STEM CELLS®

TISSUE-SPECIFIC STEM CELLS

Injury-Activated Transforming Growth Factor β Controls Mobilization of Mesenchymal Stem Cells for Tissue Remodeling

MEI WAN,[a] CHANGJUN LI,[a] GEHUA ZHEN,[a] KAI JIAO,[a,b] WENYING HE,[a,c] XIAOFENG JIA,[d] WEISHAN WANG,[c] CHENHUI SHI,[c] QIUJUAN XING,[a,e] YIU-FAI CHEN,[f] SUZANNE JAN DE BEUR,[g] BING YU,[a] XU CAO[a]

[a]Department of Orthopaedic Surgery; [d]Department of Biomedical Engineering; [g]Division of Endocrinology, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA; [f]Division of Cardiovascular Disease, Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama, USA; [b]Stomatological College, Fourth Military Medical University, Xi'an, People's Republic of China; [c]Shihezi Medical Collage, Shihezi Univeristy, Xinjiang, People's Republic of China; [e]Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, People's Republic of China

Key Words. TGFβ activation • Mesenchymal stem cells • Cell mobilization • Vascular remodeling

ABSTRACT

Upon secretion, transforming growth factor β (TGFβ) is maintained in a sequestered state in extracellular matrix as a latent form. The latent TGFβ is considered as a molecular sensor that releases active TGFβ in response to the perturbations of the extracellular matrix at the situations of mechanical stress, wound repair, tissue injury, and inflammation. The biological implication of the temporal discontinuity of TGFβ storage in the matrix and its activation is obscure. Here, using several animal models in which latent TGFβ is activated in vascular matrix in response to injury of arteries, we show that active TGFβ controls the mobilization and recruitment of mesenchymal stem cells (MSCs) to participate in tissue repair and remodeling. MSCs were mobilized into the peripheral blood in response to vascular injury and recruited to the injured sites where they gave rise to both endothelial cells for re-endothelialization and myofibroblastic cells to form thick neointima. TGFβs were activated in the vascular matrix in both rat and mouse models of mechanical injury of arteries. Importantly, the active TGFβ released from the injured vessels is essential to induce the migration of MSCs, and cascade expression of monocyte chemotactic protein-1 stimulated by TGFβ amplifies the signal for migration. Moreover, sustained high levels of active TGFβ were observed in peripheral blood, and at the same time points following injury, $Sca1^{+}CD29^{+}CD11b^{-}CD45^{-}$ MSCs, in which 91% are $nestin^{+}$ cells, were mobilized to peripheral blood and recruited to the remodeling arteries. Intravenously injection of recombinant active TGFβ1 in uninjured mice rapidly mobilized MSCs into circulation. Furthermore, inhibitor of TGFβ type I receptor blocked the mobilization and recruitment of MSCs to the injured arteries. Thus, TGFβ is an injury-activated messenger essential for the mobilization and recruitment of MSCs to participate in tissue repair/remodeling. *STEM CELLS 2012; 30:2498–2511*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Adult stem/progenitor cells including mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), and endothelial progenitor cells (EPCs) not only have the ability to differentiate into many cell types but also can be recruited to the sites of injury where they either repair the injured tissue or contribute to tissue remodeling [1–5]. In particular, the circulating MSCs in peripheral blood are increased under many circumstances, such as myocardial infarction, chronic ischemic cardiomyopathy, pulmonary fibrosis, hypoxic stress, tissue injury, and these cells can be integrated into the repairing/remodeling tissues [6–13]. It is believed that promigratory factor(s) released from injured tissue or surrounding inflammatory cells create a gradient locally to mediate the migration of stem cells from peripheral blood or surrounding tissue to the injury sites [14–17]. These migratory factor(s) may also diffuse into circulation. The high blood concentration of the factor(s) outside of the stem cell niche may lead to the mobilization of the cells from their original niche into peripheral blood. Many growth factors and cytokines have been found to regulate the mobilization of HSCs and EPCs. These factors include colony-stimulating factors, stromal cell-derived factor-1 (SDF-1),

Author contributions: M.W.: conception and design, financial support, collection and assembly of data, data analysis and interpretation, and manuscript writing, C.L., G.Z., K.J., W.H., X.J., W.W., C.S., Q.X., and B.Y.: provision of study material, collection and assembly of data, Y.-F.C. and S.J.D.B.: data analysis and interpretation; X.C.: conception and design, financial support, data analysis and Interpretation, and manuscript writing.

Correspondence: Mei Wan, MD., Ph.D., Department of Orthopaedic Surgery, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, USA. Telephone: 410-502-6598; Fax: 410-502-6414; e-mail: mwan4@jhmi.edu; or Xu Cao, Ph.D., Department of Orthopaedic Surgery, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, USA. Telephone: 410-502-6440; Fax: 410-502-6414; e-mail: xcao11@jhmi.edu Received March 26, 2012; accepted for publication July 25, 2012; first published online in STEM CELLS *EXPRESS* August 21, 2012. 1066-5099/2012/$30.00/0 doi: 10.1002/stem.1208

vascular endothelial growth factor, erythropoietin, angiopoietin-2, fibroblast growth factor, stem cell factor, and interleukin 8 [14, 15]. However, the primary endogenous factor(s) activated or released from vascular cells, vascular matrix, or surrounding inflammatory cells in response to injury to stimulate the mobilization of MSCs are largely unknown.

Transforming growth factor β (TGFβ), consists of three isoforms: TGFβ1, 2, and 3, regulates a broad spectrum of biological processes. Excessive TGFβ activation is a major contributor to a variety of diseases including cancer, autoimmune diseases, vascular diseases, and progressive fibrosis in multiple organs. TGFβs are synthesized as latent form in a complex that is maintained in a sequestered state in extracellular matrix (ECM) [18, 19], thereby preventing the release of free TGFβ for its action, a process called latent TGFβ activation. Thus, the extracellular TGFβ activity is primarily regulated by the conversion of latent TGFβ to active TGFβ, and latent TGFβ is considered as a molecular sensor that responds to specific signals by releasing active TGFβ. These signals are often perturbations of the ECM that are associated with phenomena such as angiogenesis, wound repair, inflammation, and cell growth [20]. We previously demonstrated that active TGFβ1 released from bone matrix induces MSCs migration for bone remodeling [21]. The fact that TGFβ activation occurs during tissue injury or under other stressful conditions [22–26] suggests that active TGFβ may act as a promigratory factor for MSCs to participate tissue repair/remodeling.

In vasculature, it is known that increase in shear stress and injury of the arteries induce activation of TGFβ [25, 27, 28]. TGFβs are important modulators of vascular remodeling in diseases such as atherosclerosis, restenosis, and hypertension. Importantly, TGFβ levels elevate during progressive neointimal thickening following arterial injury [29, 30]. Thus, arterial injury in animals provides a good model to examine the function of active TGFβ released from ECM during tissue remodeling. Accumulated evidence suggests that blood circulation/bone marrow-derived [31–37] or vascular wall-resident stem/progenitor cells [38–43] can be recruited to the injured sites where they may play an essential role in the development of intimal hyperplasia. In this study, using a rat model of balloon-induced injury of carotid artery [44] and a mouse model of wire-induced injury of femoral artery [45], we demonstrate that MSCs mobilized into peripheral blood and migrated to the injured sites to participate in vascular repair and remodeling. We found that active TGFβ is essential for both the mobilization of the stem cells into circulating blood and subsequent recruitment of these cells to the remodeling sites. These observations shed new light on the mechanisms by which TGFβ participates in both tissue repair/regeneration in the maintenance of tissue homeostasis and tissue remodeling in disease conditions.

Materials and Methods

Animals

Sprague–Dawley rats and wild-type C57BL/6J mice were purchased from Charles River (Wilmington, MA, http://www.criver.com). Nestin-cre and C57BL/6J-Gtrosa26 tm1EYFP mice were purchased from Jackson Lab (West Grove, PA, http://www.jacksonimmuno.com). All animals were maintained in the animal facility of the Johns Hopkins University School of Medicine. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University, Baltimore, MD.

