Goldman *et al.*, 2012). The assembly state of vimentin is regulated through posttranslational modifications (PTMs) that also modulate vimentin function. Two key PTMs linked to increased vimentin solubility are phosphorylation and citrullination (deimination). Some of the phosphorylation PTMs are known to trigger disassembly of intermediate filaments (Ando *et al.*, 1989). Vimentin citrullination, which results from the conversion of an arginine to a citrulline by peptidyl arginine deiminase (PAD) enzymes, prevents vimentin assembly and promotes vimentin filament disassembly (Inagaki *et al.*, 1989). In its soluble form, vimentin can function as an extracellular molecule that has been implicated in the repair process, as shown in a spinal cord injury model (Teshigawara *et al.*, 2013; Shigyo *et al.*, 2015; Shigyo and Tohda, 2016), and in modulating macrophage function in bacteria killing (Mor-Vaknin *et al.*, 2003). In studies with our *ex vivo* MCS-wounded-explant cultures, a model that closely replicates the features of wound repair *in vivo*, we now investigate how a resident mesenchymal repair cell population that modulates repair of the injured lens epithelium can become misdirected and become an underlying cause of the aberrant wound-healing response of fibrosis. We show, for the first time, a role for extracellular vimentin in promoting the differentiation of these mesenchymal repair cells to the myofibroblast phenotype associated with fibrosis.

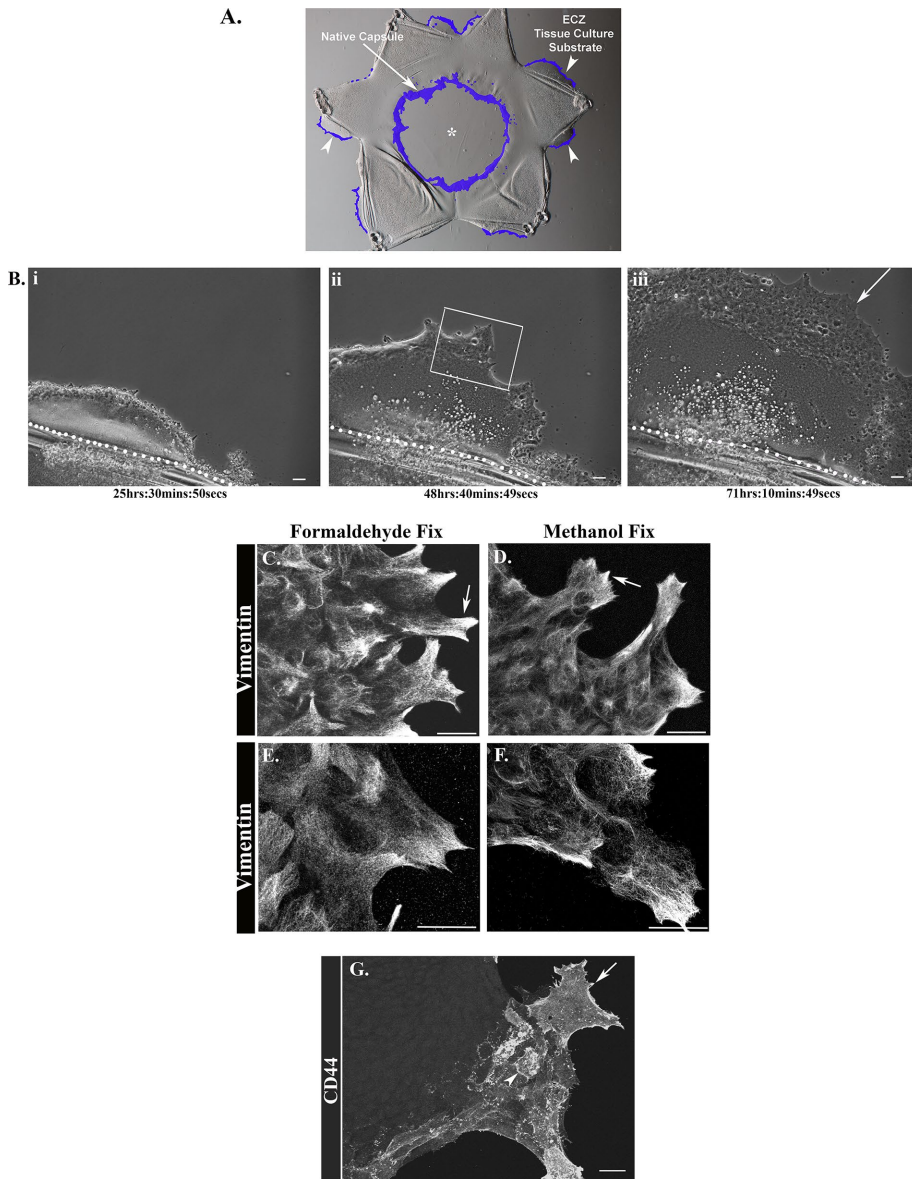
## RESULTS

### Resident repair cells of the lens direct wounded lens epithelial cells to migrate off their endogenous basement membrane and across an adjacent tissue culture substrate

Our studies investigate how mesenchymal leader cells, whose normal role it is to direct regenerative repair of epithelia following wounding, can be induced to differentiate to myofibroblasts. They were conducted with the *ex vivo* MCS culture model we originally established to investigate mechanisms of wound repair (Walker *et al.*, 2007, 2010, 2015; Menko *et al.*, 2014a). This wound model has clinical relevance to fibrosis, as cataract surgery often elicits a fibrotic response, resulting in the disease known as posterior capsule opacification (PCO) (Wormstone *et al.*, 2009; Wormstone and Eldred, 2016; Nibourg *et al.*, 2015). We focus on the resident population of vimentin-rich, mesenchymal repair cells of the lens that, on MCS injury, are activated to migrate to and rapidly populate the wound edge of the lens epithelium (Figure 1A, D1 postinjury, repair cells at the MCS wound edge colored purple, arrow) (Walker *et al.*, 2010). Here, functioning as classical leader cells, these cells direct regenerative repair of the injured epithelium, a task that is vimentin dependent (Menko *et al.*, 2014a; Bleaken *et al.*, 2016).

To create these cultures, the MCS-wounded lens is flattened on a tissue culture substrate by making cuts through the epithelium and its associated basement membrane, creating the star-shaped wounded-explant cultures. The cuts made to flatten the explants are additional injury sites that form the outside edge of the explant. These sites of injury are also rapidly populated by the wound-activated resident mesenchymal repair cells (Figure 1A, repair cells at the cut edge colored purple, arrowheads). These mesenchymal cells lead the lens epithelial cells from their original position on their native lens basement membrane capsule onto and across the adjacent, rigid tissue culture substrate. We refer to the region on the tissue culture plastic that the cells occupy postwounding as the extracapsular zone (ECZ). Formation of the ECZ in response to injury is best observed by time-lapse microscopy (Supplemental Video 1, representative still images shown in Figure 1, Bi–Biii). Live imaging revealed that the mesenchymal cells at the leading edge extend and retract lamellipodial protrusions along the substrate as they lead the forward movement of the wounded epithelium across the culture substrate. This behavior closely parallels the injury response we have observed for these resident repair cells previously, when they populate the leading edge of the MCS-wound and direct wound closure on the endogenous basement membrane substrate of the lens (Menko *et al.*, 2014a; Bleaken *et al.*, 2016).

We have shown previously that vimentin is a principal characteristic of the repair cells that direct wound closure at the MCS wound edge (Menko *et al.*, 2014a). Here we examined whether vimentin was also a defining feature of the repair cells in their function as leader cells of the ECZ. Following immunolabeling for vimentin at D2 postinjury, the MCS-wounded-explant cultures were imaged in regions of the ECZ typical of the boxed area in Figure 1Biii. The leading-edge cells of the ECZ expressed high levels of vimentin, prominently localized to the lamellipodial protrusions that extend along their substrate surface (Figure 1, C and D, arrows). The organization of vimentin in these cells was examined in cultures fixed with either formaldehyde (Figure 1, C and E) or methanol (Figure 1, D and F), and the cells were imaged by both confocal (Figure 1, C and D) and superresolution confocal (Figure 1, E and F) microscopy. Methanol fixation, which extracts membrane lipids, highlighted the organized vimentin



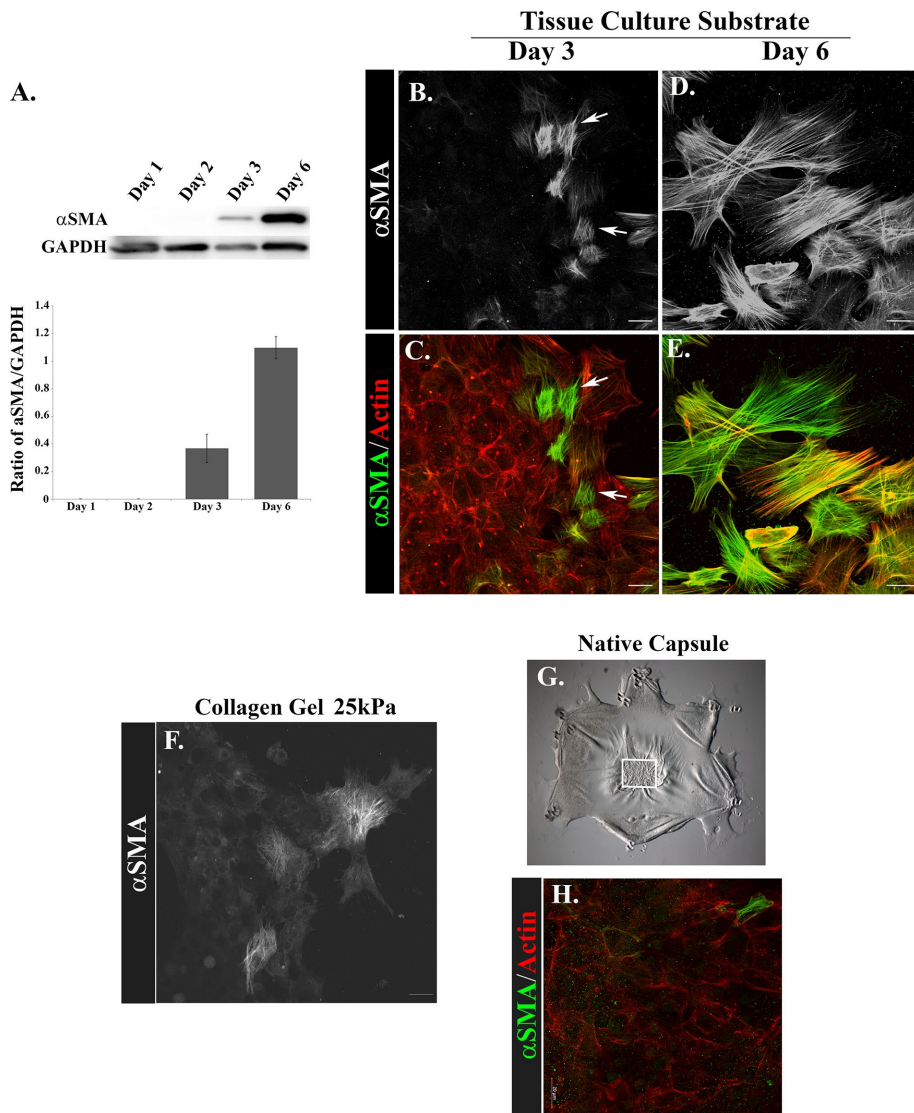
**FIGURE 1:** Properties of leader cells activated on injury when they encounter a profibrotic microenvironment. (A) Ex vivo wound healing/fibrosis culture model, created by performing mock cataract surgery on isolated chick embryo lenses, which removes the lens fiber cell mass from the lens native basement membrane (BM) capsule (\*). Mesenchymal repair cells (shaded purple) migrate to both the leading edge of the MCS wound (arrow) and to the cut edges created to flatten the lens capsule on the substrate (arrowhead). Repair cells at the cut edge lead the lens epithelium off the lens capsule onto and across the rigid tissue culture substrate, referred to the extracapsular zone (ECZ). (Bi–Biii) Representative still images from a time-lapse movie (Supplemental Video 1) taken over 3 d postinjury show the collective migration of the lens epithelial cells across the ECZ led by mesenchymal leader cells (arrow). The dotted white line in i, ii, and iii indicates the border of the native capsule and the ECZ. (C–F) Ex vivo MCS wounded-explant cultures were fixed in formaldehyde (C, E) or in methanol (D, F) 2 d postinjury, labeled for vimentin, and imaged by confocal microscopy (C, D) or superresolution confocal microscopy (E, F) in the ECZ, in a region similar to the boxed area in Bii. Vimentin was enriched in the lamellipodial protrusions extended by leader cells (C–F, arrows). When cultures were fixed with formaldehyde, both filamentous and diffuse populations of vimentin were observed at the tips of lamellipodia extensions (C, E, arrow). Methanol fixation conditions highlighted the vimentin cytoskeleton (D, F). (G) At 2 d postinjury the ex vivo explant cultures were labeled for CD44 and imaged by confocal microscopy in the ECZ. Mesenchymal leader cells were distinguished by their expression of the CD44 receptor (arrow), which was enhanced at cell borders (arrowhead). Magnification (Mag) bars = 20  $\mu\text{m}$ . Images in C–G are presented as projections.

