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# Extracellular Matrix Degradation Products and Low-Oxygen Conditions Enhance the Regenerative Potential of Perivascular Stem Cells

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Tissue and organ injury results in alterations of the local microenvironment, including the reduction in oxygen concentration and degradation of the extracellular matrix (ECM). The response of perivascular stem cells to these microenvironment changes are of particular interest because of their wide distribution throughout the body and their potential involvement in tissue and organ response to injury. The chemotactic, mitogenic, and phenotypic responses of this stem cell population were evaluated in response to a combination of decreased oxygen concentration and the presence of ECM degradation products. Culture in low-oxygen conditions resulted in increased proliferation and migration of the cells and increased activation of the ERK signaling pathway and associated integrins without a change in cell surface marker phenotype. The addition of ECM degradation products were additive to these processes. Reactive oxygen species within the cells were increased in association with the mitogenic and chemotactic responses. The increased proliferation and chemotactic properties of this stem cell population without any changes in phenotype and differentiation potential has important implications for both *in vitro* cell expansion and for *in vivo* behavior of these cells at the site of injury.

## Introduction

**S**TEM CELL MIGRATION, proliferation, and differentiation are required for tissue and organ regeneration. The factors that induce or facilitate these events are largely unknown, but changes in the microenvironment associated with tissue and organ injury would logically play important roles.<sup>28</sup> Stem cells have been shown to migrate *in vivo* to sites of injury,<sup>1,2</sup> and wounding has been shown to be required for both *de novo* hair follicle regeneration in adult mouse skin<sup>3</sup> and for limb regeneration in the salamander.<sup>4</sup> Two prominent factors in the microenvironment of injured tissue are decreased oxygen concentration and the degradation of the extracellular matrix (ECM). The degradation products of biologic scaffolds composed of ECM can recruit multipotent cells to the site of tissue injury in a mouse model of digit amputation,<sup>5</sup> and the *in vivo* ECM degradation and remodeling process result in the formation of molecules with potent *in vitro* chemotactic properties for selected stem cells.<sup>6-8</sup> In part, these properties are believed to be due to the release of matricryptic peptides derived from the ECM itself.<sup>9-11</sup> Oxygen concentration also affects the survival, proliferation, and trafficking of stem cells<sup>12-17</sup> with the general

view that low-oxygen conditions increase the proliferation of stem cells and aid in their survival.

A regenerative medicine approach for the replacement of tissues and organs frequently requires the isolation of stem cells from a patient and their subsequent culture *in vitro* on a scaffold. There is a concern, however, about the ability of the cells to survive the transfer from the laboratory to the patient, with as many as 99% of transferred cells dying within the first 4 days.<sup>18</sup> An alternative method for placement of stem cells to the required site for tissue and organ regeneration is the recruitment of endogenous stem cells from either the circulation or local tissue reservoirs. A population of human perivascular stem cells has been recently described<sup>19</sup> that have been postulated to be the precursors of mesenchymal stem cells (MSCs). This population of cells may be of particular importance to tissue regeneration and the constructive and functional remodeling of injured tissue because of their wide anatomic distribution.

The objectives of the present study were to determine the response of these perivascular stem cells to the degradation products of ECM and the influence of a low-oxygen environment. The ability of these cells to maintain their multipotential differentiation state after proliferation in a

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low-oxygen environment and the potential role of reactive oxygen species in this process were also evaluated.

## Materials and Methods

### Source of cells and culture conditions

Perivascular stem cells that had been isolated by flow cytometry from fetal muscle<sup>19–21</sup> were used in all experiments. These cells have been previously shown to represent a homogeneous population of perivascular cells obtained after positive selection and stringent exclusion of hematopoietic, endothelial, and myogenic cells, and be able to differentiate into mesodermal lineages. Isolated cells were cultured in high-glucose Dulbecco's modified Eagle's medium (Invitrogen) containing 20% fetal bovine serum (FBS; Thermo), 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma) at 37°C in 5% CO<sub>2</sub>. Oxygen levels were maintained at 21% in a Hera Cell<sub>150</sub> incubator or at 6% in Hera Cell<sub>150</sub> containing a sealed, gas-regulated chamber (Biospherix). Oxygen levels in the body ranging from 3% to 12% have been reported, with considerable local variation.<sup>22</sup> Six percent oxygen has been previously used as a level representing a decreased oxygen level.<sup>23</sup> Manipulation of cells at 6% oxygen was performed in a gas-regulated glovebox. Cells were grown for at least two passages at their respective oxygen concentration before being used in each assay.

### Preparation of ECM degradation products

ECM was harvested from porcine urinary bladder matrix (UBM) as previously described.<sup>24</sup> The basement membrane and tunica propria of the bladder were isolated from the overlying urothelial cells and all subjacent connective tissue, including muscle. The remaining tissue was then decellularized with 0.1% peracetic acid/4% ethanol. The resulting ECM was referred to as UBM. Decellularized material was defined as material having no visible nuclei under neither hematoxylin and eosin nor 4',6-diamidino-2-phenylindole staining, <50 ng DNA/mg dry weight material, and any residual DNA being smaller than 200 bp. UBM was digested at 10 mg/mL dry weight with 1 mg/mL pepsin (Sigma) in 0.01N HCl for 48 h at 20°C before dialysis at 4°C into phosphate-buffered saline (PBS). Protein concentration was quantified using the BCA protein assay (Thermo) against bovine serum albumin. The resultant material was referred to as UBM degradation products.

### Cell proliferation assay

Perivascular stem cells were inoculated at a concentration of  $1 \times 10^4$  cells per well in standard 12-well plate (well area 3.8 cm<sup>2</sup>) at 6% or 21% oxygen. Cell growth was monitored every 24 h using a Coulter Particle Counter (Beckman). Cell proliferation was also monitored via immunohistochemical methods using the thymidine analog 5-bromo-2'-deoxyuridine (BrdU) ELISA (Roche). BrdU incorporation was detected using the supplied anti-BrdU antibody. Cells were plated at  $5 \times 10^4$  cells per well in a standard 96-well plate with 0, 2, 5, or 15 µg/mL UBM digest material and labeled with BrdU for 18 h. Relative proliferation was quantified at 370 and 492 nm in a plate reader (Molecular Devices). Each assay was performed in triplicate on three separate occasions. Statistical significance

was evaluated by the Student's *t*-test and a *p*-value of 0.05 was considered significant.