In Vitro Assays for MSCs Migration

To prepare vascular conditioned medium (CM), male Sprague–Dawley rats (250–300 g) were perfused with the 200 ml 0.9% saline, and their ascending thoracic aortae were isolated from the peri-adventitial tissue under a dissection microscope. The isolated aortae were cut into three pieces with equal length and were flushed with sterilized phosphate-buffered saline (PBS). Endothelium injury was achieved by rubbing the luminal surface three times with a cotton-tipped applicator as described previously [46]. Each segment of injured aorta and the uninjured control were cultured in 24-well tissue culture plates with 600 μl serum-free Dulbecco's modified Eagle's medium (DMEM) at 37°C. After 2 days, the conditioned media were collected and stored at −80°C. In some experiments, neutralizing antibodies against TGFβ1, TGFβ2, TGFβ3, SDF-1 or monocyte chemotactic protein-1 (MCP-1) at 500 ng/ml or TGFβ type I receptor (TβRI) inhibitor (SB-505124, 2 μM) were individually added to the CM. Cell migration was assessed in 96-well Transwells (Corning, Inc., Acton, MA, http://www.corning.com/lifesciences) as described previously [21]. The 8 μm pore membrane between the upper and lower chambers was precoated with 0.5 μg/ml type I collagen (BD Biosciences, San Diego, CA, http://www.bdbiosciences.com). 1×10^4 MSCs in 50 μl serum-free DMEM were plated in the upper chambers, and 150 μl undiluted conditioned media from the cultured aortae was added to the lower chambers. After 10 hours of incubation, cells were fixed with 10% formaldehyde for 4 hours, and then the MSCs that remained on the upper chamber membrane were removed with cotton swabs. The cells that had migrated through the pores to the bottom surface of the membrane were stained with hematoxylin (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com). Five fields at ×20 magnification were selected. Micrographic images were obtained and the cell number on each image was counted. Experiments were performed in triplicate.

Rat Model of Balloon-Induced Injury of Common Carotid Artery

Balloon-induced injury of common carotid artery was conducted as described previously [44]. Briefly, rats were anesthetized by i.p. injection of ketamine (80 mg/kg) and xylazine (5 mg/kg), and the right carotid artery was isolated by a middle cervical incision. The distal right common carotid artery and region of the bifurcation were exposed. A 2F Fogarty balloon catheter (Baxter V. Mueller, Niles, IL, http://www.manta.com/c/mtw6dnp/baxter-v-mueller) was introduced through the external carotid artery and advanced into the thoracic aorta. The balloon was inflated with saline to distend the common carotid artery and was then pulled back to the external carotid artery. After six repetitions of this procedure, the endothelium was removed completely, and some injury to medial smooth muscle layers throughout the common carotid artery. After removal of the catheter, the external carotid artery was ligated and the wound closed. The left carotid artery was not damaged and served as a control.

Mouse Model of Wire-Induced Injury of Femoral Artery

Transluminal mechanical injury of the femoral artery was conducted as described previously [45]. Briefly, either the left or right femoral artery was subjected to blunted dissection. A straight spring wire (0.38 mm in diameter, No. C-SF-15-15, COOK, Bloomington, IN, http://www.cookgroup.com) was inserted toward the iliac artery to reach 5-mm distance from the incision. The wire was left in place for 1 minute to denude endothelium and dilate the artery. Blood flow in the

femoral artery was restored by releasing the sutures placed in the proximal and distal portions. To interrupt TGFβ1 signaling or MCP-1 signaling gradient in vivo, some mice were pretreated with TβRI inhibitor (SB-505124, 5 mg/kg, i.p. injection), a MCP-1-specific antagonist (RS 504393, 2 mg/kg, s.c.) or vehicle (50% Dimethyl sulfoxide (DMSO) in 0.9% saline) once daily, started at 3 days before the surgery and continued until mice were sacrificed. At 3 days or 2 weeks after surgery, mice were euthanized by i.p. administration of ketamine/xylazine. Five hundred microliters of peripheral blood from each mouse was collected by cardiac puncture, the blood or plasma was used for following studies. The femoral artery was excised, fixed with 4% paraformaldehyde, and embedded in paraffin. Thin sections (5 μm) were used for histological and immunohistochemical analyses.

ELISA of TGFβ1, TGFβ3, and MCP-1 in CM and Plasma

Levels of active and total TGFβ1 and TGFβ3 in CM of aorta and plasma of wire-injured mice were determined using a DuoSet ELISA Development kit (R&D Systems, Minneapolis, MN, http://www.rndsystems.com). Levels of MCP-1 in CM of aorta and plasma were determined using a Mouse MCP-1 ELISA MAX (Biolegend, San Diego, CA, http://www.biolegend.com).

Analysis of Biotinylated TGFβ1-Bound Cells in Bone Marrow

Biotinylated TGFβ1 (60 ng/kg b.wt., R&D System, Minneapolis, MN, http://www.rndsystems.com) was injected into mouse via tail vein. After 24 hours, bone marrow was collected by flushing 1 ml mesencult® MSC medium (Stem Cell Technologies, Vancouver, BC, Canada, http://www.stemcell.com) through the femurs. Cells were planted on coverslip and cultured with mesencult® MSC medium for 2 days. Double immunofluorescence staining of the cells attached to the slides was conducted using streptavidin-FITC for biotinylated TGFβ1 and Texas red for antibodies against nestin, Sca1, CD29, and CD11b.

Characterization of Human MSCs

Green fluorescence protein-labeled human MSCs were purchased from Texas A&M, Institute for Regenerative Medicine. Cells were sorted by fluorescence activated cell sorting (FACS) using antibodies against CD29, Sca-1, CD45, and CD11b (Biolegend, San Diego, CA, http://www.biolegend.com). The sorted cells were enriched, cultured, and further confirmed by flow cytometry. For osteogenic differentiation, cells were seeded at a density of 5×10^3 per centimeter square with α-minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 0.1 μM dexamethasone (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com), 10 mM β-glycerol phosphate (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com), and 50 μM ascorbate-2-phosphate (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com). Cultures in α-MEM supplemented with 10% FBS served as a negative control. After 3 weeks of differentiation, the mineralization capacity of the cells was evaluated by Alizarin Red staining (2% of Alizarin Red S dissolved in water at pH 4.2). For adipogenic differentiation, cells were seeded at a density of 1×10^4 per centimetre square with α-MEM supplemented with 10% FBS, 1 μM dexamethasone, 0.5 μM 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com), and 10 ng/ml of insulin (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com) for 2 weeks. Cultures of cells in α-MEM supplemented with 10% FBS served as a negative control. Lipid accumulation was identified by oil red O staining (0.5 g of oil red O [Sigma-Aldrich] dissolved in 100 ml of isopropanol [Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com], and diluted to 60% with distilled water). For chondrogenic differentiation, cells (1×10^6) were seeded in polypropylene tubes with high-glucose D-MEM supplemented with 0.1 μM dexamethasone, 1% insulin-transferrin-sodium selenite mix (ITS) (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com), 50 μM ascorbate-2-phosphate, 1 mM sodium pyruvate (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com), 50 μg/ml of proline (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com), and 20 ng/ml of TGF-β3 (R&D Systems, Minneapolis, MN, http://www.rndsystems.com). Culture cells in high-glucose D-MEM supplemented with 10% FBS served as a negative control. After 3 weeks in culture, the pellets were fixed in 10% buffered formalin for 2 days and embedded in paraffin. Then 4-μm-thick sections were processed for toluidine blue staining (1 g of toluidine blue [Sigma-Aldrich] was dissolved in 100 ml of 70% alcohol and diluted to 10% with 0.9% NaCl, pH adjusted to 2.3).

Flow Cytometry Analysis

Blood samples were collected from mice by cardiac puncture. After the process of RBC lysis with commercial ACK lysis buffer (Quality Biological, Inc., Gaithersburg, MD, http://www.qualitybiological.com), cells were washed with 0.1% bovine serum albumin (BSA) in PBS and then counted. 1×10^6 cells per milliliter were permeabilized in 0.1% Triton X-100 prior to blocking in 2.5% BSA for 1 hour, incubated with Alexa Fluor 647-conjugated Nestin antibody (BD Pharmingen, San Diego, CA, http://www.bdbiosciences.com/index) or isotype control for 1 hour at 37°C in dark room, and then washed twice with 0.1% BSA in PBS. $Sca1^+CD29^+CD11b^-CD45^-$ were considered as MSC fraction [21, 47], $c\text{-}Kit^+Sca\text{-}1^+Lin^-$ cells were considered as the primitive HSC fraction [33], and $VE\text{-}cadherin^+CD133^+CD31^+CD11b^-$ cells were considered as the EPC fraction [48]. For analysis of MSCs, HSCs, and EPCs in peripheral blood, the cells were stained with the combination of antibodies against cell surface markers. Specifically, for MSCs, phycoerythrin (PE)-conjugated anti-Sca1, PE/Cy7-conjugated anti-CD29, PerCP-conjugated anti-CD45, and allophycocyanin (APC)-conjugated anti-CD11b antibodies were used. For HSCs, cells were stained with the combination of cocktail biotinylated monoclonal antibodies against lineage markers, PE-conjugated anti-Sca-1, PerCP-conjugated c-kit, and then stained with streptavidin-allophycocyanin. For EPCs, the cells were stained with the combination of PE-conjugated anti-vascular endothelial (VE)-cadherin, FITC-conjugated anti-CD133, APC-conjugated anti-CD11b, and PerCP-conjugated anti-CD31, all commercially available from Bio-Legend. Probes were analyzed using a FACSCalibur flow cytometer and CellQuest software (Becton Dickinson, Franklin Lakes, NJ, http://www.bd.com).