cytoskeleton in the leader cell population. Immunostaining of formaldehyde fixed cultures, permeabilized with Triton X-100 postfixation, revealed both a diffuse pattern of vimentin labeling and organized vimentin filaments. This suggests that formaldehyde fixation may enhance preservation of soluble vimentin populations.

We have shown previously that another defining feature of the vimentin-rich, leader cell population activated in response to MCS wounding was its expression of CD44 (Bleaken *et al.*, 2016). While widely expressed, this cell-surface receptor for hyaluronic acid (HA) is characteristic of leukocytes and known for its role in cell adhesion and migration (McDonald and Kubers, 2015; Bano *et al.*, 2016; Senbanjo and Chellaiiah, 2017). Immunolabeling of the wounded-lens explant cultures with an antibody to the CD44 extracellular domain at D2 postinjury showed that, in the ECZ, CD44 is expressed exclusively by the repair cells at the leading edge (Figure 1G, arrow). CD44 is enriched at the plasma membrane of these leader cells, especially along their cell borders (arrowhead), and was not expressed by the lens epithelial cells that migrate behind the CD44+/vimentin+ leader cell population of the ECZ.

#### **$\alpha\text{SMA}$ + myofibroblasts appear at the leading edge of the ECZ, with their emergence linked to rigidity of the microenvironment**

Substrate rigidity is a known factor in inducing cells that mediate regenerative repair to become myofibroblasts (Arora *et al.*, 1999; Li *et al.*, 2007; Liu *et al.*, 2010; O'Connor *et al.*, 2015). Reprogramming of cells to a myofibroblast phenotype is characterized by the induction of  $\alpha\text{SMA}$  and its organization into stress fibers (Skalli *et al.*, 1989; Schmitt-Graff *et al.*, 1994). Western blot analysis of protein extracts from cells isolated from the ECZ of MCS-wounded-explant cultures from D1 to D6 postinjury showed that  $\alpha\text{SMA}$  was first expressed by ECZ cells at culture D3 and increased greatly by D6 (Figure 2A). Since the ECZ is composed of both lens epithelial cells and the lens-resident, vimentin-rich repair cells that populate the wound edge postinjury, we performed immunofluorescence analysis to determine where in the ECZ the  $\alpha\text{SMA}$ -expressing cells were localized. Confocal image analysis at culture D3 revealed that  $\alpha\text{SMA}$  stress-fiber-positive myofibroblasts emerged exclusively at the leading edge of the ECZ (Figure 2, B and C), the area populated by the vimentin-rich



**FIGURE 2:** Myofibroblasts emerge at the leading edge of the ECZ and their differentiation is influenced by the rigidity of the microenvironment. (A) Total cell lysates prepared from isolated ECZ regions at D1–D3, and D6 were immunoblotted for  $\alpha$ SMA, with GAPDH as loading control. Results were quantified for three independent experiments and presented as a ratio of  $\alpha$ SMA to GAPDH for each fraction. Results demonstrate that  $\alpha$ SMA is first expressed on D3 and increases threefold by D6. (B–E, H) Ex vivo explant cultures were fixed D3 (B, C, H) and D6 (D, E) postinjury and labeled for  $\alpha$ SMA (B–E, H). In C, E, and H  $\alpha$ SMA is green and F-actin is red. Cultures were imaged by confocal microscopy in the ECZ (B–E) or on the native capsule (H).  $\alpha$ SMA-positive myofibroblasts emerged at the leading edge of the ECZ at D3 (B, C, arrows) and by D6 myofibroblasts had expanded in number and formed extensive  $\alpha$ SMA stress fibers (D, E). (F) To examine induction of myofibroblast differentiation in a physiological relevant profibrotic microenvironment, the ex vivo MCS-wounded explants were cultured for 3 d postinjury on 2.5 mg/ml collagen gels, corresponding to 25 kPa, and immunolabeled for  $\alpha$ SMA. The result shows that leader cells are induced to acquire a myofibroblast phenotype in a microenvironment within the physiological range of fibrotic tissues. In contrast, few  $\alpha$ SMA+ cells were observed on the native capsule at culture D3 (H), at which time wound healing was just completed (G, white box). Results show that the rigidity of the tissue culture substrate is a factor in the induction of myofibroblast differentiation. The results presented represent a minimum of three independent studies. Mag bar = 20  $\mu$ m; images in B–E and G are presented as projections. Image in H is presented as single optical section.

leader cells (Figure 1, C and D). The presence of myofibroblasts at the leading edge of the ECZ had increased greatly by D6 post-wounding (Figure 2, D and E), in part due to their proliferation (Supplemental Figure 1).

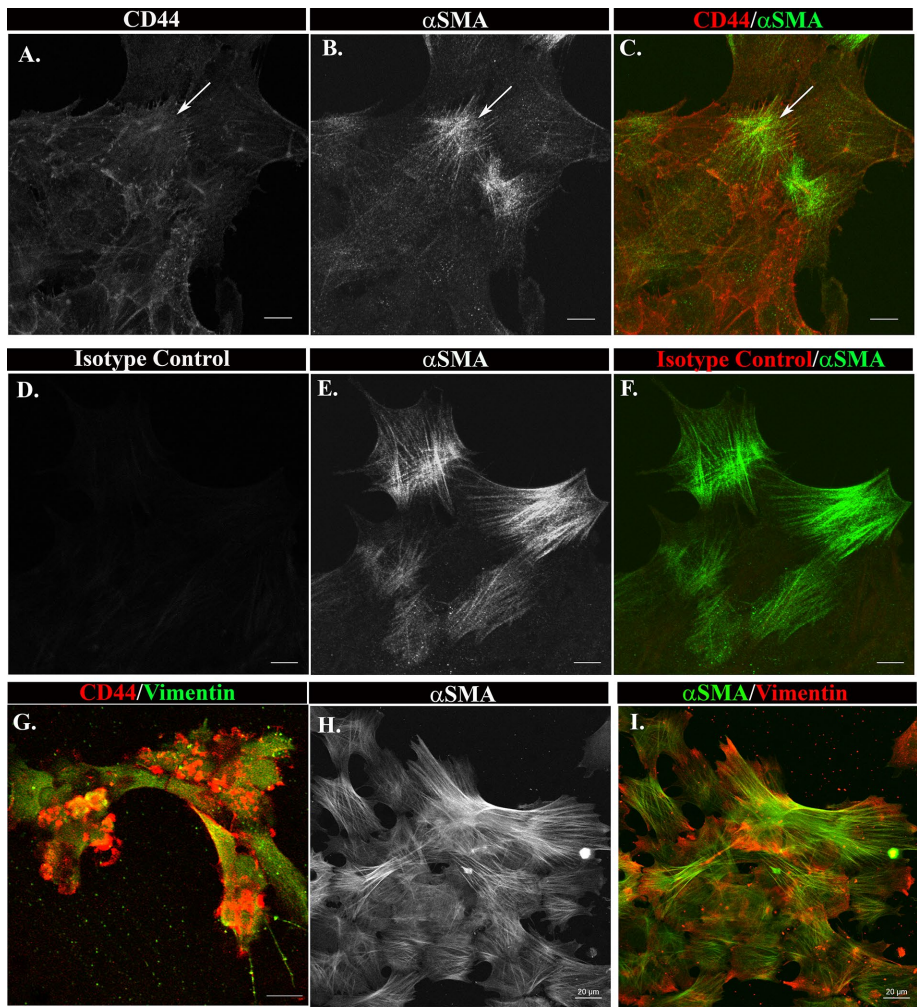
in the ECZ. To determine whether the cells labeled with CD44 at D1 had differentiated to myofibroblasts at D3, the cultures were fixed, the CD44-antibody-tagged cells labeled with a fluorescent-labeled secondary antibody, and the cells in the ECZ coimmunolabeled for

We also examined whether a substrate in the physiological range of a profibrotic tissue (Wells, 2013) induces repair-modulating cells to alter their fate to differentiate into a myofibroblast phenotype. The ex vivo, MCS-wounded explants were placed on tissue culture dishes coated with collagen I gels of 2.5 mg/ml, corresponding to a Young's modulus of 25 kPa to examine whether rigidity of the microenvironment is a factor in inducing cells involved in repair of the wounded-lens epithelium to alter their fate and differentiate to myofibroblasts. The stiffness of the collagen gel is in range of the rigidity of fibrotic tissues (Wells, 2013). Appearance of myofibroblasts in the ECZ of MCS-wounded explants cultured on the collagen I gel substrates was determined by confocal microscopy imaging following immunolabeling for  $\alpha$ SMA. The results show that on collagen gels of 25 kPa, the leading-edge cells had differentiated to  $\alpha$ SMA+ stress-fiber-containing myofibroblasts on D3 (Figure 2F).