### Metabolic activity assay

Cells were plated to a density of  $1 \times 10^5$  cells/well in a standard 96-well plate (well area 0.32 cm<sup>2</sup>) in the medium containing 10% AlamarBlue and 0, 10, 25, 50, or 100 µg/mL UBM degradation products at either 6% or 21% oxygen concentration. Metabolic activity was quantified using AlamarBlue (Biotium). AlamarBlue is a nontoxic, cell-permeable compound. Upon entering cells, the active ingredient resazurin is reduced to resorufin. The amount of absorbance change corresponds to the cells metabolic activity.<sup>25</sup> Cells were exposed to AlamarBlue over a period of 6 h and quantified at 600 nm. Each assay was performed in triplicate on three separate occasions. Statistical significance of the results was evaluated by the Student's *t*-test and a *p*-value of 0.05 was considered significant.

### Quantification of superoxide dismutase

Perivascular stem cells at  $2.5 \times 10^5$  cells/well in a standard 96-well plate were exposed to 0 or 20 µg/mL UBM degradation products at either 6% or 21% oxygen concentration for 6 h. Cells were then lysed in buffer (10 mM Tris pH 7.5, 150 mM NaCl, 0.1 mM EDTA, and 0.5% triton), and the amount of superoxide dismutase present per cell was analyzed using the Oxiselect activity assay (Cell Biolabs, Inc.) or via native gel (11% gel) fractionation of protein extract from  $4 \times 10^4$  cells followed by nitro blue tetrazolium and riboflavin detection.<sup>26</sup> Each assay was performed in triplicate on three separate occasions.

### Reactive oxygen species analysis

Cells grown in either oxygen concentration were loaded with 2'-7'-dichlorofluorescein diacetate (Invitrogen) at 10 µM in PBS for 30 min. Loaded cells were centrifuged (5 min, 300g), resuspended in media, and aliquoted at a concentration of  $5 \times 10^4$  cells/well in a standard 96-well plate into 0 or 20 µg/mL UBM degradation products. Fluorescence intensity was quantified at excitation wavelength (Ex) 485 nm and emission wavelength (Em) 538 nm at 5 min intervals. The assay was repeated on three separate occasions.

### Chemotaxis assay

Chemotactic assays were performed in chemotaxis chambers using 8 µm filters (Neuro Probe Inc.) as described previously.<sup>8</sup> Cells at 6% or 21% oxygen concentration were grown to ~80% confluence before being incubated overnight in Dulbecco's modified Eagle's medium containing 0.5% FBS. Trypsinized cells were resuspended and the effect of 0, 25, 50, 100, and 250 µg/mL UBM degradation products on the chemotaxis of  $3 \times 10^4$  cells was determined. Each chamber was incubated at the corresponding oxygen concentration for 3 h. Migrated cells were stained by 4',6-diamidino-2-phenylindole and quantified with ImageJ (NIH). All of the data are means of quadruplicate determinations with standard deviation. Each assay was performed in quadruplicate on three separate occasions. Migration of cells in UBM was compared to cells in UBM-free media at the same oxygen concentration. Statistical analysis was performed using the Student's *t*-test where  $*p < 0.05$ .

### Adipogenesis, osteogenesis, and chondrogenesis

Perivascular stem cells that had been passaged the same number of times at their respective oxygen concentration were differentiated toward fat, bone, or cartilage using commercial differentiation media (Thermo). For adipogenic and osteogenic differentiation, the cells were plated at  $10^5$  cells per well in a standard 12-well plate and incubated at either 6% or 21% oxygen concentration in the differentiation medium for 21 days. Adipogenic differentiation was determined by incubating fixed cells in oil red O (Sigma) for 10 min to detect lipids. Osteogenic differentiation was determined by fixing cells in 4% formaldehyde before observation of calcium deposits by incubation with alizarin red (Sigma). For chondrogenic differentiation, a pellet culture assay was performed by centrifuging  $3 \times 10^5$  cells at  $300g$  for 5 min. The supernatant was then discarded and the pellet was cultured in the chondrogenic differentiation medium for 3 weeks at the respective oxygen concentration. Chondrogenic differentiation was determined by the fixation of the cell pellet in 10% formalin, before embedding in paraffin and sectioning. Sulfated glycosaminoglycan presence and cell nuclear morphology were examined on hydrated sections with alcian blue and nuclear fast red (both Sigma), respectively.

### Flow cytometry

The presence of MSC markers after culture in 6% or 21% oxygen was determined using monoclonal antibodies CD105-PE (1:50), CD90-APC (1:50), and CD44-PECy5 (1:100) (all Invitrogen) and CD73-PE (1:50; BD). About  $2 \times 10^5$  cells were stained with the specific combination of antibodies. The same amount of cells was used for the analysis of the unstained controls. Unstained cells and isotype control antibodies were used as negative controls. Cells were incubated at  $4^\circ\text{C}$  for 20 min and then washed in PBS, resuspended in 20% FBS medium, and analyzed with a FACS-Calibur flow cytometer (Becton Dickinson).