Morphometric and Histological Analysis of Neointima

To study the morphology of the arteries, vessels were perfused with PBS followed by 4% paraformaldehyde by cannulating the left ventricle. Five μm sections were stained with hematoxylin and eosin (H&E). Morphometric analysis of neointima formation after 14 days consisted of the measurement of intimal area (I), medial area (M), and I/M ratios with a computerized morphometric analysis system (Image Pro Plus 6.0, Olympus) by an investigator blinded to the treatment. For each artery section, five random, noncontiguous microscopic

fields were examined. All measurements performed on the four sections of this aorta were averaged.

Immunohistochemical Analysis of the Vessel Sections

For immunohistochemical analysis of active TGFβ1 expression, paraffin-embedded sections (5 μm) were incubated with LC [1–30], a polyclonal antibody raised against the amino-terminal 30 amino acids of the mature TGFβ1 and specifically reacts with active TGFβ1 [49–51]. A horseradish peroxidase (HRP)-streptavidin detection system (Dako, Glostrup, Denmark, http://www.dakocytomation.com) was used to detect the positive staining followed by counterstaining with hematoxylin (Dako). Double immunofluorescence staining was performed as described previously [52]. After blocking in 0.5% horse serum, sections were incubated with first antibodies (anti-α smooth muscle actin [anti-α-SMA], anti-nestin, or anti-Sca1, Abcam, Cambridge, U.K., http://www.abcam.com) followed by incubation with fluorescein isothiocyanate (FITC) or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Sigma). The sections were mounted with the ProLong Antifade Kit (Molecular Probes, Grand Island, NY, http://www.invitrogen.com) and observed under a confocal microscope (FLUOVIEW FV300, Olympus).

Statistics

Statistical analysis was done using SAS software. The unpaired two-tailed Student's t test and χ^2 test were used to calculate p values.

Results

MSCs Are Mobilized to Peripheral Blood and Recruited to the Remodeling Arteries in Response to Vascular Injury

Mobilization of the stem cells/progenitor cells from bone marrow to peripheral blood is a prerequisite for the involvement of the cells in tissue repair and remodeling. To assess whether $Sca1^+CD29^+CD11b^-CD45^-$ MSCs [21, 47] can be mobilized in response to arterial injury, we used a mouse model of wire-induced injury of femoral artery [45], in which the arterial changes following injury mimic neointimal formation in restenosis. The numbers of $Sca1^+CD29^+CD11b^-CD45^-$ cells were significantly elevated in peripheral blood compared to their sham control group within 3 days postinjury, and the elevation lasted for 2 weeks (Fig. 1A). Bone marrow-derived $nestin^+$ cells are MSC-enriched cell population [53]. A similar increase in $nestin^+$ cells in peripheral blood was also observed after wire injury of femoral artery (Fig. 1B). These results showed that MSCs were mobilized into blood circulation following arterial injury.

The mobilization of MSCs to peripheral blood in response to injury indicated that they may participate in arterial remodeling. We then examined whether the mobilized MSCs were recruited to the injured artery in a rat model of balloon injury of carotid artery [44] and mouse model of wire injury of femoral artery [45]. Neointimal tissue was observed at 1 week postinjury, became much thicker at 2 weeks postinjury in rat carotid artery (Fig. 1C–1E) and in mouse femoral artery (Supporting Information Fig. S1A, S1B). Neointima hyperplasia continued to grow up to 6 weeks postinjury until the re-endothelialization is completed [44, 45]. We examined the recruitment of the MSCs at 1 week and 2 weeks following injury during the active phase of neointima formation. $Nestin^+$ cells were detected in the neointima of injured carotid arteries of rats (Fig. 1E) and injured femoral arteries of mice (Supporting Information Fig. S1C) but were undetectable in uninjured arteries. Of note, 90.1% ± 5.8% of the cells in the single layer of the endoluminal side of the neointimal tissue are $nestin^+$, whereas almost no $nestin^+$ cells were detected in the deeper layers of the neointima, which consisted α-smooth muscle actin (αSMA)$^+$ myofibroblast-like cells (Fig. 1G).

Recruited MSCs Participate in Both Endothelium Repair and Neointima Thickening

To further dissect the contribution of the recruited cells to the formation of neointima, we examined the cell fate(s) of the $nestin^+$ cells by performing double immunofluorescence analysis of the artery sections. 89.6% ± 9.2% and 83.1% ± 10.1% of the $nestin^+$ cells were $Sca1^+$ cells in the single layer of the intraluminal side of the neointimal tissue at 1 week and 2 weeks, respectively, following injury (Fig. 2A, 2B), indicating that most of the newly recruited cells are MSCs. The exclusive localization of $nestin^+$ cells at the single layer of the intraluminal side indicates that the cells may participate in re-endothelialization of the injured vessels. Approximately 19.0% ± 3.4% of $nestin^+$ cells expressed CD31 at 2 weeks following injury (Fig. 2C, 2D), indicating that some $nestin^+$ cells were transformed into endothelial cells for endothelium repair. To examine whether the recruited $nestin^+$ cells are also capable of differentiation into myofibroblasts that contribute to thick neointima formation, we took advantage of a novel genetic mouse model by crossing mice carrying a nestin promoter/enhancer-driven cre-recombinase (Nestin-cre) with C57BL/6J-Gtrosa26 tm1EYFP (R26-stop-EYFP) mice in which both $nestin^+$ cells and their descendants at the neointimal tissue can be marked (Supporting Information Fig. S2). Approximately 57.0% and 44.6% of cells in all layers of neointimal tissue were $EYFP^+$ cells at 1 week and 2 weeks, respectively, postinjury, and almost none of the media smooth muscle cells were $EYFP^+$ (Fig. 2D, 2E). Consistent with Figure 1, only the cells at the layer of endoluminal side still maintained nestin expression, and the $nestin^+$ cell-derived myofibroblastic cells in deeper layers lost the nestin marker. Collectively, these results suggested that the MSCs were mobilized into the peripheral blood in response to vascular injury and recruited to the injured sites where they give rise to both endothelial cells for re-endothelialization and myofibroblastic cells to form thick neointima.

Active TGFβ1 Is Released from Injured Arteries to Recruit MSCs to the Injured Sites

We have shown that the active TGFβ1 induces MSCs' migration during bone remodeling [21]. We therefore investigated whether TGFβ1 also plays a role in recruiting MSCs in response to arterial injury. We measured the levels of active TGFβ1 in injured arteries. Immunohistostaining analysis showed that active TGFβ1 expression increased in intimal and medial layers of the injured arteries at 3 days, 1 week, and 2 weeks postinjury in both rats (Supporting Information Fig. S3A) and mice (Supporting Information Fig. S3B). In contrast, active TGFβ1 was undetectable in uninjured arteries. Active TGFβ1 levels in injury arteries detected by ELISA were elevated at 8-hour postinjury, reached peak at 3–14 days (Fig. 3A). The ratios of active to total TGFβ1 levels were in a similar pattern (Fig. 3B), indicating that the activation of TGFβ1 from latent form to free form was induced by the injury of vascular matrix.