The ex vivo MCS-injury model provides a unique opportunity to follow, concurrently, the function of resident repair cells in regulating wound healing in their native microenvironment and how these same repair-modulating cells may be induced to differentiate to myofibroblasts in the controlled environment of the ECZ. Therefore, we examined whether myofibroblasts also appeared on the native lens basement membrane capsule undergoing regenerative repair post-MCS (Figure 2, G and H). In contrast to the induction of myofibroblasts on the ECZ substrates, very few  $\alpha$ SMA+ cells were induced to form on the native substrate of the explant where the mesenchymal leader cells effectively direct closure of the lens epithelial wound by culture D3.

### Resident lens mesenchymal cells activated to modulate repair of the wounded lens epithelium are myofibroblast progenitors

Cell fate-tracking studies were performed to examine whether the leader cells of the ECZ had the potential to become myofibroblasts. This approach took advantage of our ability to specifically tag the vimentin-rich repair cells at the leading edge of the ECZ with the extracellular domain CD44 antibody (Figure 1G). For these studies, the mesenchymal leader cells were tagged with the CD44 antibody by live labeling for 20 min at D1 post-injury. Then the cultures were grown through culture D3 when myofibroblasts first appear



**FIGURE 3:** CD44 positive leader cells are precursors of myofibroblasts. (A–F) ECZ cells were live labeled for 20 min at D1 postinjury with antibody to the cell surface antigen CD44 (A–C) or an isotype antibody control (D–F). (A–C) The fate of the antibody-tagged cells was followed for 2 more days until myofibroblasts first emerge, at which point the cultures were fixed and the CD44-tagged cells detected with fluorescent-tagged secondary antibody, the cells colabeled for  $\alpha$ SMA and imaged by confocal microscopy. (D–F) Control study in which cells were labeled with an isotype control antibody and then treated under the same experimental conditions as in A–C. (G–I) Ex vivo MCS explants were placed cell side down in the top chamber of Matrigel transwell invasion chambers, and the properties of the cells that had invaded to the filter side facing the bottom chamber determined by immunofluorescence analysis. (G) At 2 d postinjury, the wound-activated CD44+/vimentin+ leader cells had invaded through the Matrigel and populated the bottom side of the filter, confirming the efficacy of this approach to isolate the repair cell population. (H, I) The cells on the bottom filter were cultured through D10 postinjury and the cells co-immunolabeled for vimentin and  $\alpha$ SMA. The results show that the wound-activated repair cells that had invaded to the bottom filter were  $\alpha$ SMA+, demonstrating that they had acquired a myofibroblast phenotype. Together these studies show that the CD44+/vimentin+ leader cell population activated on injury are myofibroblast progenitors. A–F, Mag bar = 10  $\mu$ m. G–I, Mag bar = 20  $\mu$ m. Images shown are single optical sections (A–F, H, and I) or projections (G).

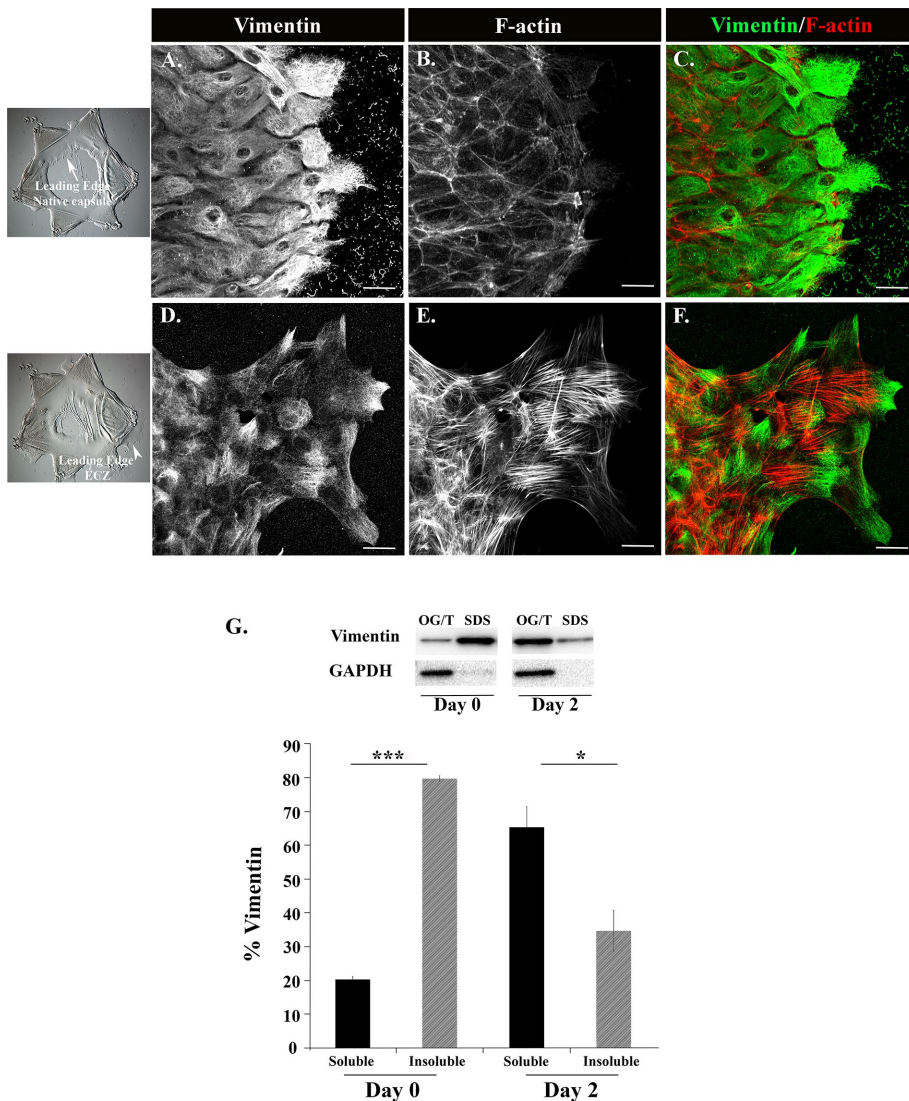
$\alpha$ SMA. Confocal image analysis showed that all  $\alpha$ SMA+ stress-fiber-expressing cells in the ECZ were positive for CD44 (Figure 3, A–C, arrow). As a control, the cultures were labeled with a nontargeting immunoglobulin G (IgG) of the same isotype as the CD44 antibody. The  $\alpha$ SMA+ cells that emerge in these control cultures were not labeled by the nontargeting IgG (Figure 3, D–F), confirming the specificity of the CD44-tracking studies. These results demonstrate that the CD44+ leader cells, a vimentin-rich cell population, are progenitors of the  $\alpha$ SMA+ myofibroblasts that emerge in the ECZ in response to wounding.

As an alternative approach, we used a Boyden chamber invasion assay to examine the potential of vimentin-rich repair cells to differentiate to myofibroblasts. We have previously used this assay to reveal the invasive properties of the CD44+/vimentin+ mesenchymal subpopulation of the lens following their activation by MCS injury (Bleaken et al., 2016). For these studies, MCS-wounded-explant cultures are placed, cell-side down, on the Matrigel-coated filter of the top Boyden chamber immediately postinjury. Serum-free medium was placed in the top chamber and serum-containing medium in the bottom chamber. As shown in our previous study, at D2 postinjury the wound-activated CD44+/vimentin+ repair cells have invaded through Matrigel to the bottom filter surface of the Matrigel-coated transwell chamber (Figure 3G). The wounded-lens epithelial cells remain associated with their native basement membrane capsule in the top chamber (Bleaken et al., 2016). The vimentin-rich leader cells activated following injury that had invaded to the bottom of the Boyden chamber filter were fixed at culture D10 and immunolabeled for  $\alpha$ SMA. Confocal image analysis showed that these cells expressed both vimentin and  $\alpha$ SMA (Figure 3, H and I), evidence that the vimentin+/CD44+ repair cells of the lens can be targeted to become myofibroblasts.

### Distinct organization of actin and vimentin when leader cells are in located in proregenerative and profibrotic microenvironments

We found that rigidity is a factor in inducing wound-activated resident repair cells of the lens to become myofibroblasts (Figure 2). This suggests that the change in their differentiation state involves a mechanotransduction-signaling event. The cytoskeleton is a key factor in transmitting mechanotransduction signals. Therefore, we compared the organization of the actin and vimentin cytoskeletons in wound-activated repair cells at the leading edge of the MCS wound on the native lens basement membrane capsule to their organization in the leading-edge cells of the ECZ that forms on the tissue-culture

substrate. The cultures were colabeled with antibody to vimentin and with fluorescent-tagged phalloidin that labels F-actin. Cytoskeletal organization was analyzed by confocal microscopy imaging (Figure 4, A–F). The vimentin-rich repair cells at the leading edge of the wound on the cells' endogenous basement membrane capsule have a well-organized vimentin cytoskeleton (Figure 4, A and C, D1), but this leading-edge population is remarkably F-actin poor (Figure 4, B and C). In contrast, there is a significant population of vimentin that is unorganized in the repair cells at the leading edge of the ECZ, most highly localized to lamellipodial processes these cells extend



**FIGURE 4:** Distinct organization of vimentin and actin filaments when leader cells are in proregenerative and profibrotic microenvironments. (A–F) Ex vivo MCS wounded-explant cultures were labeled for vimentin (A, C, D, F; green in C, F) and F-actin (B, C, E, F; red in C, F) on day 1 (A–C) and day 2 (D–F) postinjury, and imaged by confocal microscopy. Vimentin-rich leader cells at the MCS wound edge on the lens native basement membrane capsule exhibited a highly organized vimentin cytoskeleton (A, C) but were notably poor in F-actin (B, C). In contrast, in leader cells associated with the profibrotic environment of the ECZ there was a prominent population of vimentin with a diffuse labeling pattern that was highly enriched at the tips of lamellipodial extensions extended along the tissue culture substrate of the ECZ, in addition to a more classical vimentin cytoskeletal network (D, F). These cells contained abundant F-actin stress fibers (E, F). (G) Solubility of vimentin was determined at the time of wounding and in the ECZ cells on D2 postinjury. Cells were extracted sequentially in Triton X-100/octyl glucoside (Triton/OG) and SDS-containing solutions to separate the soluble vimentin populations, including unassembled vimentin and short vimentin filaments, from the highly insoluble vimentin cytoskeleton. Extracts were immunoblotted for vimentin and GAPDH and the percentage of total vimentin (G) in each fraction was quantified over three independent experiments. Results show that at the time of wounding vimentin was largely a detergent-insoluble cytoskeletal network, in contrast to its largely soluble state of organization in the ECZ at D2 postinjury, a finding consistent with the diffuse state of organization of vimentin in leader cells of the ECZ at this time (D, F). GAPDH fractionated with the Triton/OG soluble fraction. The histogram shows the percentage of vimentin  $\pm$  SEM. The  $p$  values were generated by Student's  $t$  test,  $*p < 0.05$ ,  $***p < 0.001$ . Mag bar = 20  $\mu$ m. Images in A–F are presented as projections.