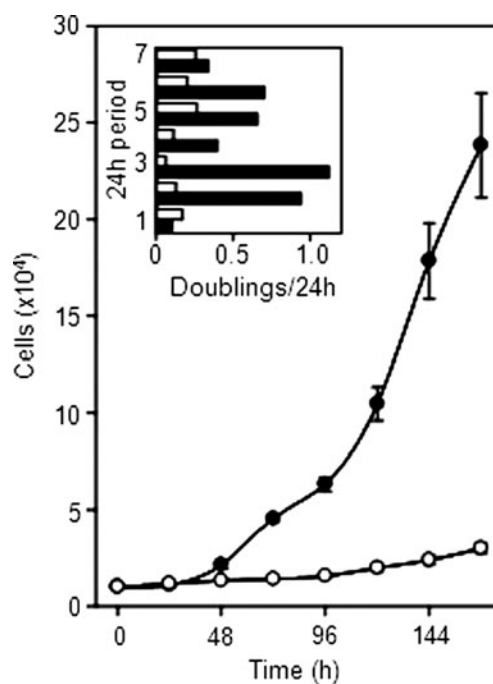
### Immunoblots

Perivascular stem cells grown at 6% or 21% oxygen were seeded at  $1.75 \times 10^5$  cells per well in a standard 12-well plate. After 6 h, the medium was removed, and a fresh medium containing 0, 50, or 100  $\mu\text{g}/\text{mL}$  UBM degradation products was added for 16 h before cell lysis in radio-immuno precipitation assay (RIPA) buffer (Cell Signal Technology) containing a dissolved complete mini protease inhibitor cocktail tablet (Roche). Protein samples were resolved on a 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel before being transferred to a polyvinylidene fluoride membrane (Millipore).<sup>27</sup> The membrane was blocked in 5% milk before being exposed to 1:1000 anti- $\beta$ -actin mouse monoclonal antibody (Santa Cruz Biotechnology) and polyclonal goat anti-mouse secondary antibody (Dako), or 1:500 anti-p-ERK1/2 rabbit (Santa Cruz Biotechnology) or 1:1000 anti-ERK1/2 rabbit (Assay Designs) primary antibodies and 1:5000 goat anti-rabbit secondary antibody (Dako). The immunoblot was observed using SuperSignal West Pico Chemiluminescent Substrate (Thermo). The amount of protein loaded per well was normalized based on  $\beta$ -actin expression. Immunoblots were repeated with three separate isolates.

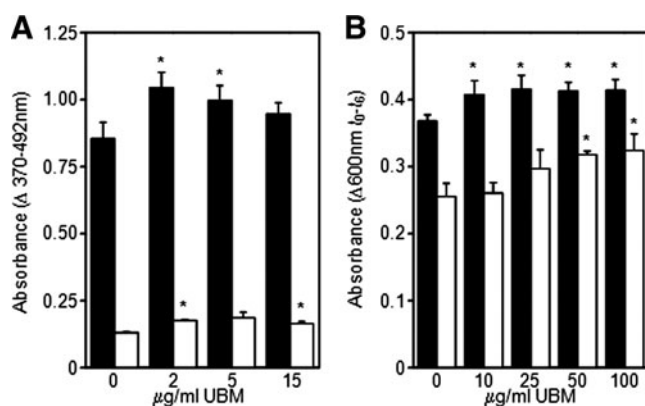
### Results

Perivascular stem cells show increased proliferation after exposure to 6% oxygen and UBM degradation products. To determine the effect of oxygen concentration on the growth rate of perivascular stem cells, cells were plated at either reduced oxygen ( $\sim 6\%$ ) or under the oxygen concentration normally used in tissue culture ( $\sim 21\%$ ). Cell growth was monitored via total cell counts every 24 h for 7 days (Fig. 1). Cells cultured at 21% oxygen concentration tripled in number over the 7 day time period. Cells cultured at 6% oxygen concentration increased 24-fold over the same time period. Cells cultured at the 21% oxygen concentration reached a maximum of 0.26 doublings per 24 h period, whereas cells cultured at 6% oxygen concentration reached a maximum of 1.1 doublings per 24 h period (Fig. 1 inset). At the 6% oxygen culture condition, cell populations usually showed  $>0.5$  doublings per 24 h period, confirming the sustained increase in cell proliferation at this oxygen concentration.

The effect of oxygen concentration on the proliferation of perivascular stem cells was also evaluated by the incorporation of the pyrimidine analog BrdU into newly synthesized DNA. Cells were cultured at 6% or 21% oxygen for 18 h and the incorporation of BrdU was determined by colorimetric immunoassay (Fig. 2a). Cells cultured in 6% oxygen synthesized seven times more DNA than cells cultured in 21%



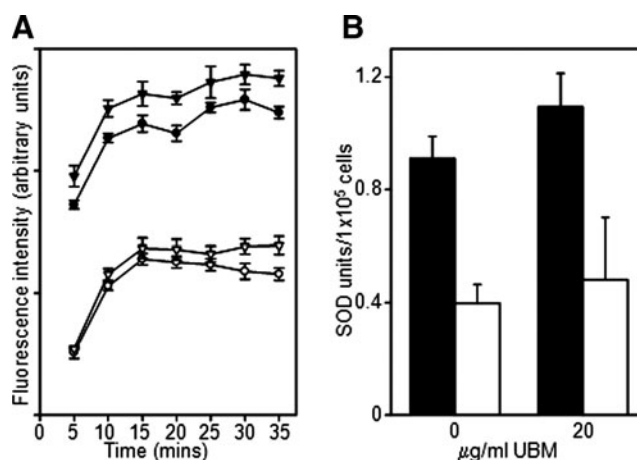
**FIG. 1.** Perivascular stem cells exhibit increased growth in 6% compared with 21% oxygen. Growth of perivascular stem cells plated at 6% (filled circles) or 21% (open circles) oxygen. Cells were plated at an initial seeding density of  $1 \times 10^4$  per well and duplicate wells harvested every 24 h with total cells per well counted. All of the data are means of triplicate determinations with SD (some values are too small to be visible). Equivalent trends have been observed on two further occasions. Inset: the doubling rate of perivascular stem cells per 24 h period under 6% (filled bars) or 21% (open bars) oxygen. SD, standard deviation.