Increased active TGFβ1 level in the injured arteries indicates that TGFβ1 may stimulate the migration of MSCs to the

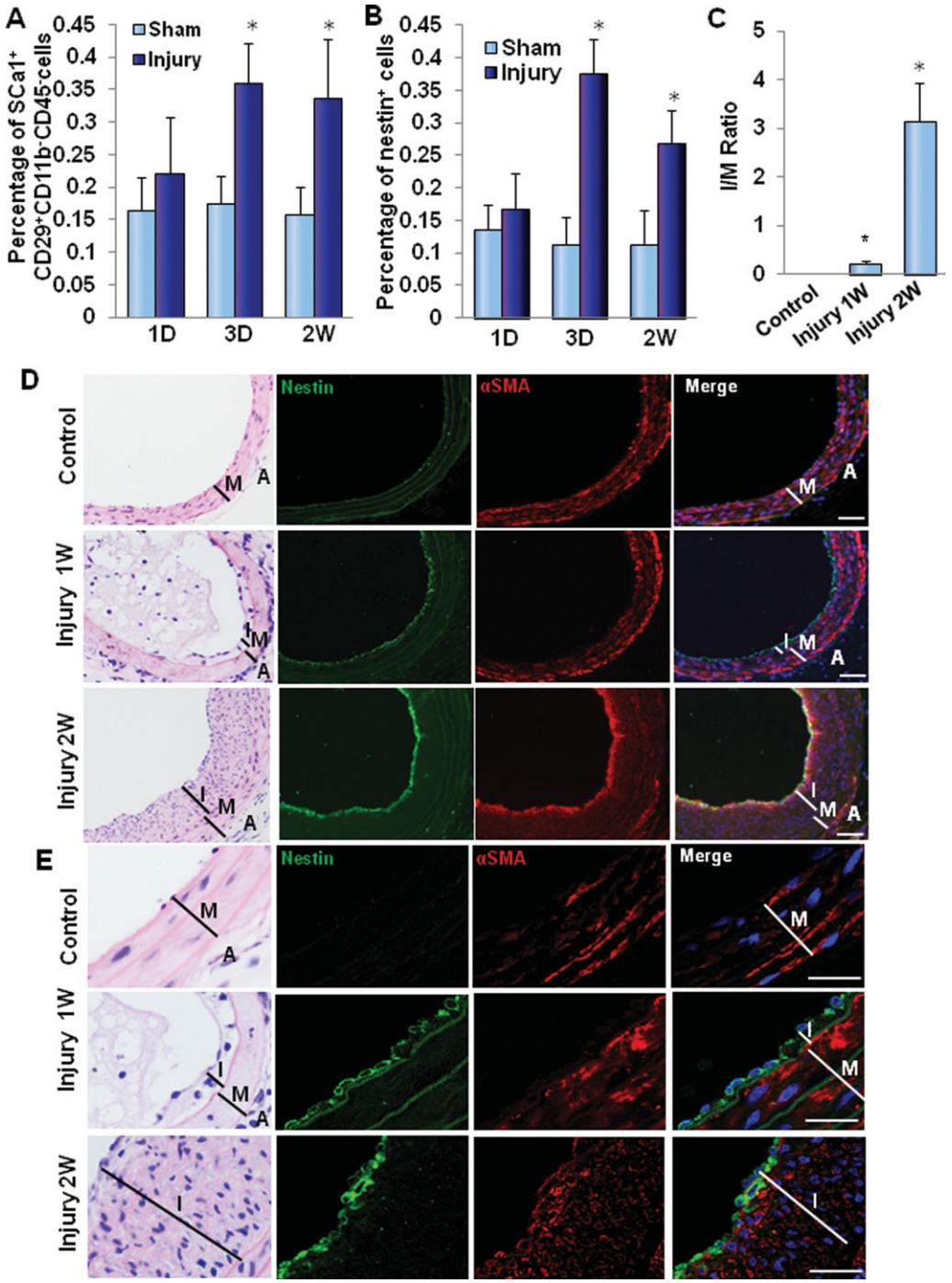

Figure 1. Mesenchymal stem cells were mobilized to peripheral blood and recruited to the remodeling arteries in response to vascular injury. **(A, B):** Percentages of $Sca1^+CD29^+CD11b^-CD45^-$ cells or $nestin^+$ cells, respectively, in peripheral blood at 1-day (1D), 3 days (3D), and 2 weeks (2W) after femoral arterial injury or sham surgery in mice. Results are the mean ± SD, $n = 4$ mice per group per time point. *, $p < .05$ versus respective Sham groups. **(C):** Calculation of the I/M ratio of rat carotid artery from uninjured artery (Control), and 1 week (1W) or 2 weeks (2W) after balloon injury. Results are the mean ± SD, $n = 4$ mice per group per time point. *, $p < .001$ versus uninjured control group. **(D, E):** Representative H&E and immunofluorescence images of tissue sections of rat carotid artery from uninjured artery (Control), and 1 week (1W), or 2 weeks (2W) after balloon injury. Left column of (D): H&E staining; right columns of (D): Triple-immunofluorescence staining for nestin (green), α-SMA (red), and DAPI (blue) in carotid artery of uninjured control, 1 week, or 2 weeks postinjury. Scale bars = 100 μm. Left column of (E): H&E staining; right columns of (D): Triple-immunofluorescence staining for nestin (green), α-SMA (red), and DAPI (blue) in carotid artery of uninjured control, 1 week, or 2 weeks postinjury. Scale bars = 50 μm. Abbreviations: A, adventitia layer; I, intima layer; I/M ratio, ratio of intima/media areas; M, media smooth muscle layer; α SMA, α smooth muscle actin. DAPI, 4′,6-diamidino-2-phenylindole, dihydrochloride.

injured arterial sites. We developed an Aorta-CM-based cell migration assay, in which MSCs were placed in the upper chamber and the Aorta-CM was placed in the lower chamber of a transwell chamber (Fig. 3C). Aorta-CM prepared from ex vivo injured aorta significantly enhanced cell migration (Fig. 3D, 3E) compared to CM prepared from uninjured aorta.