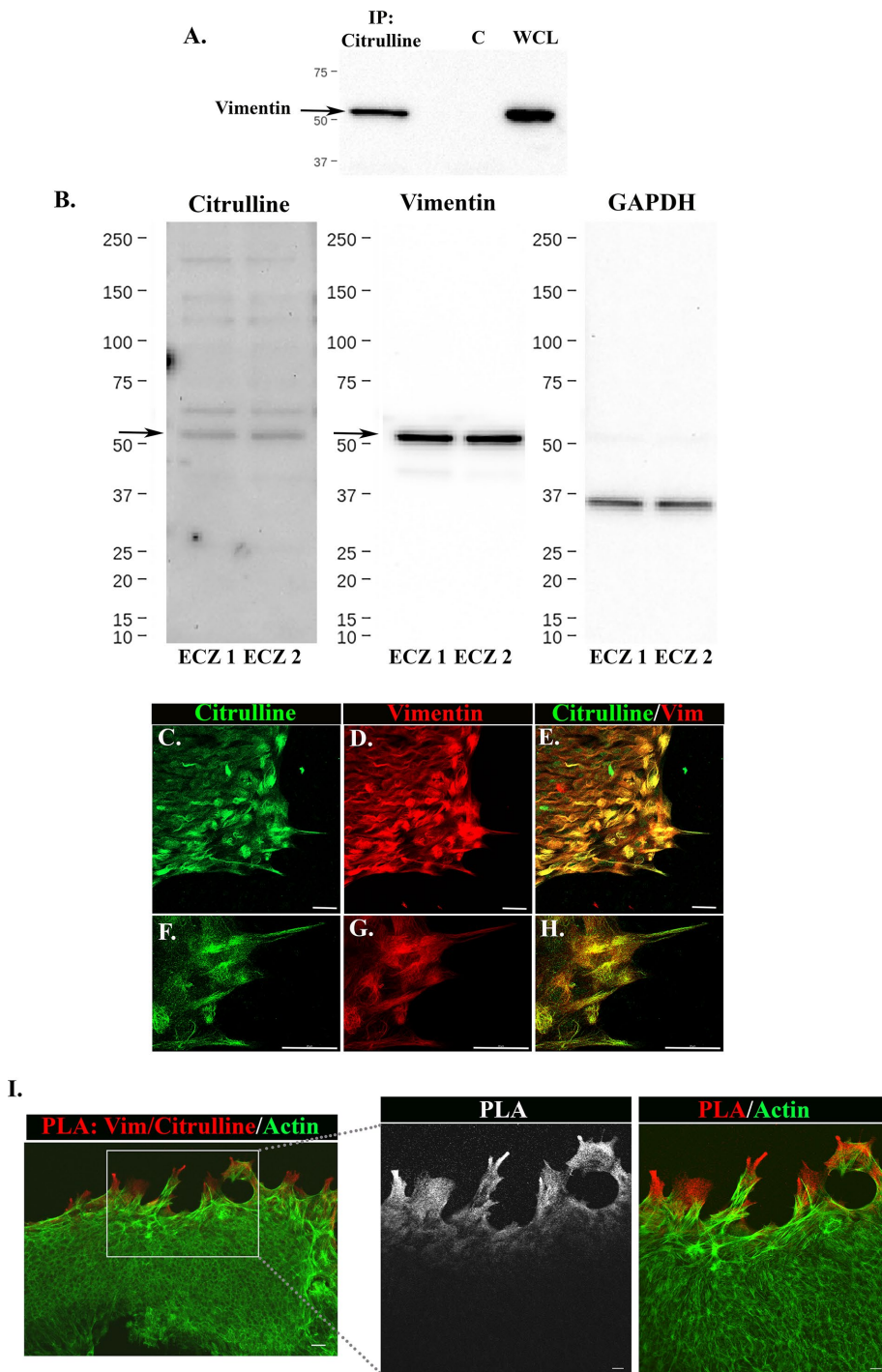
along the substrate surface (Figure 4, D and F, D2). In contrast to the MCS-wound edge, the leading-edge cells in the ECZ are rich in actin stress fibers (Figure 4, E and F).

### Increase in vimentin solubility postwounding precedes leader cell differentiation to myofibroblasts

The diffuse vimentin-labeling pattern in the lamellipodia of the highly migratory reparative cells at the leading edge of the ECZ indicated that the organization of this vimentin population was distinct from the more classical vimentin filament structures in these cells. While the vimentin cytoskeletal network present in most cell types is insoluble to Triton X-100 detergent extraction (Osborn and Weber, 1977; Blikstad and Lazarides, 1983; Gilbert and Fulton, 1985; Soellner *et al.*, 1985), vimentin protein that is not organized into filaments, or present in the short filament structures that often appear as squiggles (Chou *et al.*, 2007), is Triton X-100 soluble. Using a differential detergent extraction approach, we compared vimentin solubility in MCS-wounded explants at the time of injury to cells in ECZ at D2 postwounding (Figure 4G). Cells were extracted sequentially with a Triton X-100/octyl glucoside buffer (Brown and Rose, 1992) that isolates soluble forms of vimentin, including unassembled unit or short vimentin filaments, and a 4% SDS-containing buffer that extracts the highly insoluble vimentin cytoskeletal network. The extracted proteins were separated by gel electrophoresis and Western blotted for vimentin (Figure 4G). We immunoblotted for the Triton-soluble protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as control. The Western blot results were quantified and represented graphically as percentage of total vimentin that is soluble (Triton/OG) and insoluble (SDS) (Figure 4G). While at the time of wounding, vimentin was largely present as a detergent-insoluble cytoskeletal network, at D2 postinjury in the ECZ, vimentin had become highly soluble, with the majority of the vimentin protein fractionating together with GAPDH in the Triton X-100/OG extracts (Figure 4G). This high level of soluble vimentin was consistent with the diffuse labeling for vimentin observed in the leader cell population of the ECZ (see Figure 4D).

### Posttranslational modification of vimentin following injury

We investigated the possibility that posttranslational modifications (PTMs) of vimentin linked to vimentin solubility, including citrullination and phosphorylation (Ando *et al.*, 1989; Inagaki *et al.*, 1989; Sihag *et al.*, 2007; Hyder *et al.*, 2008; Snider and Omary, 2014; Gudmann *et al.*, 2015), were associated with the repair-modulating leader cell population activated by MCS injury. The presence of citrullinated proteins in MCS-wounded-explant cultures



**FIGURE 5:** Citrullinated vimentin is detected postinjury and associated with repair-modulating leader cells. (A) Proteins from D2 MCS-wounded-explant cultures were extracted, immunoprecipitated with a citrulline antibody, and immunoblotted for vimentin. The results showed the presence of vimentin in these citrulline immunoprecipitates. Whole cell lysates (WCL) of MCS-wounded explants were immunoblotted for vimentin as a control. (B) Lysates of cells in the ECZ regions at D2 postwounding were immunoblotted for citrulline and reprobbed for vimentin. Results shown for two independent studies (ECZ1 and ECZ2) demonstrate that vimentin comigrates with the principal citrullinated band at 55 kDa (arrow), supporting the conclusion that vimentin is citrullinated in cells of the ECZ. GAPDH is shown as a loading control. (C–H) MCS-wounded explant cultures were coimmunolabeled at D2 postinjury with antibodies to citrulline (C, E, F, H) and vimentin (D, E, G, H). The cultures were imaged in the ECZ by confocal microscopy (C–E), and at the leading edge of the same ECZ by superresolution confocal microscopy (F–H). While citrullinated proteins localized throughout the ECZ, they were enriched in leading edge cells, including many regions where citrulline colocalized with vimentin. (I) Proximity ligation assay (PLA) was performed in the ECZ at 2 d postinjury with antibodies to

was examined using an immunoprecipitation approach. At 2 d postinjury, protein extracts were immunoprecipitated with antibody to citrulline and the immunoprecipitated, citrullinated proteins immunoblotted for vimentin (Figure 5A). The results showed that vimentin is present in these citrulline immunoprecipitates, indicating that vimentin is citrullinated in the MCS-wounded-explant cultures. Next, we extracted the proteins from the ECZ region at D2 postinjury, electrophoretically separated the proteins, immunoblotted the ECZ extracts for the citrulline PTM, and reprobbed for vimentin (Figure 5B). This study demonstrated the citrullinated protein of greatest abundance migrated at 55 kDa and was labeled with antibody to vimentin; this supports the conclusion that vimentin is citrullinated in cells that form the ECZ postwounding.

Next, cell-based studies were performed to examine the localization of citrullinated proteins in ECZ region at D2 postwounding. The cultures were coimmunolabeled for citrulline and vimentin (Figure 5, C–H). While there are citrullinated proteins throughout the ECZ, they are enriched in the cells at the leading edge (Figure 5, C–E). Superresolution confocal imaging of the protrusions extended along the substrate by the leader cells of the ECZ showed coincident immunolabeling of citrulline with a subset of the vimentin protein in these cells (Figure 5, F–H). To further examine vimentin citrullination in the lamellipodial protrusions of the leader cells in the ECZ we performed a proximity ligation assay (PLA) with antibodies to vimentin and the citrulline PTM and postlabeled the cells for F-actin. The results showed a strong vimentin/citrulline PLA signal specific to the cells at the leading edge of the ECZ (Figure 5I).

Another PTM associated with vimentin solubility is its phosphorylation at sites such as Ser38. Immunolabeling for pSer38-vimentin at D2 post-MCS wounding showed that pSer38-vimentin+ cells were associated with cells remaining on the wounded explant but that there were very few pSer38-vimentin+ cells at the leading edge of the ECZ (Supplemental Figure 2). As there are multiple potential sites for vimentin phosphorylation, phosphorylation PTM cannot

vimentin and citrulline and then the cultures were labeled for F-actin with fluorescent-conjugated phalloidin. A positive PLA signal (red) was detected in the lamellipodia tips of the leading-edge cells of the ECZ, shown at two levels of magnification. Mag bars, C–H, and low-magnification image in I = 20 μm. In high-magnification images in I the Mag bar = 10 μm; images are presented as projections.



be ruled out as a factor in regulating vimentin organization and function in the leader cells of the ECZ.