**FIG. 2.** Cells have elevated DNA synthesis and metabolism in 6% compared with 21% oxygen, which addition of UBM further elevates. **(A)** Cells were plated at 6% (filled columns) or 21% (open columns) oxygen at  $5 \times 10^4$  cells per well containing 0, 2, 5, or 15  $\mu\text{g}/\text{mL}$  UBM and incubated for 18 h. New DNA synthesis was monitored via 5-bromo-2'-deoxyuridine incorporation. The presence of 5-bromo-2'-deoxyuridine in DNA was determined via immunoassay and quantified colormetrically at 370 and 492 nm. All of the data are means of triplicate determinations with SD. Proliferation of cells in UBM was compared with cells in UBM-free media at the same oxygen concentration. Statistical analysis was performed using the Student's *t*-test where  $*p < 0.05$ . Equivalent trends have been observed on two further occasions. **(B)** About  $1 \times 10^5$  cells per well were plated at 6% (filled columns) or 21% (open columns) oxygen in media containing 10% alamarBlue and 0, 10, 25, 50, or 100  $\mu\text{g}/\text{mL}$  UBM. The metabolism of alamarBlue was quantified colormetrically at 600 nm at time 0 and after 6 h incubation. All of the data are means of triplicate determinations with SD. Metabolism of cells in UBM was compared with cells in UBM-free media at the same oxygen concentration, where  $*p < 0.05$ . Equivalent trends have been observed on two further occasions. UBM, urinary bladder matrix.

oxygen during 18 h, confirming the increased proliferation of the cells cultured in the lower oxygen concentration. To determine the effect of the ECM degradation products upon the stem cells, UBM degradation products were incubated with the cells and DNA synthesis determined. In both oxygen conditions DNA synthesis was significantly increased by the presence of the UBM degradation products digest material. The increase in proliferation observed in the 6% oxygen-cultured cells after the addition of the UBM degradation products was greater than the total proliferation observed in the cells cultured in 21% oxygen condition during the same time period.

The increase in cell growth observed by culturing the perivascular stem cells in 6%, as opposed to 21%, oxygen raises the possibility that low-oxygen culture increases the metabolic rate of the stem cells. Metabolic activity was determined by culturing the cells at 6% or 21% oxygen and determined using the alamarBlue assay (Fig. 2b). Cells were exposed to alamarBlue at either 6% or 21% oxygen concentration and the metabolic rate monitored for 6 h. As expected, 6% oxygen culture resulted in an increased metabolic rate compared with cells cultured in 21% oxygen conditions. The addition of UBM degradation products caused a significant further increase in the metabolic rate in both oxygen condi-



**FIG. 3.** Perivascular stem cells in 6% oxygen have elevated reactive oxidative species and greater superoxide dismutase activity. **(A)** Cells grown in 6% (filled symbols) or 21% (open symbols) oxygen were loaded with dichlorofluorescein diacetate before plating into media containing 0 (circles) or 20  $\mu\text{g}/\text{mL}$  (triangles) UBM digest material. Fluorescence intensity was quantified at excitation wavelength (Ex) 485 nm and emission wavelength (Em) 538 nm at 5 min intervals. **(B)** Cells grown in 6% (filled columns) or 21% (open columns) oxygen were exposed to 0 or 20  $\mu\text{g}/\text{mL}$  UBM digest material for 6 h before quantification of cellular superoxide dismutase activity against a superoxide dismutase standard curve.

tions. The cells cultured at 6% oxygen showed a significantly increased metabolism with the addition of 10  $\mu\text{g}/\text{mL}$  UBM degradation products. Cells cultured in 21% oxygen required 50  $\mu\text{g}/\text{mL}$  UBM degradation products before a significant increase of metabolic activity was observed.

#### *Increased reactive oxygen species in perivascular stem cells at 6% oxygen concentration*

A possible explanation for the altered growth rates observed under different oxygen concentrations is that cells cultured in 21% oxygen suffer greater oxidative damage. To test this possibility, oxidative stress was analyzed in cells cultured at 6% or 21% oxygen using the cell-permeable indicator dichlorofluorescein diacetate. Using this probe it was possible to determine whether cells cultured in 6% oxygen concentration have greater amounts of intracellular reactive oxygen species than cells cultured in 21% oxygen (Fig. 3a). In addition, cells grown at either oxygen concentration and exposed to UBM degradation products have further increased amounts of reactive oxygen species.

If perivascular stem cells cultured in 6% oxygen have higher levels of reactive oxidative species than cells cultured at 21% oxygen concentration, it would be expected that 6% oxygen-cultured cells have greater levels of superoxide dismutase activity. The amount of superoxide dismutase present per cell of cultures grown under the two different oxygen conditions was therefore determined. The level of superoxide dismutase doubled at 6% oxygen concentration (Fig. 3b and Supplemental Fig. S1, available online at [www.liebertonline.com/ten](http://www.liebertonline.com/ten)) compared with 21% oxygen. The addition of UBM degradation products to the stem cells caused a modest reproducible increase in the average amount of superoxide dismutase present per cell.

### Perivascular stem cells maintain MSC features at 6% oxygen concentration

It has been previously shown that perivascular stem cells display features typical of MSCs.<sup>19</sup> We investigated whether the exposure of perivascular stem cells to a 6% oxygen concentration affects cells' expression of MSC surface markers and their capacity to differentiate into multiple lineages. Expression of the hallmark surface markers CD44, CD90, CD105, and CD73 was analyzed via flow cytometry on cells cultured at 6% or 21% oxygen. Cells cultured in 21% oxygen showed all tested MSC markers (>90%) and no difference in surface marker expression was observed in perivascular stem cells cultured in 6% oxygen concentration (Fig. 4a). We evaluated the maintained ability of the perivascular stem cells to differentiate into mesodermal lineages after culture at 6% or 21% oxygen concentration. We found that the cells can be differentiated into adipogenic, osteogenic, and chondrogenic phenotypes (Fig. 4b). These results show that perivascular stem cells retain their MSC characteristics after exposure to either 6% or 21% oxygen concentration.