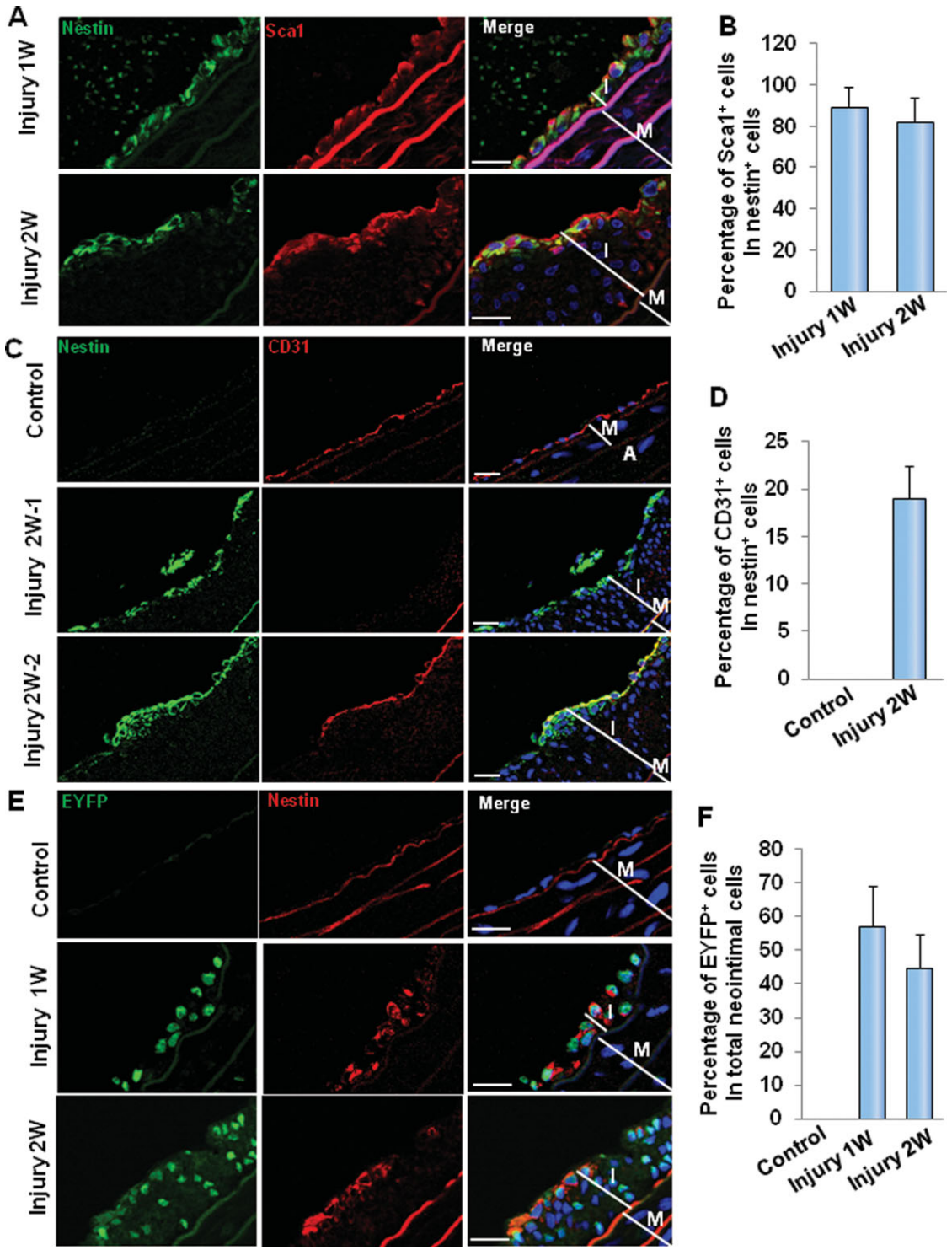

Figure 2. The differentiation fates of the recruited cells in neointimal tissue. **(A):** Colocalization of nestin and Sca1 in the recruited cells. Triple-immunofluorescence staining for nestin (green), Sca1 (red), and DAPI (blue) in balloon injured rat carotid artery 1 week (1W) or 2 weeks (2W) postinjury. Scale bars = 50 μm. **(B):** Calculation of the percentages of Sca1$^+$ cells out of nestin$^+$ cells in the layer near to luminal side. Results are the mean ± SD, $n = 4$ mice per group per time point. **(C):** Colocalization of nestin and CD31 in the recruited cells. Triple-immunofluorescence staining for nestin (green), CD31 (red), and DAPI (blue) in balloon injured rat carotid artery 2W postinjury (Injury 2W-1 & Injury 2W-2 indicate two different artery samples). Scale bars = 50 μm. **(D):** Calculation of the percentages of CD31$^+$ cells out of nestin$^+$ cells in the layer near to luminal side. Results are the mean ± SD, $n = 4$ mice per group per time point. **(E):** Localization of EYFP$^+$ and nestin$^+$ cells in neointimal tissue. Triple-immunofluorescence staining for EYFP (green), nestin (red), and DAPI (blue) in wire-injured femoral arteries 1 week (1W) or 2 weeks (2W) postinjury in Nestin-cre and R26-stop-EYFP mice. Scale bars = 50 μm. **(F):** Calculation of the percentages of EYFP$^+$ cells out of total neointimal cells. Results are the mean ± SD, $n = 4$ mice per group per time point. Abbreviations: A, adventitia layer; I, intima layer; M, media smooth muscle layer. DAPI, 4′,6-diamidino-2-phenylindole, dihydrochloride.

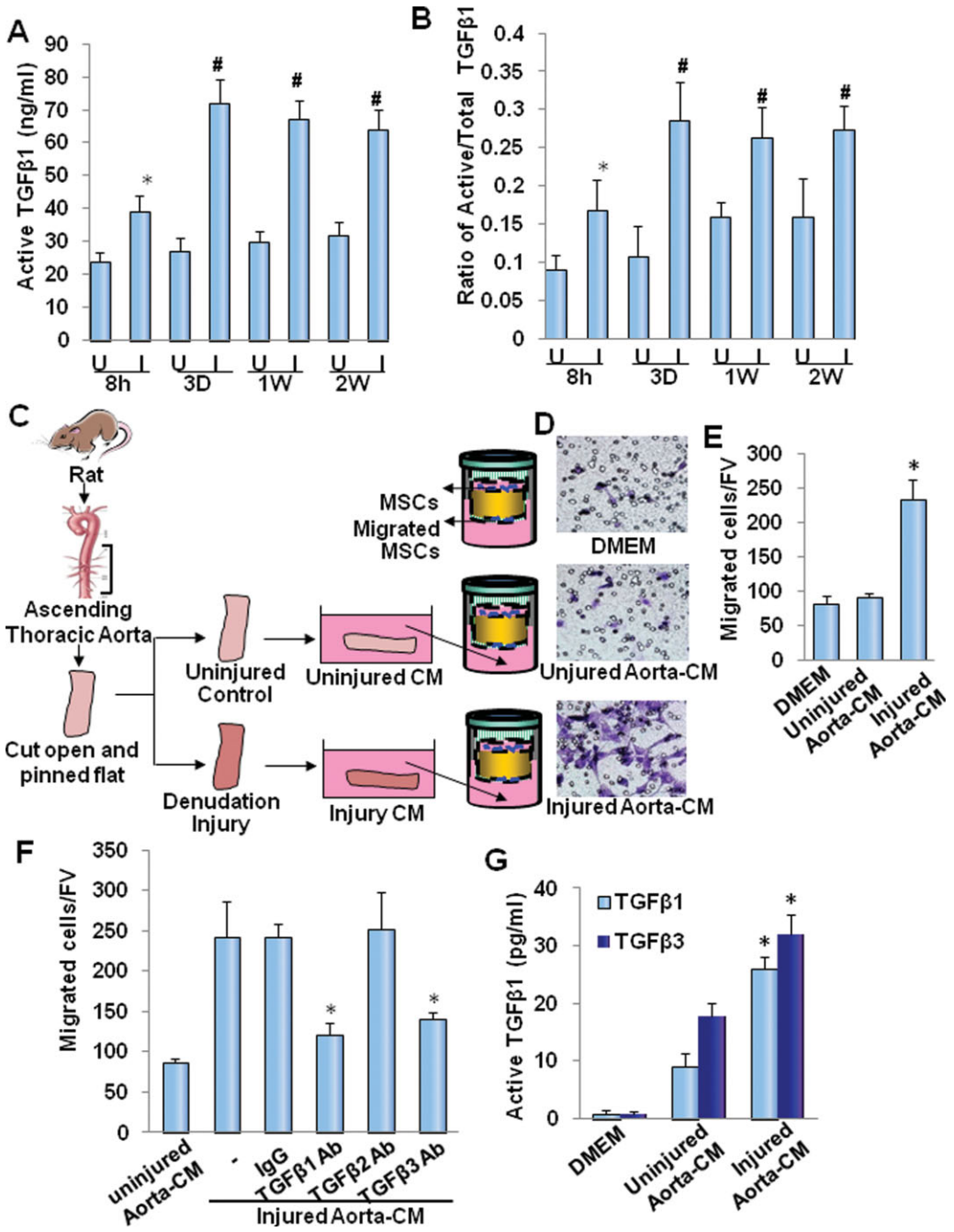

Figure 3. Active TGFβ1 is released from injured arteries to recruit MSCs to the injured sites. **(A, B):** ELISA analysis of active TGFβ1 levels and ratios of active/total TGFβ1, respectively, in wire-injured mouse femoral arteries at 8 hours, 3 days, 1 week, and 3 weeks postinjury (I). 0-hour sample was uninjured control (U). Results are the mean $\pm$ SD, $n = 5$ mice per group, *, $p < .05$; #, $p < .01$, versus uninjured control. **(C–G):** Active TGFβ released from injured vessels is a primary factor to stimulate migration of MSCs. (C): Diagrams show the preparation of Aorta-CM. Ascending thoracic aortae isolated from rats were either injured by rubbing the luminal surface three times with a cotton-tipped applicator or left uninjured. The aortae were cultured in serum-free DMEM for 48 hours, and culture media were collected as injured Aorta-CM or uninjured Aorta-CM. (D): Transwell assay for migration of MSCs using DMEM, uninjured Aorta-CM, or injured Aorta-CM collected from (C). (E): Numbers of migrated cells in (D) were counted per FV (magnification $\times 20$). Results are mean $\pm$ SD, $n = 4$ preparation per group, *, $p < .001$ versus uninjured aorta-CM. (F): Transwell assay for MSCs migration using injured Aorta-CM with addition of neutralizing antibodies (Ab) as indicated. Numbers of migrated cells were counted per FV (magnification $\times 20$). Results are mean $\pm$ SD, $n = 4$ per group, *, $p < .01$ versus IgG control. (G): Active TGFβ1 and β3 levels measured by ELISA in CM prepared in (D). Results mean $\pm$ SD, $n = 4$ per group, *, $p < .01$ versus uninjured aorta-CM. Abbreviations: Aorta-CM, aorta-conditioned medium; DMEM, Dulbecco's Modification of Eagle's Medium; FV, field of view; MSC, mesenchymal stem cell; TGFβ1, transforming growth factor β1.