### **On injury extracellular vimentin is released into the extracellular space and associated with the surface of wound-activated leader cells and their underlying matrix substrate**

Although best known as a component of the intermediate filament cytoskeleton, vimentin has also been shown to function as an extracellular protein. In this form, vimentin binds to the cell surface at distinct sites, including specific cell-surface receptors, where it initiates a cellular response (Garg *et al.*, 2006; Shigyo *et al.*, 2015). Among the roles identified for extracellular vimentin are the regulation of 1) immune cell function (Mor-Vaknin *et al.*, 2003), 2) viral/bacterial entry into cells (Koudelka *et al.*, 2009; Chi *et al.*, 2010; Das *et al.*, 2011; Liang *et al.*, 2011; Du *et al.*, 2014; Mak and Brugge *et al.*, 2016; Yu *et al.*, 2016), and 3) tumor progression (van Beijnum *et al.*, 2006; Satelli and Li, 2011). In studies with our clinically relevant MCS-injury model, we examined whether extracellular vimentin was also a factor in the tissue response to wounding. We began with an immunoblot analysis of conditioned media collected from the MCS-wounded-explant cultures at D1 postinjury, which showed that on injury vimentin was released into the culture media during the repair process (Figure 6A). Medium alone does not contain vimentin. Since in a previous study we found no evidence of cell death following MCS (Walker *et al.*, 2010), we considered this an unlikely source of the vimentin released into the medium. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay analysis performed at D1 postinjury showed that very few cells were TUNEL positive in the MCS-wounded-explant cultures (Supplemental Figure 3), supporting this conclusion. These findings imply that the release of vimentin into the media is an active process of the wounding-healing response.

The impact of extracellular vimentin on cell behavior following wounding is expected to involve its association with the cell surface. To determine whether vimentin is on the surface of cells, we labeled the cells within the ECZ with a cell-impermeable biotinylation reagent at D2 postwounding. Labeling with fluorescent-conjugated streptavidin, which directly binds to biotin, confirmed that the biotin label in this study was limited to the cell surface (Figure 6B). Following biotinylation, the ECZ cells were extracted and their biotin-tagged surface proteins isolated on an avidin column. The isolated, biotin-tagged proteins were immunoblotted for vimentin (Figure 6C), for the surface receptor N-cadherin (positive control), and for the cytoplasmic proteins GAPDH and Akt (negative controls) (Figure 6C). The results showed that extracellular vimentin was linked to the surface of cells in the ECZ. Cell-surface-linked vimentin may associate directly with the cell surface as it is released from the cells or become associated with the cell surface after it is released to the medium.

To determine whether there was cell-type specificity of vimentin cell-surface association, MCS-wounded-explant cultures were fixed with paraformaldehyde and not permeabilized prior to immunolabeling for vimentin. We examined both the MCS wound edge on the native basement membrane capsule (Figure 6E) and the leading edge of the cells migrating across the ECZ (Figure 6F) by confocal imaging for the presence of cell-surface-associated vimentin. The results show that extracellular vimentin was bound exclusively to the wound-activated repair cells at the leading edge of both the MCS wound and the ECZ. Z-stacks collected at high magnification in the ECZ revealed that extracellular vimentin was widely distributed all along these cells' basal surfaces, with higher levels at the filopodia/lamellipodial processes extended at the cells' periphery (Figure 6H).

At these cells' apical domains, extracellular vimentin was mostly located to the surface of the filopodia/lamellipodial processes extended between the cells (Figure 6G).

Using a biochemical approach, we investigated if vimentin released in response to wounding was associated with matrix substrate underlying the leader cells in the ECZ. For this study, the wounded explants were detached from the tissue culture substrate and the ECZ cells removed by gentle scraping at D2 postinjury, leaving behind proteins linked to the substrate surface of the ECZ. These substrate-associated proteins were extracted and immunoblotted for vimentin, which was shown to be a component of this protein fraction (Figure 6I). Immunoblotting for GAPDH confirmed that these substrate-associated extracellular lysates were largely cell free. In parallel, extracts from the wounded explants that were detached from the dish were immunoblotted for vimentin and GAPDH. In a different approach, the cells in the ECZ were exposed to the membrane-impermeable cross-linker bis (sulfosuccinimidyl) suberate (BS<sup>3</sup>) at D2 postinjury to link cell-surface-associated proteins to proteins in their ECM microenvironment. After radioimmunoprecipitation assay (RIPA) buffer extraction of cellular proteins the extracellular proteins were coimmunolabeled for vimentin and the ECM protein tenascin-C that forms a provisional matrix on the underlying substrate of ECZ cells migrating in response to wounding. Tenascin-C has been implicated in the induction of fibrosis (Kasprzycka *et al.*, 2015; Bhattacharyya *et al.*, 2016; Fu *et al.*, 2017). Confocal imaging showed codistribution of tenascin-C and vimentin on the substrate surface in an area underneath the leader cells of the ECZ (Figure 6, J and K). No F-actin was detected in these cross-linking/extraction studies (Figure 6L).

### **Extracellular vimentin signals leader cell behavior in response to wounding and the differentiation of these cells to a myofibroblast phenotype**

Our results showed that a soluble, extracellular, vimentin pool was released by the cells into the culture medium during the repair process, so we examined whether the timing of myofibroblast emergence could be enhanced by the addition of soluble vimentin into the explant culture medium. Those results showed that increasing the concentration of vimentin over that produced by the cells did not alter the timing of myofibroblast emergence or significantly alter the degree of myofibroblast induction in this clinically relevant wound-repair culture model (Supplemental Figure 4). Therefore, we examined the functional roles of extracellular vimentin produced by the cells in response to MCS wounding in both wound repair and the induction of fibrosis. For these studies, the wounded cultures were grown in the presence of monoclonal antibodies to vimentin. To analyze effects on wound closure and emergence of myofibroblasts, we used two distinct vimentin antibodies, AMF17B and H5, with IgG of the same isotype as control. In our previous studies, we have shown that wound closure on the native substrate of the basement membrane capsule is typically complete by D3 post-MCS (Menko *et al.*, 2014a,b). For these studies, antibodies to vimentin were added to the explant cultures at the time of wounding (D0), the cultures imaged daily through culture D3, and open-wound area quantified. Wound repair in the presence of the vimentin antibodies slowed wound closure (Figure 7A), with dynamics and phenotype similar to what we observed previously with small interfering RNA vimentin knockdown (Menko *et al.*, 2014a).

To investigate whether extracellular vimentin regulates leader cell function in the ECZ and signals differentiation of leader cells to myofibroblasts, we exposed the cells in the ECZ to antibodies to vimentin from D1 postinjury, after removing the original lens explants from the culture dish, and examined them for effects on

leader cell behavior and emergence of myofibroblasts at D3 in culture. Both vimentin antibodies suppressed the extension of lamellipodial processes by the leader cells and interfered with the movement of leader cells across the ECZ (Figure 7B). The H5 monoclonal antibody (mAb) had a more potent effect on lamellipodia extension than AMF17B. Biochemical analysis revealed that blocking the extracellular vimentin signal inhibited expression of the myofibroblast protein  $\alpha$ SMA by cells in the ECZ, with H5 being slightly more efficacious at blocking  $\alpha$ SMA expression than AMF17B (Figure 7C). Immunolabeling confirmed that these vimentin antibodies suppressed the differentiation of leader cells to  $\alpha$ SMA+ myofibroblasts (Figure 7, E and F and H and I). The isotype IgG control had no effect on either cell migration or the development of fibrosis (Figure 7, D and G). We also treated the ex vivo MCS cultures with low doses of Withaferin A (WFA), which targets vimentin-soluble pools to inhibit vimentin function (Bargagna-Mohan *et al.*, 2013) but can also have off-target effects (Vanden Berghe *et al.*, 2012). We found that WFA, added to the media at D1 postinjury, decreased soluble pools of vimentin, altered the motility of the leader suppressing the extension of lamellipodial protrusions along the substrate surface, and blocked emergence of  $\alpha$ SMA+ myofibroblasts in a dose-dependent manner (Supplemental Figure 5). Together, these studies show that the extracellular population of vimentin has the potential to regulate leader cell function in the repair process and to signal their differentiation to myofibroblasts.

## DISCUSSION

### Fate of mesenchymal repair cells is determined by their microenvironment

In studies of wound repair, there is growing evidence that mesenchymal cell populations activated to direct regenerative repair become the progenitors of the myofibroblasts that underlie the aberrant repair processes of fibrosis and scarring. We have discovered that the cytoskeletal protein vimentin, which is essential to the normal function of these repair-modulating cells in tissue regeneration, can tip the balance from regenerative to fibrotic repair when these cells encounter a profibrotic microenvironment. Our studies examined this dichotomous role for repair cells in the clinically relevant ex vivo MCS wound-repair/fibrosis culture model. In their native microenvironment of the lens basement membrane capsule, these vimentin-rich mesenchymal repair cells rapidly migrate to the leading edge of the MCS wound where they direct lens epithelial tissue repair in a vimentin-dependent manner. When these same repair cells move to the outside edges of the MCS explant cut to flatten the lens capsule on the substrate, they lead lens-epithelial cells off the lens capsule onto and across the culture dish, where these vimentin-rich leader cells are induced to differentiate to  $\alpha$ SMA+ myofibroblasts, the cell type associated with fibrotic disease. This same change in cell phenotype was induced when these repair cells encounter a collagen gel microenvironment whose stiffness is in the physiological range of fibrotic tissues.

### Cell-surface-associated vimentin is a factor in determining repair cell fate and function

In our wound healing/fibrosis model, using vimentin antibodies that blocked the function of extracellular vimentin, we found that vimentin plays a role in promoting fibrosis beyond its classically described structural functions. Our studies show for the first time that an extracellular, cell-surface-associated vimentin population released in response to injury is linked specifically to the wound-activated mesenchymal repair cells that localize to the leading edge of the MCS wound. This extracellular vimentin pool is required

for the function of these mesenchymal leader cells in directing the regenerative repair process in the cells' native basement membrane microenvironment (modeled in Figure 8). Our data suggest that the fate of these cells is altered to that of a myofibroblast when they associate with a profibrotic ECM microenvironment. This interaction has the potential to signal a change in cell fate to a myofibroblast and alter the function of these repair cells from pro-regenerative to agents that promote fibrotic disease. It is possible that this extracellular vimentin population functions by binding to a putative receptor for extracellular vimentin located on the cell surface of the repair cells.