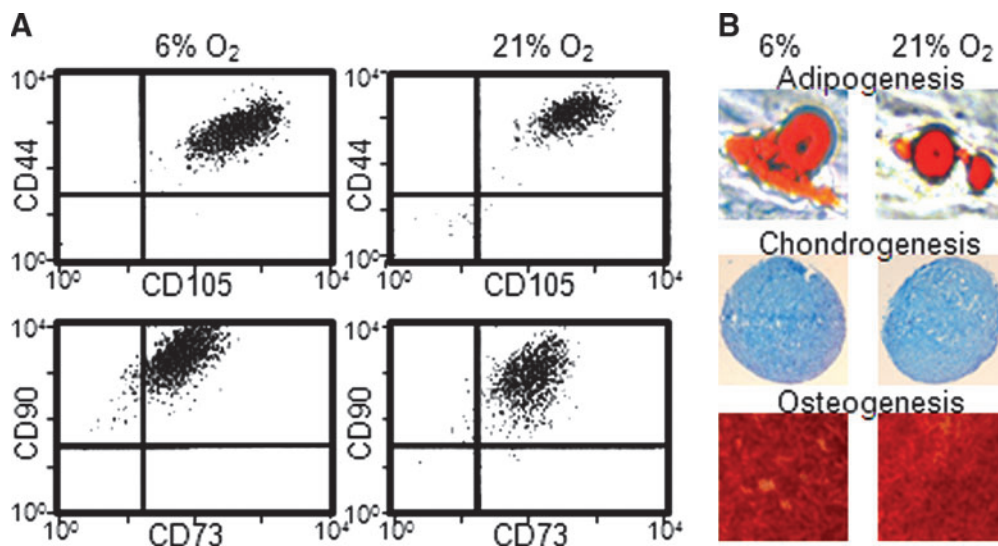
### Increased activation of the ERK signaling pathway

Cell proliferation is regulated in part via the ERK pathway.<sup>30</sup> Since growth in either or both 6% oxygen and UBM digest material causes an increase in perivascular stem cell proliferation, we investigated whether these same stimuli cause an increase in ERK phosphorylation. Immunoblots were performed using protein extracted from cells after 16 h of exposure to increasing concentrations of UBM degradation products at either oxygen concentration (Fig. 5).

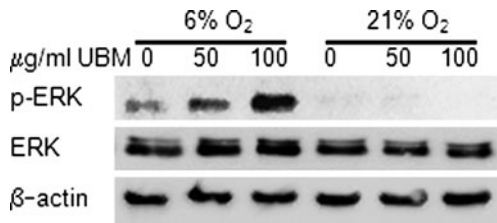
The immunoblot showed a clear increase in the phosphorylation of ERK upon exposure to 6% oxygen concentration. Exposure to UBM degradation products caused a further increase in ERK phosphorylation at both oxygen concentrations (Fig. 5 and overexposed Supplemental Fig. S2, available online at [www.liebertonline.com/ten](http://www.liebertonline.com/ten)). The bands for total ERK1/2 show similar intensity across treatments, suggesting that ERK protein abundance does not change due to oxygen concentration or exposure to UBM degradation products.

### Low-oxygen exposure results in greater migration of perivascular stem cells

It has been noted previously that UBM degradation products are chemotactic for perivascular stem cells.<sup>19</sup> With low-oxygen culture conditions increasing the metabolic rate of the stem cells, it is possible that these low-oxygen-cultured cells have increased migratory ability compared with cells cultured at 21% oxygen. To test this hypothesis, cells cultured at 6% or 21% oxygen were evaluated for their migratory capacity using a chemotaxis chamber. After 3 h of incubation, greater cell migration toward the UBM degradation products was observed in the 6% oxygen condition compared with migration at 21% oxygen (Fig. 6). Most concentrations of UBM degradation products tested resulted in double the number of cells migrating at 6% oxygen compared with 21% oxygen. The number of cells migrating without added UBM degradation products was also increased under 6% oxygen conditions compared with cells migrating under 21% oxygen conditions showing the general improvement of migratory capacity by the low-oxygen-cultured cells.



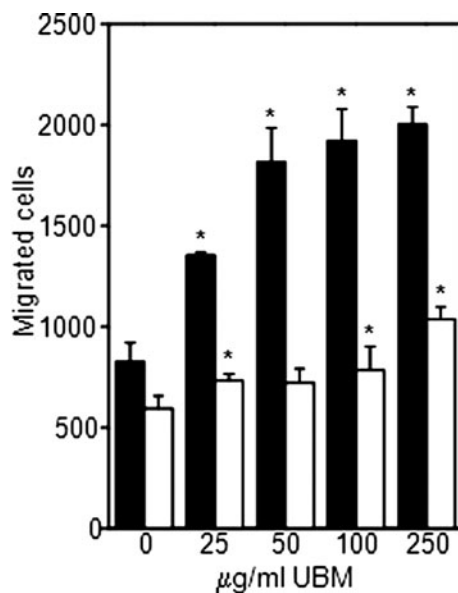
**FIG. 4.** Cells cultured in 6% oxygen maintain mesenchymal stem cell markers and multilineage potential. **(A)** Cells cultured at 21% (left panels) or 6% oxygen (right panels) were analyzed for mesenchymal stem cell markers CD105 coexpressed with CD44 (top) and CD90 coexpressed with CD 73 (bottom). **(B)** Perivascular stem cells were cultured in 6% (left) or 21% (right) oxygen and tested for the ability to differentiate. Cells were maintained in adipogenic medium, fixed, and stained for lipids with oil red O (Top). Stem cells were centrifuged into a pellet and cultured in the chondrogenic medium containing TGF- $\beta$ 1. Pellets were then sectioned, and stained with Alcian blue and nuclear fast red for detection of sulfated glycosaminoglycans and nuclei, respectively (middle). Cells cultivated in the osteogenic medium were stained with alizarin red at pH 4.2, staining calcium deposits red (bottom). The high degree of differentiation is shown by the almost solid blue (sulfated glycosaminoglycans, middle image) and solid red (calcium, bottom image) observed. Images at 40 $\times$ .