When neutralizing antibodies against TGFβ1 or TGFβ3 were added to the Aorta-CM, the migration of MSCs was inhibited, whereas the TGFβ2 neutralizing antibody had minimal inhibition (Fig. 3F). Consistently, high levels of active TGFβ1 and TGFβ3 were detected in the Aorta-CM from injured vessels (Fig. 3G). These results suggested that TGFβ is a primary factor released by injured arteries to stimulate MSCs migration. We also examined the downstream signaling that mediate the

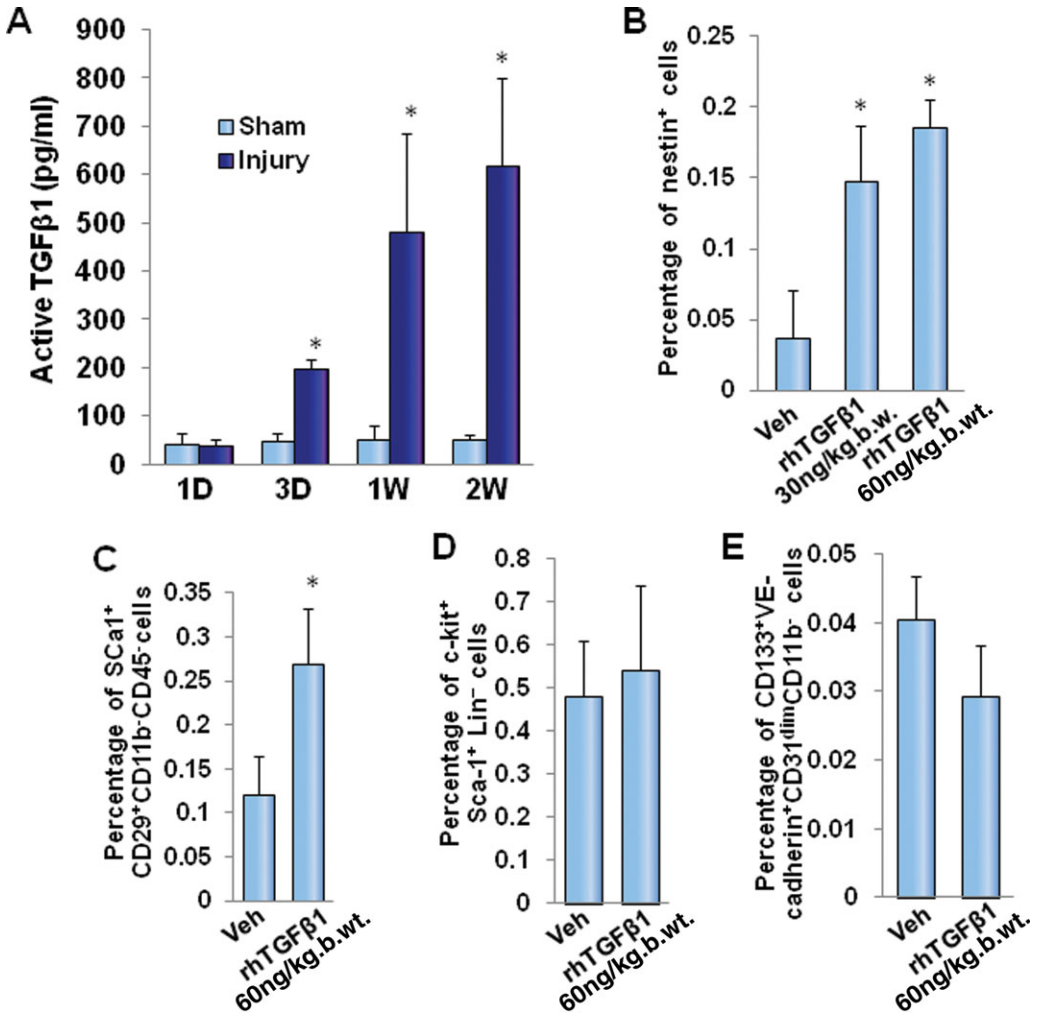

Figure 4. Active TGFβ1 is sufficient to mobilize mesenchymal stem cells into peripheral blood. **(A):** Active TGFβ1 levels in peripheral blood of mouse 1-day, 3 days, 1 week, and 2 weeks postfemoral artery injury. Sham controls were mouse without injury. Results are the mean ± SD, $n = 5$ mice per group, *, $p < .01$ versus respective Sham controls. **(B–E):** Mobilization of stem cells into peripheral blood by rhTGFβ1 transfusion in uninjured mice. Flow cytometry measured percentages of nestin$^+$ cells (B), Sca1$^+$CD29$^+$CD11b$^-$CD45$^-$ cells (C), Sca1$^+$c-kit$^+$lin$^-$ cells (D), or CD133$^+$VE-cadherin$^+$ CD31^{dim}CD11b$^-$ (E) in peripheral blood at 1-day after i.v. injection of rhTGFβ1 or vehicle. Results are the mean ± SD, $n = 4$ mice per group, *, $p < .01$ versus respective Veh groups. Abbreviations: rhTGFβ1, recombinant human TGFβ1; TGFβ1, transforming growth factor β1.

effect of TGFβ. The roles of canonical and noncanonical TGFβ signaling pathways in mediating MSCs migration stimulated by injured Aorta-CM were detected. Addition of SB505124 (TβRI/Smads inhibitor) or SP600125 (c-Jun N-terminal kinases [JNK] inhibitor) in the injured Aorta-CM significantly suppressed the migration of MSCs, whereas U0126 (extracellular signal-regulated kinases [ERK] inhibitor), SB202190 (p38 inhibitor), or Y27632 (RhoA inhibitor) had marginal effect (Supporting Information Fig. S4). These results suggest that both Smads and JNK signaling contribute to the recruitment of MSCs to the injured arteries.

Active TGFβ1 Mobilizes MSCs into Peripheral Blood

Inflammatory cells and platelets in circulating blood produce TGFβ after tissue injury [25, 54], and active TGFβ released from the injured arteries may also diffuse into the blood circulation. Indeed, active TGFβ1 levels in blood was elevated at 3 days and reached eightfold at 1 week and 10-fold at 2 weeks postinjury (Fig. 4A). To investigate whether active TGFβ1 is sufficient to mobilize stem cells, recombinant active TGFβ1 (rhTGFβ1) was injected i.v. to uninjured mice. The numbers of both nestin$^+$ and Sca1$^+$CD29$^+$CD11b$^-$CD45$^-$ cells in peripheral blood were increased 2.5–5-fold at 1-day post-rhTGFβ1 treatment relative to that of the vehicle-treated group (Fig. 4B, 4C). In contrast, the numbers of HSCs and EPCs in peripheral blood remained unchanged post-rhTGFβ1 injection (Fig. 4D, 4E). To examine the origin(s) of the mobilized MSCs, biotinylated rhTGFβ1 was injected i.v. and its binding to MSCs at different tissue was analyzed by measuring streptavidin-FITC-bound cells. Biotinylated rhTGFβ1 bounds to bone marrow nestin$^+$Sca1$^+$CD29$^+$CD11b$^-$ cells (Fig. 5A, 5B). No specific biotinylated TGFβ1-bound cells were found in liver and lung tissue. We detected a few positive cells in adipose tissue and adventitia tissue of femoral arteries, but these cells were nestin$^-$ (Supporting Information Fig. S5). The results suggest that the biotinylated TGFβ1 mainly binds to nestin$^+$ MSCs in bone marrow. Importantly, we found 91% and 89% of the sorted Sca1$^+$CD29$^+$CD11b$^-$CD45$^-$ cells from bone marrow and peripheral blood, respectively, following rhTGFβ injection were nestin$^+$ (Fig. 5C, 5D), indicating that