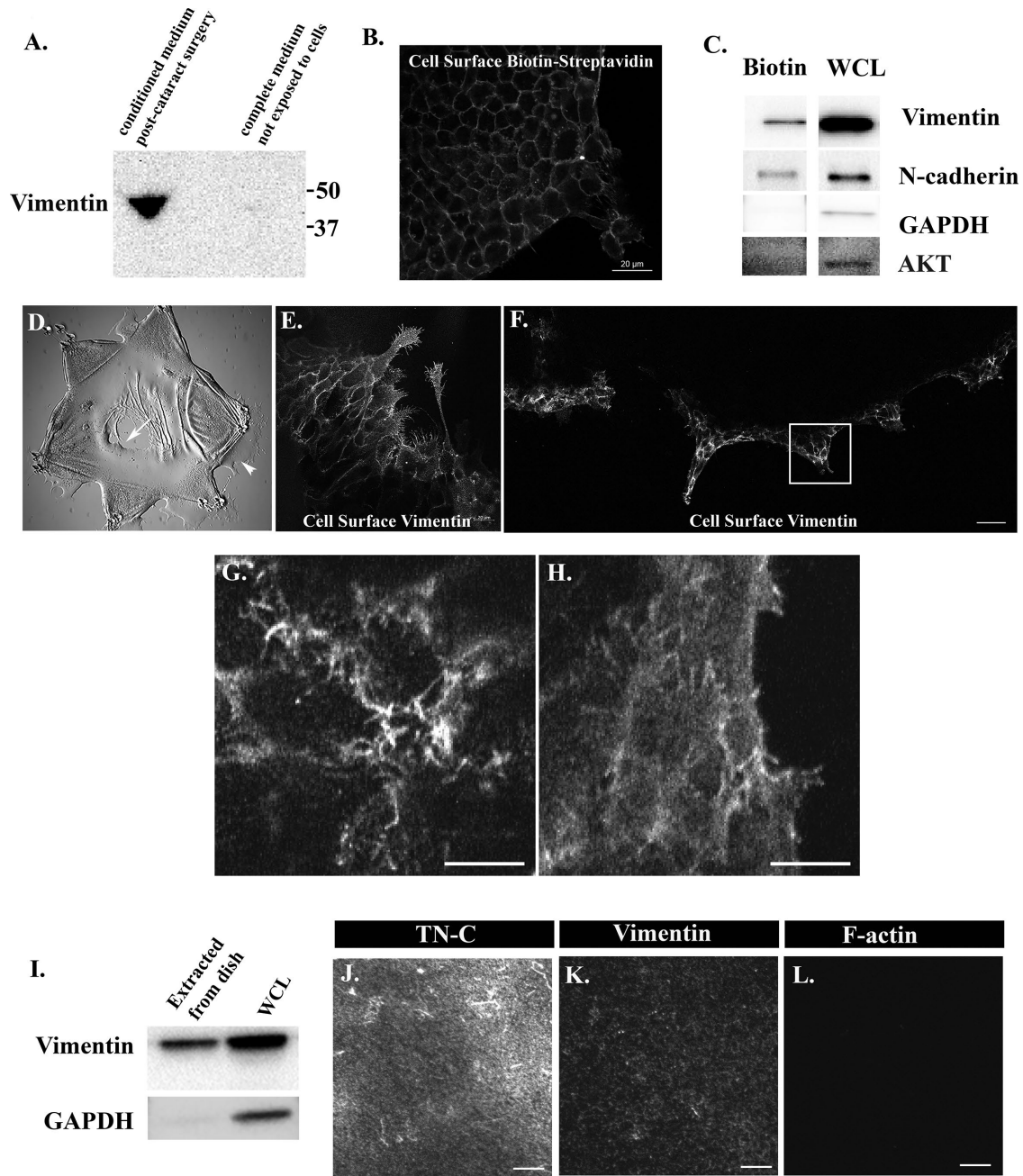
### Correlation between vimentin solubility and citrullination following injury

A high percentage of the vimentin pool in the cells of the ECZ following wounding was Triton/OG detergent soluble, contrasting the highly detergent-insoluble vimentin cytoskeletal network of the lens before wounding. Detergent insolubility is a feature of this intermediate filament protein typical of most cell and tissue types (Osborn and Weber, 1977; Blikstad and Lazarides, 1983; Gilbert and Fulton, 1985; Soellner *et al.*, 1985). It is likely that the soluble pool of vimentin in the ECZ includes both cytoplasmic and extracellular forms of this protein. Our data suggest that maintaining a large, soluble pool of vimentin in response to the MCS wound is important to the mechanism by which the mesenchymal leader cells function in directing regenerative repair.

Vimentin solubility is typically associated with PTMs of this protein, with both citrullination and phosphorylation believed to be primary PTMs that lead to and/or maintain vimentin protein-soluble states (Ando *et al.*, 1989; Inagaki *et al.*, 1989; Sihag *et al.*, 2007; Hyder *et al.*, 2008; Snider and Omary, 2014). Our studies show that vimentin citrullination was a feature of the mesenchymal leader cells located at the leading edge of the cells migrating across the ECZ. This finding is interesting in light of evidence that suggests citrullinated forms of vimentin play a role in fibrosis, including a report that patients with fibrosis express PAD enzymes that catalyze vimentin citrullination (Abdeen and Olusi, 2010; Gudmann *et al.*, 2015) and the finding that patients with fibrosis produce antibodies to citrullinated vimentin (Abdeen *et al.*, 2011; Vassiliadis *et al.*, 2012, 2013).

### Dynamic differences in vimentin and actin cytoskeletal organization in proregenerative and profibrotic environments

The lamellipodial protrusions that the mesenchymal leader cells extend along the rigid substrate of the ECZ were rich in vimentin, with a high level of the vimentin labeling of these cells appearing diffuse. This immunolabeling pattern likely represents a concentration of soluble forms of vimentin, which could include unassembled vimentin protein, and unit or short vimentin, likely including vimentin that is released to their cell surface. A distinguishing feature of the mesenchymal repair cells is the assembly of actin stress fibers in the more rigid ECZ microenvironment, but not when these cells populate the MCS wound edge on their native basement membrane. It has been shown that the presence of an organized vimentin intermediate filament cytoskeletal network can negatively regulate Rho activity and block both actin stress fiber formation and myosin contractility (Jiu *et al.*, 2017). This suggests that there is a dynamic, coordinated regulation of cytoskeletal elements in these cells and indicates that the predominance of soluble vimentin forms may be necessary to establish the contractile phenotype of  $\alpha$ SMA+ myofibroblasts.



**FIGURE 6:** Extracellular vimentin is released in response to injury, associated with the cell surface of leader cells and localized along the ECM substrate beneath the cells. (A) Conditioned media was collected from ex vivo MCS cultures at 1 d postinjury and immunoblotted for vimentin. The results show that an extracellular form of vimentin is released in the media. No vimentin is present in culture media prior to exposure to the cells. (B) Cell surface proteins in the ECZ were biotinylated at culture D2 and fixed and the biotin label was tagged with fluorescent-conjugated streptavidin. The results showed that biotin labeling is restricted to the cell surface of cells in the ECZ. (C) Cell surface proteins in the ECZ were biotinylated at culture D2, extracted, and immunoblotted for vimentin, N-cadherin (positive control), and GAPDH and Akt (negative controls). The results demonstrated that extracellular vimentin is associated with the surface of cells in the ECZ. (D–H) Paraformaldehyde fixed but not permeabilized ex vivo wounded cultures were immunolabeled for vimentin to examine cell type specificity for extracellular-surface-associated vimentin at both the MCS wound edge (D, arrow) and the leading edge of the ECZ (D, arrowhead). Cell-surface vimentin localized exclusively to leader cells of both the native BM capsule (E) and in the ECZ (F). Boxed region in F is shown at higher magnification along apicolateral (G) and basal (H) aspects of the cells. While extracellular vimentin was most prominent on cellular protrusions at the apicolateral surface of the leader cells in the ECZ, the extracellular vimentin pool localized all along the cells' basal surfaces of these cells where they contact the extracellular matrix and to basal membrane extensions between cells. (I) To determine whether basal extracellular vimentin also was associated with the substrate beneath the cells, cell-free extracts of substrate-associated proteins were examined by Western blot. Extracts of isolated ex vivo explants were included as a control (WCL). Results show that extracellular vimentin is associated with the proteins on the substrate. Immunoblotting for GAPDH confirmed that extracellular lysates were largely cell free. Results are representative of

### On injury vimentin is released into the extracellular space

Our discoveries that extracellular vimentin is released in these wound-repair cultures in response to injury, that it associates with the cell-surface of repair-modulating cells, and that it mediates the differentiation of the leader/repair cells to myofibroblasts are important new findings. The mechanism of vimentin release is not yet clear. While vimentin lacks a classic secretory signal sequence, it is secreted by macrophages in response to both pro- and anti-inflammatory cytokines (Mor-Vaknin *et al.*, 2003). The release in exosomes is another potential source for the extracellular vimentin pool (Chen *et al.*, 2016). Our own studies of the invasive potential of these wound-activated repair cells have shown that when these cells migrate through three-dimensional Matrigel, they are surrounded by vimentin concentrated in puncta that could represent vimentin-containing vesicles (Bleaken *et al.*, 2016).

### Potential mechanisms of extracellular vimentin function

While extracellular vimentin has been localized to the cell surface of different cell types, including macrophages (Mor-Vaknin *et al.*, 2003), endothelial cells (Xu *et al.*, 2004; Zou *et al.*, 2006; Koudelka *et al.*, 2009; Glaser-Gabay *et al.*, 2011), neutrophils (Moisan and Girard, 2006), astrocytes (Teshigawara *et al.*, 2013), and circulating tumor cells (Satelli *et al.*, 2014, 2016, 2017), its extracellular function has been poorly understood. In the mesenchymal repair cells that populate the leading edge of the ECZ of our MCS-wounded cultures, extracellular vimentin was most highly localized to the cells' basal surfaces (see model, Figure 8). Vimentin could mediate signaling binding to cell-surface receptors, transmitting a mechanotransduction signal that leads to the change in cell fate associated with fibrosis. In support of this hypothesis, studies have reported that vimentin can act as a ligand for cell-surface receptors, such as CD44 (Pall *et al.*, 2011) and IGF-1R (Garg *et al.*, 2006; Shigyo *et al.*, 2015). While future studies will be aimed at elucidating the mechanisms by which extracellular vimentin signals myofibroblast differentiation, our current studies show that the microenvironment is a critical driver of the function of the extracellular pool of vimentin. In wound repair, the microenvironment determines whether vimentin associated with the surface of the mesenchymal cells activated on wounding promotes their role in regenerative repair or their transition to myofibroblasts associated with fibrosis.