**FIG. 5.** Low oxygen and the presence of UBM digest material increase phosphorylation of ERK1/2. The phosphorylation of ERK1/2 after exposure of perivascular stem cells to 0, 50, or 100  $\mu\text{g}/\text{mL}$  UBM digest material for 16 h at 6% or 21% oxygen was monitored via immunoblot. The equivalent intensity of the bands for  $\beta$ -actin and ERK across loading conditions indicates equal loading of protein per well.

## Discussion

Stem and progenitor cells play an important role in most tissue engineering and regenerative medicine strategies for restoration of functional tissues and organs, including the use of biologic scaffold materials.<sup>5,7,8,19,31</sup> The mechanisms by which endogenous stem and progenitor cells are recruited to sites of need and then proliferate and differentiate *in situ* are poorly understood. The present study investigated the effect of two independent factors upon the proliferation, migration, and metabolic activity of a defined perivascular



**FIG. 6.** Migration of perivascular stem cells toward UBM digest material is elevated at 6% compared with 21% oxygen. The migration of cells toward 0, 25, 50, 100, and 250  $\mu\text{g}/\text{mL}$  UBM was monitored at 6% (filled columns) or 21% (open columns) oxygen. Chemotaxis chambers containing  $3 \times 10^4$  cells per well were incubated for 3 h at their respective oxygen concentration, and migrated cells counted. All of the data are means of quadruplicate determinations with SD. Migration of cells in UBM was compared with cells in UBM-free media at the same oxygen concentration. Statistical analysis was performed using the Student's *t*-test, where  $*p < 0.05$ . Equivalent trends have been observed on two further occasions.

stem cell population. Specifically, the results of this study show that increased migration and proliferation of perivascular stem cells occur *in vitro* upon exposure to degradation products of ECM, and that these properties are further enhanced under low-oxygen conditions. Low-oxygen conditions and ECM degradation products also cause an increased production of markers of reactive oxygen species within the cells and the phosphorylation of the signaling molecule ERK. Alterations in the oxygen environment did not cause the cells to change either their surface markers or their ability to differentiate into different tissue types. These findings have important implications for the recruitment of stem cells for functional tissue and organ replacement before stem cell differentiation. The effects of oxygen concentration and ECM degradation products upon the perivascular stem cells appear to be additive.

The findings of the present study are not surprising if considered in the context of an evolutionarily preferred and developed mammalian response to tissue injury. Immediately after injury, local tissue hypoxia as a result of compromised circulation and the presence of matrix metalloproteinases and other proteases as a component of the acute inflammatory response would define at least a part of the microenvironmental niche. ECM is a reservoir of not only latent and active growth factors, cytokines, chemokines, and structural proteins, but also a source of matricryptic peptides that can modulate a variety of biologic events, including the recruitment of stem cells.<sup>8,31</sup> Such matrikines are generated as a result of ECM degradation and remodeling. These tissue injury conditions define some of the signals that mobilize endogenous reserve cell populations for the purpose of replacing injured tissue.

The present study confirms the mitogenic response of a specific stem cell population to the presence of ECM degradation products and low-oxygen concentration. The oxygen concentration in which cells naturally reside within the body varies widely depending upon tissue type. Oxygen concentration in most tissues ranges from 2% to 9% (14–64 mmHg),<sup>17</sup> far below the 21%, which is present in normal atmospheric conditions and the oxygen concentration at which most cell cultures are maintained. The ability of oxygen tension to regulate stem/progenitor cell proliferation and differentiation was first noted with cytotrophoblasts.<sup>32</sup> Cytotrophoblast cells were cultured under low-oxygen conditions (2%), which allowed cell proliferation but inhibited differentiation. In contrast, 21% oxygen inhibited proliferation but allowed normal differentiation of the cytotrophoblasts. The mechanisms by which stem/progenitor cells respond to oxygen concentration is believed to involve the hypoxia inducible factor induction of stromal-derived factor-1.<sup>12</sup> Stromal-derived factor-1 is known to be expressed in injured tissue and to induce homing of stem cells.<sup>33</sup>

The specific molecules within the UBM degradation products that are responsible for the effects reported herein are unknown. ECM facilitates constructive remodeling in a variety of tissues,<sup>29,34–39</sup> and endogenous progenitor/stem cells participate in this process.<sup>6,40</sup> Bioactive degradation products of ECM promote the migration of a variety of cell types, including terminal Schwann cells,<sup>41</sup> endothelial cells,<sup>42</sup> and the perivascular stem cell population used in this present study.<sup>19</sup> Different effects of the UBM digest material have been observed for different cell types.<sup>8,42</sup> Fractionation of

hydrolyzed ECM revealed molecules in the range of 5–16kDa that were chemoattractive *in vitro* for endothelial cells and promoted vascularization *in vivo*.<sup>42</sup>

The increased migration of progenitor cells toward an ECM scaffold compared with toward an autologous tissue scaffold in a tendon repair model implies that degradation products of ECM may be favorable for the processes of tissue remodeling.<sup>7</sup> It should be noted that the ECM material was digested with the proteinase pepsin in the present study. Future experiments will evaluate the effects of ECM digestion products that result from more physiological methods, such as exposure to matrix metalloproteinases, upon the perivascular stem cells. The effect of such degradation products on additional stem cell lines also warrants investigation. Also worthy of future investigation is the possibility that oxygen concentration may alter the rate or degree of differentiation of these cells.

The present study showed the paradoxical increase of reactive oxygen species in the presence of low-oxygen concentration. The increased cell proliferation under low-oxygen conditions demonstrated in this study may result in increased metabolic end products, including reactive oxygen species. In addition, it has been suggested that reactive oxygen species themselves may play a part in activating cell cycle progression.<sup>43</sup> Oxidative stress has been linked to the increased phosphorylation of critical cell signaling pathways, for example, via the reversible inactivation of cellular tyrosine phosphatases leading to increased amounts of phosphorylated ERK1/2.<sup>44,45</sup> The phenotypic markers of the perivascular stem cell population remained unchanged after exposure to the low-oxygen concentration. The rapid expansion of the stem cell population while maintaining their undifferentiated multipotent state has important implications for both *in vitro* cell expansion and for *in vivo* accumulation of such cells at the site of injury. These findings suggest an important role for ECM and oxygen concentration in the tissue response to injury and in the potential role for biologic scaffolds composed of ECM in regenerative medicine strategies for tissue and organ reconstruction.