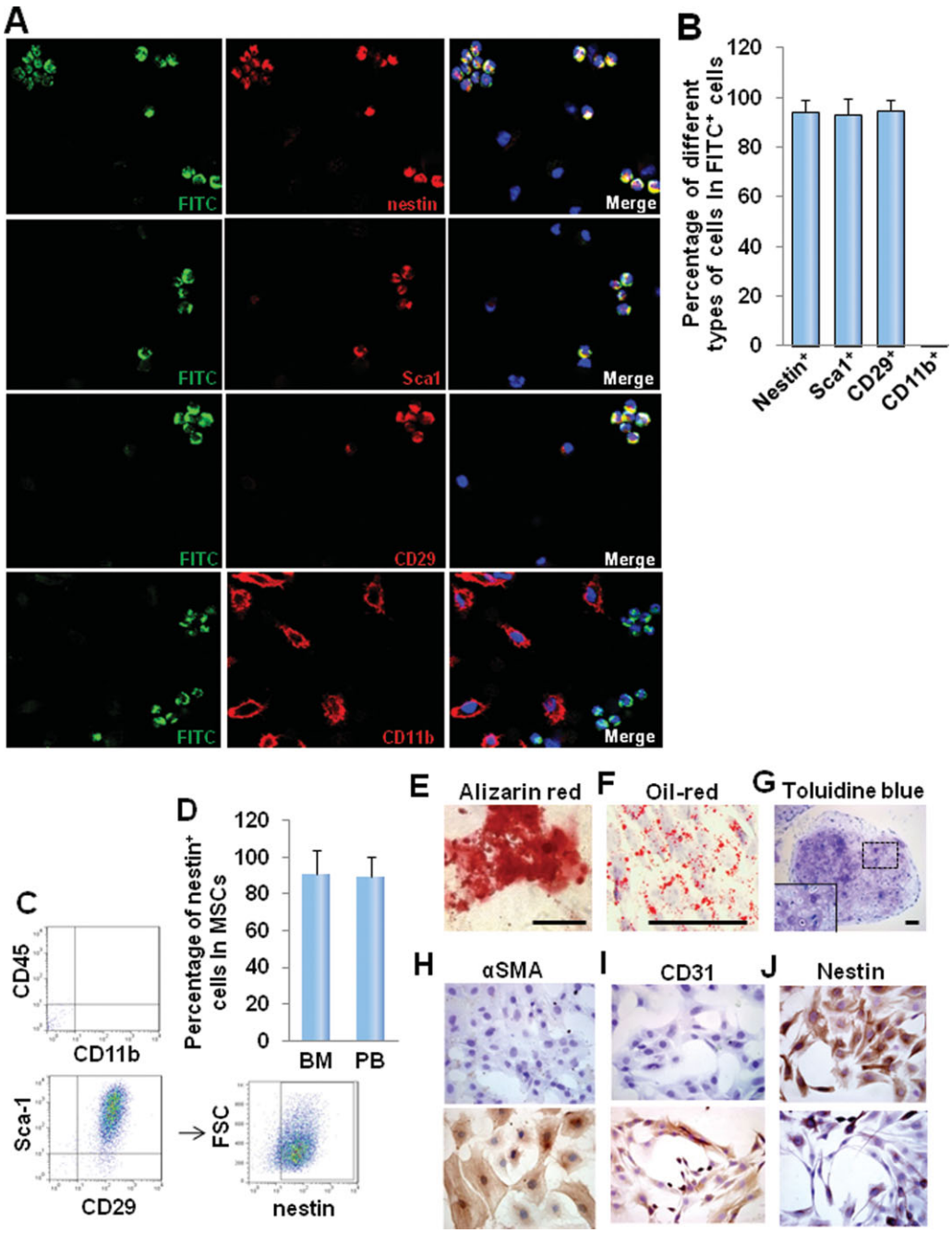

Figure 5. Transforming growth factor β (TGFβ)-mobilized cells are a subset of MSC lineage. **(A, B):** TGFβ1-bound cells in flushed mouse BM at 24 hours after the injection of biotinylated-TGFβ1 into blood. The femur was flushed with MSCs growth medium. Flushed attached cells were double immunofluorescence stained with streptavidin-FITC for biotinylated TGFβ1 and with Texas red for the indicated antibodies. Representative confocal micrographic images are shown in (A). Quantification of different cell types (in %) in total TGFβ1-bound cells is shown in (B). Results are mean ± SD calculated from three preparations of each mouse, $n = 3$ mice per group. **(C):** Sca-1^+CD29^+CD45^-CD$11b^-$ cells were sorted from murine BM cells by flow cytometry sorting, and the sorted cells were analyzed by FACS analysis using specific nestin antibody. **(D):** Calculation of the percentages of nestin$^+$ cells out of Sca-1^+CD29^+CD45^-CD$11b^-$ cells in the BM and PB. Results are the mean ± SD, $n = 4$ mice per group per time point. **(E–J):** Nestin$^+$ cells were cultured in single-cell suspensions to detect single colony forming. Individual colonies were selected and expanded by passaging. Each clonal strain derived from a single CFU-F underwent osteogenic (E, Alizarin red stain), adipogenic (F,Oil-red stain), and chondrogenic (G, Toluidine blue stain) induction by incubation with different culture media. Scale bars = 100 μm. (H, I): Nestin$^+$ cells were incubated with (bottom panels) or without (upper panels) smooth muscle differentiation medium and endothelium differentiation medium, respectively, and the cells were immunostained with anti-αSMA (H, smooth muscle marker) and anti-CD31 antibody (I, endothelial cell marker). (J): Decreased expression of nestin in cells incubated with (bottom panels) or without (upper panels) smooth muscle differentiation medium. Abbreviations: BM, bone marrow; FITC, fluorescein isothiocyanate; FACS, fluorescence activated cell sorting; MSC, mesenchymal stem cells; CFU-F, colony forming units-fibroblast; BM, bone marrow; PB, peripheral blood; αSMA, α smooth muscle actin.

the nestin$^+$ cells in peripheral blood are MSCs and are mobilized primarily from bone marrow. These cells have multilineage differentiation capacity since they were capable of osteogenesis (Fig. 5E), adipogenesis (Fig. 5F), and chondrogenesis (Fig. 5G). Furthermore, nestin$^+$ cells can differentiate into smooth muscle- or myofibroblast-like cells (Fig. 5H) and endothelial cells (Fig. 5I), and the nestin expression decreased after the differentiation (Fig. 5J). Collectively, these results suggest that active TGFβ1 is sufficient to mobilize MSCs from bone marrow to the peripheral blood.

TGFβ Activation Is Essential for the Recruitment of MSCs to the Remodeling Arteries in Response to Injury

To further validate the role of TGFβ in MSCs mobilization, TβRI inhibitor SB-505124 (SB) was injected i.v. of mice with injury of femoral arteries. Blocking of TβRI almost abolished the increase in nestin$^+$ cells in peripheral blood at 3-day and 2 weeks postarterial injury (Fig. 6A, 6B). In addition, neointima was barely formed in injured arteries of SB-treated mice compared to vehicle-treated mice (Fig. 6C, 6D, left columns and Fig. 6E). Unlike the single layer of nestin$^+$ cells on the endoluminal side of neointima in the vehicle-treated mice, nestin$^+$ cells were undetectable on the vessel wall of the SB-treated mice (Fig. 6C, 6D, right columns and Fig. 6F). These results indicate that TGFβ is required for the recruitment of MSCs during vascular remodeling.

MCP-1 Mediates TGFβ-Induced Migration and Homing of MSCs to the Injured Arteries Without Affecting the Mobilization of the Cells from Bone Marrow to Circulating Blood

MCP1 [55] and Chemokine (C-X-C motif) ligand 12 (CXCL12)/SDF1α [56–59] have been reported to be involved in the recruitment of stem/progenitor cells to the vasculature. We showed that neutralization antibody against MCP1, but not SDF1α, inhibited injured Aorta-CM-induced migration of MSCs in the transwell assays (Fig. 7A). Consistently, significantly increased MCP1 production was detected in both injured Aorta-CM (Fig. 7B) and the injured mouse femoral arteries (mainly the smooth muscle cells and neointimal cells) at 3 days to 2 weeks following injury (Fig. 7C). TGFβ has been shown to stimulate MCP1 expression in the vascular smooth muscle cells [55]. We found that MCP1 production by the injured aorta was inhibited by selective TβRI kinase inhibitor SB505124 or neutralizing antibodies against TGFβ1 or TGFβ3 (Fig. 7B), indicating that TGFβs were primary factors that regulate MCP1 expression via a TβRI-mediated pathway. To further elucidate the role of MCP1 in MSCs mobilization and recruitment during arterial remodeling, RS 504393 (RS), a selective MCP-1 receptor antagonist, was administered in mice with femoral artery injury. RS administration significantly inhibited neointima formation (Fig. 7D, left column and Fig. 7E) and the recruitment of the nestin$^+$ cells to neointima (Fig. 7D, right columns and Fig. 7F). Even though majority of the nestin$^+$ cells in peripheral blood expressed C-C chemokine receptor type 2 (CCR2), receptor of MCP1, the percentage of CCR2$^+$ cells from sham control mice and injured mice are not significantly different (Fig. 7G). Furthermore, the number of nestin$^+$ MSCs in peripheral blood of the injured mice was not reduced with the treatment of MCP1 inhibitor (Fig. 7H, 7I). These results suggest that MCP1/CCR2, a downstream target of TGFβ in injured arteries, functions as a local chemoattractant responsible for the migration and homing of MSCs to the injured vessels.