## MATERIALS AND METHODS

### Ex vivo MCS explant cultures

Ex vivo MCS explant cultures were prepared as previously described (Walker *et al.*, 2007, 2010, 2015; Menko *et al.*, 2014a,b). Cells were cultured in complete media (Media 199) (Invitrogen, Carlsbad, CA) that included 1% penicillin/streptomycin (Mediatech-Cellgro, Manassas, VA), and 1% L-glutamine (Mediatech-Cellgro) with 10% fetal calf serum (Invitrogen). The mock cataract surgery is performed after lenses are removed from E15 chick embryos (Poultry Futures, Lititz, PA) to ex vivo culture. This microsurgical procedure removes the fiber cells that comprise the mass of lens tissue (Walker *et al.*, 2007, 2015), leaving the basement membrane that surrounds the lens in vivo as a capsular bag structure containing only the wounded

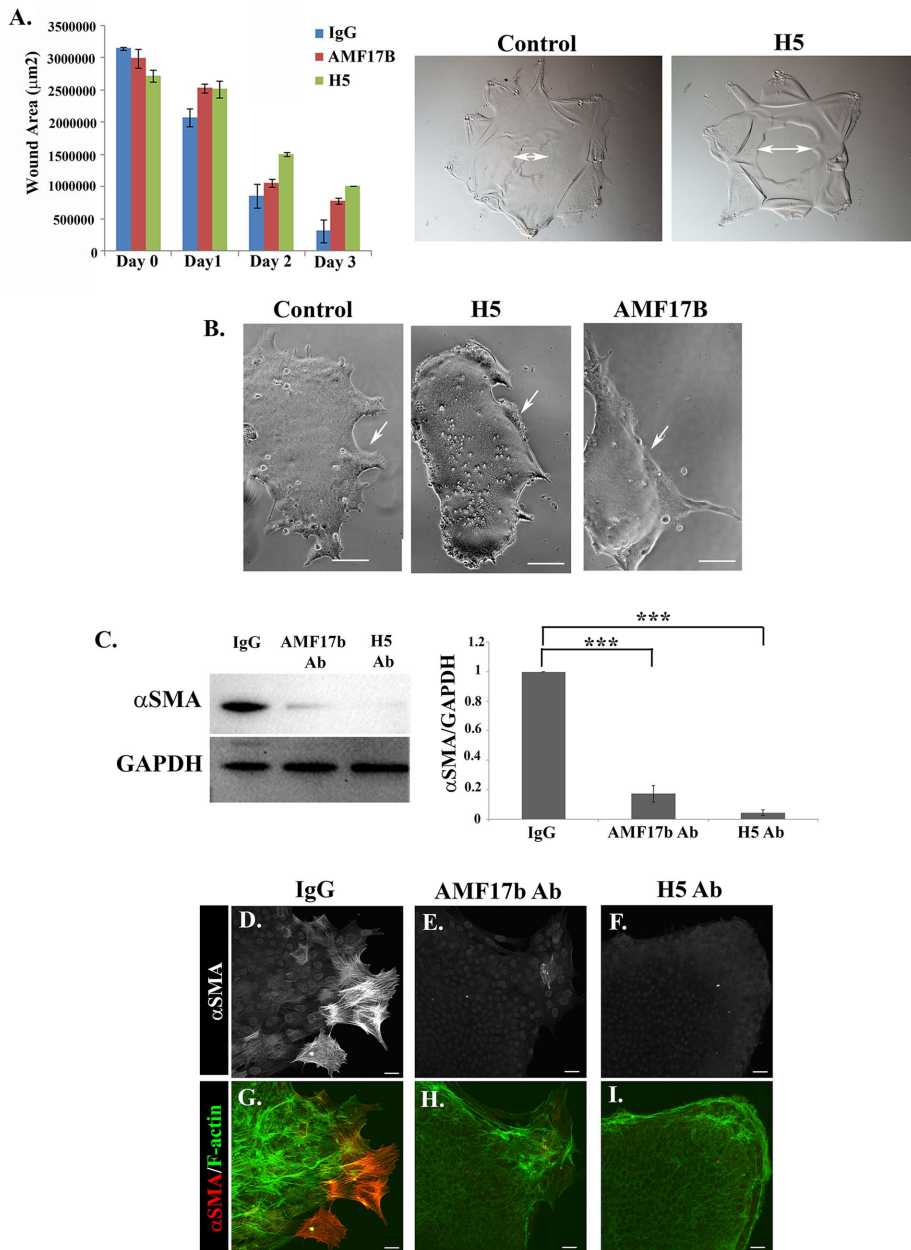
lens epithelial cells and a subpopulation of vimentin-rich mesenchymal repair cells (Walker *et al.*, 2010; Menko *et al.*, 2014a). The lens epithelium and subpopulation of mesenchymal repair cells associated with the lens capsule are placed on tissue plastic cell side up. By making cuts in the anterior aspects of the lens capsule, we flatten the postcataract surgery lens capsular bag with its lens epithelial and mesenchymal repair cells still attached. The resultant star-shaped, flattened explants are placed on the culture dish, cell side up (Figure 1A). The wounded lens epithelial cells are located at the points of the star, surrounding the denuded lens capsule in the center of the explants where the fiber cells had been attached (Figure 1A, asterisk).

Since differentiation to myofibroblasts is promoted in a rigid extracellular environment (Tomasek *et al.*, 2002; Hinz, 2007; Carver and Goldsmith, 2013), we studied myofibroblast emergence in cells that migrate from the outside cut edges of the ex vivo MCS-wounded-explant cultures onto and across the rigid microenvironment of the tissue culture plastic (Figure 1, A and B). The lens epithelial cells are led off the lens capsule by vimentin-rich mesenchymal cells that had rapidly migrated to the outside cut edge of the capsule in response to the cut wound. We refer to the region just beyond the lens capsule as the ECZ, the cells of which provide an ideal reductionist model with which to study how cells critical to regulating injury repair can be induced to change their fate and become the myofibroblasts that underlie fibrosis.

For collagen gel substrate studies, MCS-wounded-explant cultures were placed directly on tissue culture dishes coated with 1 ml of 2.5 mg/ml rat tail collagen I gels, according to the manufacturer's instructions (Millipore, Billerica, MA). For Boyden Chamber invasion studies, MCS-wounded-explant cultures were placed cell side down on Matrigel-coated membrane filters in Matrigel-Transwell assays (BD Biosciences, San Jose, CA, and Corning, Corning, NY). Serum-free media was placed in the upper chamber and serum-containing medium to the lower chamber. The underside of the filters containing invaded cells was processed for immunostaining. Phase images were acquired with a Nikon Eclipse Ti microscope using imaging software (NIS Elements; Nikon). Time-lapse video was acquired using a Nikon Eclipse Ti microscope driven by image analysis software (NIS Elements). For time-lapse studies the culture dishes were contained in a Tokai Hit stage-top incubator. Vimentin function was blocked with vimentin monoclonal antibodies, H5 (10 µg/ml) or AMF17b (20 µg/ml) (mAbs; Developmental Studies Hybridoma Bank, Bank Iowa City, IA), with mouse IgG isotype antibody (20 µg/ml) from Jackson ImmunoResearch Laboratories (West Grove, PA) as control. In supporting studies vimentin was blocked with the 0.5–1 µM Witaferin A (Tocris, Ellisville, MO) with the vehicle dimethyl sulfoxide (DMSO; Sigma Aldrich, St. Louis, MO) as control. Fresh antibody or inhibitor was added each day. To determine the effect of exogenous vimentin on myofibroblast differentiation, recombinant vimentin (catalogue no. 2105-VI-100 from R&D Systems, Minneapolis, MN) was added to ex vivo cultures on day 1. For proliferation analysis, cells were pulsed with EdU (Click-iT EdU Imaging Kits; Invitrogen, Carlsbad, CA) for 2 h and analyzed according to the manufacturer's instructions. TUNEL assay (catalogue

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three independent experiments. (J–L) To directly view the substrate-linked extracellular vimentin, extracellular proteins in the ECZ were cross-linked with the membrane-impermeable BS<sup>3</sup> cross-linker, cells extracted with RIPA, and the cross-linked proteins fixed and labeled for tenascin-C (TN-C; J), vimentin (K), and F-actin (L). The results show that extracellular vimentin was associated with the extracellular matrix substrate organized beneath the leader cells of the ECZ (J, K). No staining for F-actin was detected in the cross-linked/extracted cultures (L) confirming effective removal of the cells. Mag bars B, E = 20 µm; F = 50 µm; G, H, J–L = 10 µm. Images in B, E, F, G, and H are presented as single optical sections. Images in J–L are presented as projections.



**FIGURE 7:** Extracellular vimentin mediates leader cell behavior associated with wound healing and their transition to myofibroblasts. To examine the role of extracellular vimentin in both wound repair and fibrosis, the MCS-wounded-explant cultures were grown in the presence of two distinct antibodies to vimentin, AMF7b, and H5 or in the presence of a nontargeting IgG of the same isotype as control. (A) For the wound-healing studies, the MCS explants were treated with the vimentin antibodies from the time of wounding (D0) through D3, by which time the wound is typically healed. Open wound area was quantified each day and the results presented graphically. Representative phase-contrast images are shown at culture D3 (double arrow indicates open wound area). The presence of either vimentin antibody during wound-repair slowed wound closure. (B–I) Isolated ECZ regions were exposed to the vimentin antibodies (AMF17b and H5) or IgG isotype control for 2 d beginning at D1 postinjury to determine the effect of blocking extracellular vimentin function on myofibroblast emergence. (B) Phase-contrast imaging showed that lamellipodia extension at the leading-edge of the ECZ was impaired by treatment with either the AMF17B or the H5 vimentin antibodies (see arrows). (C) Total cell lysates prepared from ECZ regions exposed to AMF17B, H5, or isotype control antibodies were immunoblotted for  $\alpha$ SMA and GAPDH, the results quantified and presented as a ratio of  $\alpha$ SMA to GAPDH. This study showed that exposure to either of the vimentin antibodies inhibited emergence of  $\alpha$ SMA+ myofibroblasts. (D–I) ECZ cells were treated with either the vimentin antibody AMF17b, the vimentin antibody H5, or an isotype-specific IgG beginning at D1 postinjury and fixed at culture D3. The cultures were immunolabeled for  $\alpha$ SMA (D–I, red in G–I) and colabeled for F-actin (G–I, green). Emergence of  $\alpha$ SMA+ myofibroblasts at

no. 12156792910) from Sigma Aldrich (St. Louis, MO) was performed according to the manufacturer's instructions.