### Authors Contributions

S.T.: Conception and design, data analysis and interpretation, collection and/or assembly of data, and article writing.

M.C.: Collection and/or assembly of data, and data analysis and interpretation.

E.M.J.: Collection and/or assembly of data.

R.L.: Collection and/or assembly of data.

B.P.: Provision of study material or patients, and final approval of the article.

S.F.B.: Conception and design, financial support, data analysis and interpretation, article writing, and final approval of the article.

### Disclosure Statement

No competing financial interests exist.

### References

- Ji, J.F., He, B.P., Dheen, S.T., and Tay, S.S. Interactions of chemokines and chemokine receptors mediate the migration of mesenchymal stem cells to the impaired site in the brain after hypoglossal nerve injury. *Stem Cells* **22**, 415, 2004.
- Satake, K., Lou, J., and Lenke, L.G. Migration of mesenchymal stem cells through cerebrospinal fluid into injured spinal cord tissue. *Spine (Phila Pa 1976)* **29**, 1971, 2004.
- Ito, M., Yang, Z., Andl, T., Cui, C., Kim, N., Millar, S.E., and Cotsarelis, G. Wnt-dependent *de novo* hair follicle regeneration in adult mouse skin after wounding. *Nature* **447**, 316, 2007.
- Satoh, A., Gardiner, D.M., Bryant, S.V., and Endo, T. Nerve-induced ectopic limb blastemas in the *Axolotl* are equivalent to amputation-induced blastemas. *Dev Biol* **312**, 2007.
- Agrawal, V., Johnson, S.A., Reing, J., Zhang, L., Tottey, S., Wang, G., Hirschi, K.K., Braunhut, S., Gudas, L.J., and Badylak, S.F. Epimorphic regeneration approach to tissue replacement in adult mammals. *Proc Natl Acad Sci USA* **107**, 3351, 2010.
- Zantop, T., Gilbert, T.W., Yoder, M.C., and Badylak, S.F. Extracellular matrix scaffolds are repopulated by bone marrow-derived cells in a mouse model of achilles tendon reconstruction. *J Orthop Res* **24**, 1299, 2006.
- Beattie, A.J., Gilbert, T.W., Guyot, J.P., Yates, A.J., and Badylak, S.F. Chemoattraction of progenitor cells by remodeling extracellular matrix scaffolds. *Tissue Eng Part A* **15**, 1119, 2009.
- Reing, J.E., Zhang, L., Myers-Irvin, J., Cordero, K.E., Freytes, D.O., Heber-Katz, E., Bedelbaeva, K., McIntosh, D., Dewilde, A., Braunhut, S.J., and Badylak, S.F. Degradation products of extracellular matrix affect cell migration and proliferation. *Tissue Eng Part A* **15**, 605, 2009.
- Davis, G.E., Bayless, K.J., Davis, M.J., and Meiningner, G.A. Regulation of tissue injury responses by the exposure of matricryptic sites within extracellular matrix molecules. *Am J Pathol* **156**, 1489, 2000.
- Davis, G.E., and Senger, D.R. Extracellular matrix mediates a molecular balance between vascular morphogenesis and regression. *Curr Opin Hematol* **15**, 197, 2008.
- Zaidi, S.H., You, X.M., Ciura, S., O'Blenes, S., Husain, M., and Rabinovitch, M. Suppressed smooth muscle proliferation and inflammatory cell invasion after arterial injury in elafin-overexpressing mice. *J Clin Invest* **105**, 1687, 2000.
- Ceradini, D.J., Kulkarni, A.R., Callaghan, M.J., Tepper, O.M., Bastidas, N., Kleinman, M.E., Capla, J.M., Galiano, R.D., Levine, J.P., and Gurtner, G.C. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. *Nat Med* **10**, 858, 2004.
- Copland, I.B., Lord-Dufour, S., Cuerquis, J., Coutu, D.L., Annabi, B., Wang, E., and Galipeau, J. Improved autograft survival of mesenchymal stromal cells by plasminogen activator inhibitor 1 inhibition. *Stem Cells* **27**, 467, 2009.
- Wang, J.A., Chen, T.L., Jiang, J., Shi, H., Gui, C., Luo, R.H., Xie, X.J., Xiang, M.X., and Zhang, X. Hypoxic preconditioning attenuates hypoxia/reoxygenation-induced apoptosis in mesenchymal stem cells. *Acta Pharmacol Sin* **29**, 74, 2008.
- Malda, J., Klein, T.J., and Upton, Z. The roles of hypoxia in the *in vitro* engineering of tissues. *Tissue Eng* **13**, 2153, 2007.
- Rosova, I., Dao, M., Capoccia, B., Link, D., and Nolte, J.A. Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells. *Stem Cells* **26**, 2173, 2008.
- Simon, M.C., and Keith, B. The role of oxygen availability in embryonic development and stem cell function. *Nat Rev Mol Cell Biol* **9**, 285, 2008.