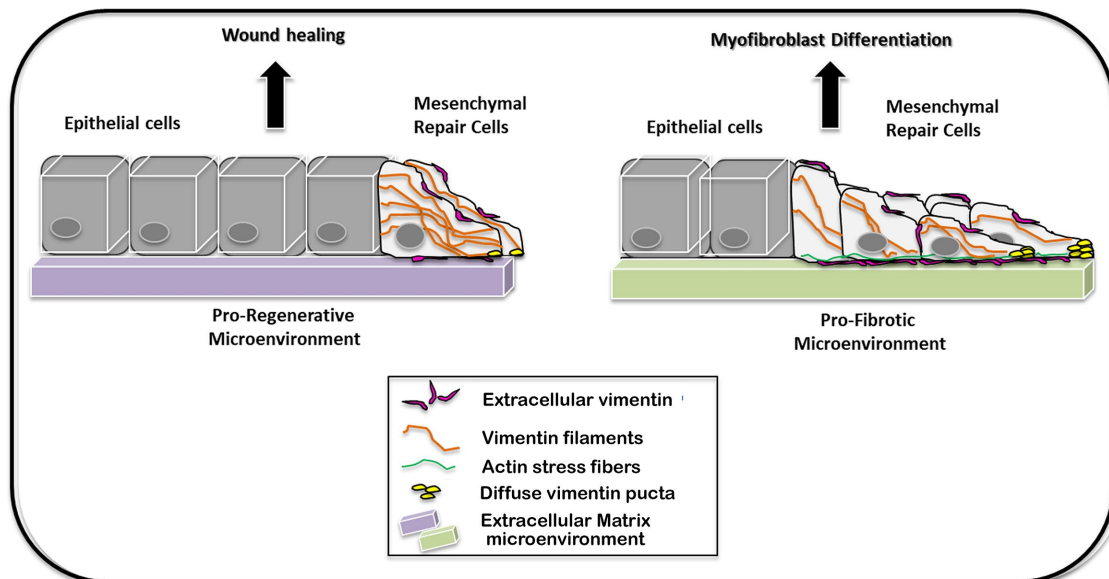
### Sequential detergent extraction

As we have described previously (Leonard *et al.*, 2011; Menko *et al.*, 2014a), for sequential extraction studies samples were extracted with Triton X-100/octyl glucoside (Triton/OG) extraction buffer (44.4 mM *n*-octyl  $\beta$ -D-glucopyranoside, 1% Triton X-100, 100 mM NaCl, 1 mM MgCl<sub>2</sub>, 5 mM EDTA, 10 mM imidazole, pH 7.4). The Triton/OG-insoluble proteins were solubilized in 2x sample buffer (125 mM Tris-HCl, 4% SDS, 20% glycerol, 2%  $\beta$ -mercaptoethanol, 0.5% bromophenol blue). To determine the relative distribution of Triton/OG soluble and insoluble proteins the Triton/OG soluble fractions were loaded at equal protein concentration and the SDS fractions were loaded at volumes equal to the respective Triton/OG soluble fractions.

### Western blot analysis

Samples were extracted in Triton/OG except for studies of  $\alpha$ -SMA expression for which the cells were extracted in RIPA buffer (5 mM EDTA, 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 1% SDS 20% solution, 50 mM Tris-HCl, pH 7.4) containing protease/phosphatase inhibitor cocktail (Cell Signaling, Danvers, MA). All protein concentrations were determined by a BCA protein assay (BCA Assay; Pierce, Rockford, IL). All proteins were separated on Tris-glycine gels (Novex, San Diego, CA), under reducing conditions followed by electrophoretically transferring to membranes (Immobilon-P; Millipore), and subsequently immunoblotted as described previously (Walker and Menko, 1999). Enhanced chemiluminescence (ECL) and ECL Plus Western blotting substrate (Pierce; ThermoFisher, Waltham, MA) were used for detection. Antibodies used for Western blotting (see Table 1) included vimentin (AMF17b mAb; Developmental Studies Hybridoma Bank, Iowa City, IA), vimentin polyclonal antibody (a generous gift from Paul FitzGerald, University of California, Davis, CA), GAPDH (catalogue no. sc-25778) from Santa Cruz Biotechnology (Santa Cruz, CA),  $\alpha$ -smooth muscle actin (catalogue no. ab5694) from Abcam (Cambridge, United Kingdom), N-cadherin (catalogue no. 610921) from BD

the leading edge of the ECZ was inhibited by blocking extracellular vimentin function. *p* values generated by Student's *t* test \*\*\**p* < 0.001. Mag bar B = 10  $\mu$ m and D–I = 20  $\mu$ m. Images in D–I are presented as projection images.



**FIGURE 8:** Model depicting differences in vimentin and actin cytoskeleton organization in mesenchymal leader cells in proregenerative and profibrotic environments. The mesenchymal leader cells that locate to the wound edge in a proregenerative environment organize an extensive vimentin intermediate filament cytoskeleton that extends into the cells' lamellipodial processes. These repair-modulating cells are remarkably poor in actin stress fibers. The cytoskeletal organization is altered when these mesenchymal leader cells migrate into a profibrotic environment. Here, these cells organize extensive actin stress fibers and vimentin is most striking for its high expression in the cells' lamellipodia where it is present in a diffusely organized form. On injury, vimentin is also released to the extracellular space and becomes linked to the cell surface of the mesenchymal repair cells at the leading edge of both environments where it plays a role in both wound repair and altering these cells' fate to myofibroblasts.

Biosciences (San Jose, CA) and Akt (catalogue number 9272) from Cell Signaling (Danvers, MA). Densitometry analysis was performed using AlphaView Software (Protein Simple; San Jose, CA).  $\alpha$ -SMA expression in the time course is presented as a ratio to GAPDH. For the WFA and vimentin antibody studies results were normalized to control and presented as a ratio to GAPDH. Sequential detergent extractions are shown as the percentage of total vimentin in each fraction. Histograms are shown  $\pm$  SEM, with *p* values generated by Student's *t* test.

#### Cell-surface biotinylation

For biotinylation of cell-surface proteins we used the Cell Surface Protein Isolation Kit (Pierce; ThermoFisher, Waltham, MA) according to manufacturer's directions. Briefly, cells were incubated with the membrane-impermeable Sulfo-NHS-SS-Biotin reagent. After the reaction was stopped, cells were gently scraped, lysed, and transferred to a NeutrAvidin agarose column for isolation of biotin-labeled cell-surface proteins. Finally, biotin labeled cell-surface proteins were eluted from the column using DTT solution and

Antibody name	Source	Product number	Dilution factor
Alpha smooth muscle actin	Millipore Sigma	CBL171	1/200 (IF)
Alpha smooth muscle actin	Abcam	Ab5694	1/100 (IF); 1/1000 (WB)
Akt	Cell Signaling	9272	1/1000 (WB)
CD44	Developmental Studies Hybridoma Bank (DSHB)	1D10	1/10 (IF)
Citrulline	Abcam	Ab6464	1/100 (IF)
Citrulline	Millipore Sigma	MABN328	1/200 (IF); 1/500 (WB)
GAPDH	Santa Cruz	Sc-25778	1/1000 (WB)
N-cadherin	BD Transduction Lab	610921	1/2000 (WB)
Tenascin	Millipore Sigma	AB19013	1/200 (IF)
Vimentin	DSHB	AMF17B	1/10 (IF); 1/100 (WB)
Vimentin	DSHB	H5	1/10 (IF)
Vimentin	Abcam	Ab92547	1/2500 (WB)
Vimentin (phosphoS38)	Abcam	Ab52942	1/100 (IF)
Vimentin	Gift, Paul FitzGerald, University of California, Davis		1/100 (IF)

**TABLE 1:** Antibody sources, product numbers, and dilutions used in these studies.

SDS–PAGE buffer and analyzed by Western blot analysis. Biotin labeled cultures were also fixed and labeled with a fluorescent-conjugated streptavidin (catalogue no. 21832) from Invitrogen (Carlsbad, CA) to confirm that biotin labeled cell surfaces.

### Immunofluorescence

Immunostaining of epithelial explants was performed as previously described (Walker *et al.*, 2007, 2010). Briefly, prior to immunostaining, explants were fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) and permeabilized in 0.25% Triton X-100 (Sigma-Aldrich) in PBS. For immunostaining of nonpermeabilized cells to detect only surface proteins cultures were fixed with either 1 or 2% methanol-free paraformaldehyde, as indicated. The following primary antibodies were used for the immunofluorescence studies (see Table 1): vimentin (H5 and AMF17b mAb; Developmental Studies Hybridoma Bank, Iowa City, IA), vimentin polyclonal antibody (a generous gift from Paul FitzGerald), vimentin polyclonal antibody (catalogue no. ab92547) from Abcam (Cambridge, MA), CD44 (1D10 mAb, Developmental Studies Hybridoma Bank, Iowa City, IA), tenascin-C (catalogue no. AB19013) from Millipore (Burlington, MA), pSerine 38 vimentin rabbit (catalogue no. ab115150),  $\alpha$ SMA (catalogue no. ab5694), and citrulline antibody (catalogue no. ab6464) from Abcam (Cambridge, MA), and a citrulline antibody F95 (catalogue no. MABN328) from MilliporeSigma (Burlington, MA). Cells were incubated with primary antiserum followed by rhodamine-conjugated (Jackson ImmunoResearch Laboratories and Millipore), fluorescein-conjugated (Jackson ImmunoResearch Laboratories, Westgrove, PA), or Alexa Fluor 488-conjugated (ThermoFisher, Waltham, MA), secondary antibodies. As indicated, cells were counterstained with Alexa Fluor 488-conjugated phalloidin, Alexa Fluor 633 or Alexa Fluor 647 phalloidin (ThermoFisher, Waltham, MA) to label filamentous actin (F-actin), and/or the nuclear stain TO-PRO 3 (Invitrogen, Carlsbad, CA). To track the fate of CD44 leader cells to become myofibroblasts, cultures were live labeled with CD44 antibody for 20 min. Antibody was removed and media was replaced for 2 d subsequent to fixation and processing for immunostaining. Immunostained samples were examined by confocal microscopy (LSM 510, LSM 800 with Super Resolution Airyscan, Zeiss, Thornwood, NY). Single images or Z-stacks were collected and analyzed; the data presented represent single optical planes or maximal intensity projection images.

### Proximal ligation assay

To determine whether vimentin was citrullinated, we performed proximal ligation assay (PLA) according to manufactures guidelines (Duolink; Sigma-Aldrich). Briefly, fixed cultures were incubated with antibody to citrulline (catalogue no. ab6464) from Abcam and vimentin antibody H5 from Developmental Studies Hybridoma Bank simultaneously, followed by PLA affinity probes for rabbit and mouse (Duolink; Sigma-Aldrich) that binds to the target antibodies. Cells were then incubated with Detection Reagents Red (Duolink; Sigma-Aldrich), which includes a ligation solution and a connector oligonucleotide that hybridizes to the probes that allows amplification of DNA to produce a signal to be detected by fluorescence imaging.

### Cross-link extraction

To examine whether vimentin associated with the extracellular matrix deposited beneath the cells on the substrate, we performed cross-link extractions as previously described (Enomoto *et al.*, 1993). Cells were then incubated with the cell-impermeable cross-linker BS<sup>3</sup> (ThermoFisher, Waltham, MA) in PBS with phenylmethylsulfonyl fluoride (PMSF) protease inhibitor (Sigma-Aldrich). The reaction was

quenched with (50 mM Tris-HCl), and the cells were extracted with RIPA buffer containing protease/phosphatase inhibitor cocktail (Cell Signaling, Danvers, MA) and the substrate-associated proteins fixed with 3.7% formaldehyde in PBS.

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