18. Toma, C., Pittenger, M.F., Cahill, K.S., Byrne, B.J., and Kessler, P.D. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation* **105**, 93, 2002.
19. Crisan, M., Yap, S., Casteilla, L., Chen, C.W., Corselli, M., Park, T.S., Andriolo, G., Sun, B., Zheng, B., Zhang, L., Norotte, C., Teng, P.N., Traas, J., Schugar, R., Deasy, B.M., Badylak, S., Buhning, H.J., Giacobino, J.P., Lazzari, L., Huard, J., and Peault, B. A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* **3**, 301, 2008.
20. Crisan, M., Deasy, B., Gavina, M., Zheng, B., Huard, J., Lazzari, L., and Peault, B. Purification and long-term culture of multipotent progenitor cells affiliated with the walls of human blood vessels: myoendothelial cells and pericytes. *Methods Cell Biol* **86**, 295, 2008.
21. Crisan, M., Huard, J., Zheng, B., Sun, B., Yap, S., Logar, A., Giacobino, J.P., Casteilla, L., and Peault, B. Purification and culture of human blood vessel-associated progenitor cells. *Curr Protoc Stem Cell Biol* Chapter 2, Unit 2B 2 1, 2008.
22. Csete, M. Oxygen in the cultivation of stem cells. *Ann NY Acad Sci* **1049**, 1, 2005.
23. Morrison, S.J., Csete, M., Groves, A.K., Melega, W., Wold, B., and Anderson, D.J. Culture in reduced levels of oxygen promotes clonogenic sympathoadrenal differentiation by isolated neural crest stem cells. *J Neurosci* **20**, 7370, 2000.
24. Freytes, D.O., Tullius, R.S., and Badylak, S.F. Effect of storage upon material properties of lyophilized porcine extracellular matrix derived from the urinary bladder. *J Biomed Mater Res B Appl Biomater* **78**, 327, 2006.
25. Al-Nasiry, S., Geusens, N., Hanssens, M., Luyten, C., and Pijnenborg, R. The use of Alamar Blue assay for quantitative analysis of viability, migration and invasion of choriocarcinoma cells. *Hum Reprod* **22**, 1304, 2007.
26. Beauchamp, C., and Fridovich, I. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal Biochem* **44**, 276, 1971.
27. Livak, K.J., and Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>-Delta Delta C(T)</sup> method. *Methods* **25**, 402, 2001.
28. Alam, N., Goel, H.L., Zarif, M.J., Butterfield, J.E., Perkins, H.M., Sansoucy, B.G., Sawyer, T.K., and Languino, L.R. The integrin-growth factor receptor duet. *J Cell Physiol* **213**, 649, 2007.
29. Badylak, S.F., Freytes, D.O., and Gilbert, T.W. Extracellular matrix as a biological scaffold material: structure and function. *Acta Biomater* **5**, 1, 2009.
30. Assoian, R.K., and Schwartz, M.A. Coordinate signaling by integrins and receptor tyrosine kinases in the regulation of G1 phase cell-cycle progression. *Curr Opin Genet Dev* **11**, 48, 2001.
31. Brennan, E.P., Tang, X.H., Stewart-Akers, A.M., Gudas, L.J., and Badylak, S.F. Chemoattractant activity of degradation products of fetal and adult skin extracellular matrix for keratinocyte progenitor cells. *J Tissue Eng Regen Med* **2**, 491, 2008.
32. Genbacev, O., Zhou, Y., Ludlow, J.W., and Fisher, S.J. Regulation of human placental development by oxygen tension. *Science* **277**, 1669, 1997.
33. Askari, A.T., Unzek, S., Popovic, Z.B., Goldman, C.K., Forudi, F., Kiedrowski, M., Rovner, A., Ellis, S.G., Thomas, J.D., DiCorleto, P.E., Topol, E.J., and Penn, M.S. Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. *Lancet* **362**, 697, 2003.
34. Akhyari, P., Kamiya, H., Haverich, A., Karck, M., and Lichtenberg, A. Myocardial tissue engineering: the extracellular matrix. *Eur J Cardiothorac Surg* **34**, 229, 2008.
35. Brigido, S.A., Boc, S.F., and Lopez, R.C. Effective management of major lower extremity wounds using an acellular regenerative tissue matrix: a pilot study. *Orthopedics* **27**, s145, 2004.
36. Badylak, S., Obermiller, J., Geddes, L., and Matheny, R. Extracellular matrix for myocardial repair. *Heart Surg Forum* **6**, E20, 2003.
37. Boruch, A.V., Nieponice, A., Qureshi, I.R., Gilbert, T.W., and Badylak, S.F. Constructive remodeling of biologic scaffolds is dependent on early exposure to physiologic bladder filling in a canine partial cystectomy model. *J Surg Res* **161**, 217, 2010.
38. Parekh, A., Mantle, B., Banks, J., Swarts, J.D., Badylak, S.F., Dohar, J.E., and Hebda, P.A. Repair of the tympanic membrane with urinary bladder matrix. *Laryngoscope* **119**, 1206, 2009.
39. Gilbert, T.W., Agrawal, V., Gilbert, M.R., Povirk, K.M., Badylak, S.F., and Rosen, C.A. Liver-derived extracellular matrix as a biologic scaffold for acute vocal fold repair in a canine model. *Laryngoscope* **119**, 1856, 2009.
40. Badylak, S.F., Park, K., Peppas, N., McCabe, G., and Yoder, M. Marrow-derived cells populate scaffolds composed of xenogeneic extracellular matrix. *Exp Hematol* **29**, 1310, 2001.
41. Agrawal, V., Brown, B.N., Beattie, A.J., Gilbert, T.W., and Badylak, S.F. Evidence of innervation following extracellular matrix scaffold-mediated remodelling of muscular tissues. *J Tissue Eng Regen Med* **3**, 590, 2009.
42. Li, F., Li, W., Johnson, S., Ingram, D., Yoder, M., and Badylak, S. Low-molecular-weight peptides derived from extracellular matrix as chemoattractants for primary endothelial cells. *Endothelium* **11**, 199, 2004.
43. Boonstra, J., and Post, J.A. Molecular events associated with reactive oxygen species and cell cycle progression in mammalian cells. *Gene* **337**, 1, 2004.
44. Janssen-Heininger, Y.M., Mossman, B.T., Heintz, N.H., Forman, H.J., Kalyanaraman, B., Finkel, T., Stamler, J.S., Rhee, S.G., and van der Vliet, A. Redox-based regulation of signal transduction: principles, pitfalls, and promises. *Free Radic Biol Med* **45**, 1, 2008.
45. Seth, D., and Rudolph, J. Redox regulation of MAP kinase phosphatase 3. *Biochemistry* **45**, 8476, 2006.

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