Discussion

Upon secretion, TGFβs are deposited in the matrices in various tissues as a latent form in a complex with its precursor molecule, latency-associated peptide (LAP) [18, 19]. The association of the mature domain with LAP prevents active TGFβ from binding to its receptor and subsequent action. Latent TGFβ is considered as a molecular sensor that releases active TGFβs in response to the perturbations of the ECM by various mechanisms at the situations of mechanical stress, wound repair, tissue injury, and inflammation [20, 60-62]. Our results show that TGFβ1 is activated in vascular tissue in response to injury, and active TGFβ1 stimulates the migration and homing of MSCs to the injured sites for vascular repair and remodeling. The specific inhibitor of TβRI blocked the recruitment of the cells to form neointima at the remodeling sites. Previously, we have shown that TGFβ activated from bone matrix during osteoclastic bone resorption induces migration of MSCs for normal bone remodeling [21]. Thus, TGFβ may act as an injury/stress-activated messenger to recruit MSCs for tissue repair, regeneration, and pathological remodeling in various organs. Excessive TGFβ activation is a major contributor to progressive fibrosis in many organs including lung, liver, kidney, heart, skin, and arteries [63, 64]. Sustained active TGFβ released locally due to chronic repetitive tissue injury may cause aberrant excessive recruitment of stem/progenitor cells, leading to excessive accumulation of myofibroblasts and progressive fibrosis. In Camurati-Engelmann disease and Marfan Syndrome, two hereditary disorders with mutations either in the LAP or in extracellular protein fibrillin-1, abnormal release of active TGFβ leads to fibrotic bone formation [21, 65, 66] and skin fibrosis [67, 68], respectively. Thus, tempo-spatial regulation of TGFβ activation is essential in the maintenance of tissue homeostasis and the regeneration of the damaged tissue by the recruitment of stem/progenitor cells, whereas sustained abnormal activation of TGFβ may lead to fibrosis development due to excessive recruitment of stem/progenitor cells and their subsequent differentiation.

Our results suggest that high levels of active TGFβ1 in peripheral blood primarily account for the mobilization of MSCs from bone marrow to peripheral blood. Thus, we consider that TGFβ acts as both a systemic factor to induce the mobilization of MSCs and a local factor to increase the production of MCP1 to induce the migration and homing of MSCs to the injured tissue (Supporting Information Fig. S6). One of the sources of active TGFβ in blood may be the injured vascular matrix. It is known that the ECM serves to concentrate latent TGFβ at the sites of intended function to influence the bioavailability and/or function of TGFβ activators [18–20]. Indeed, in the injured vessels, we observed rapid and sustained elevation of active TGFβ1, which may diffuse into the blood circulation. Platelets and inflammatory cells are major players in the initial thromboinflammatory response following arterial injury [69]. These cells within circulating blood or adhere to the injured tissue release large amount of active TGFβ1 into blood circulation [25, 54] and may also contribute to the high active TGFβ1 level in peripheral blood after vascular injury. Our data indicate that bone marrow is a primary origin of MSCs mobilized by TGFβ1, but we could not exclude the possibility of the mobilization of MSCs from other niches even though we did not observe biotinylated TGFβ-bound MSCs in several other tissues. MSCs seems a major cell type in bone marrow that is mobilized by TGFβ1 as we did not observe significant number increase for HSCs and EPCs in peripheral blood. However, in the genetic mouse model in which nestin$^+$ cells and their descendents were permanently marked, approximately 44.6% of the neointimal cells were

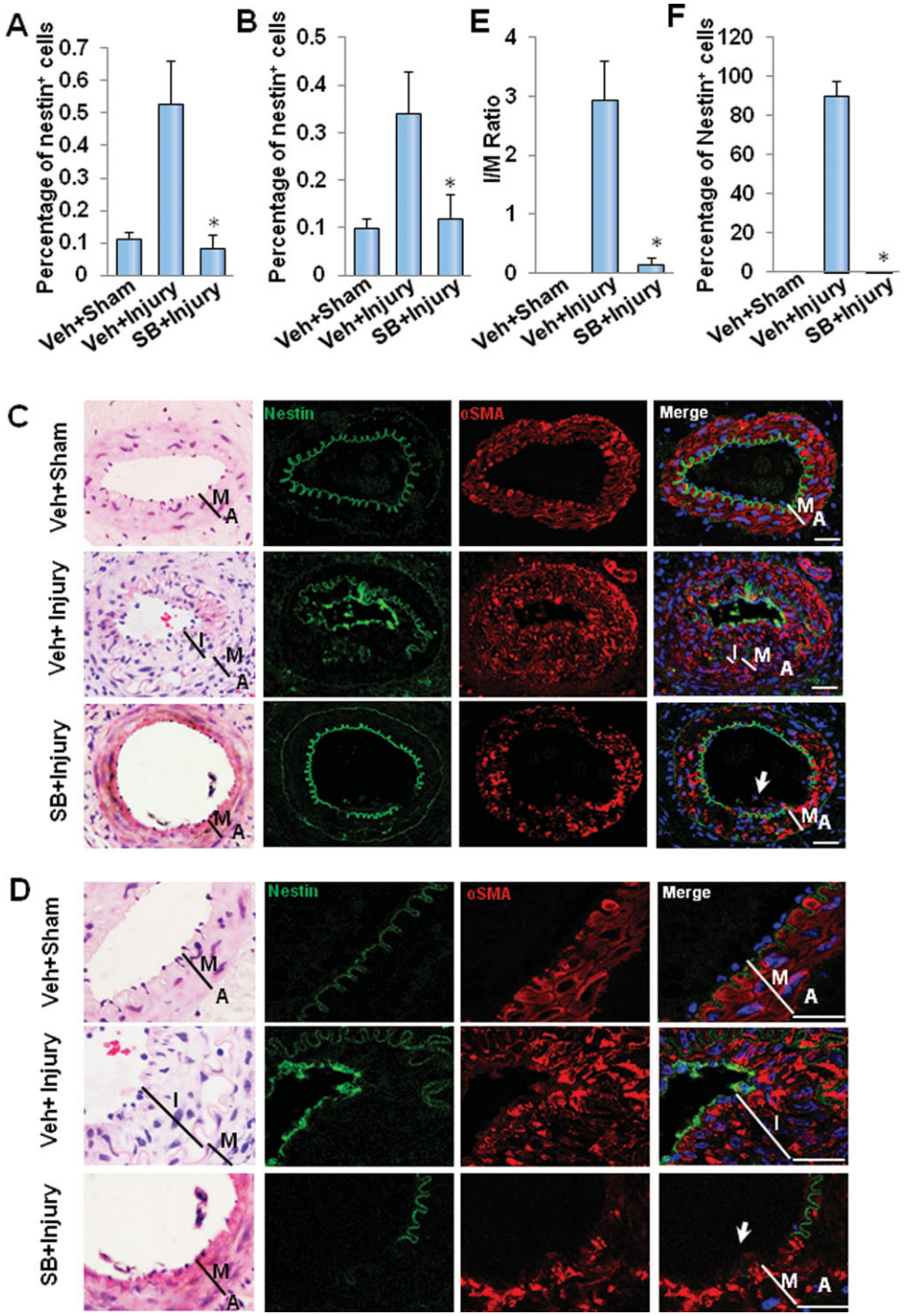

Figure 6. TGFβ was required for the mobilization and recruitment of MSCs to the remodeling arteries in response to injury. **(A, B):** Injection of TβRI blocker SB-505124 (SB, 5 mg/kg-b.wt., i.p. daily) blocked mobilization of MSCs following arterial injury. Flow cytometrical quantitation of the numbers of nestin$^+$ cells (% of total cell counted) in blood at (A) 3 days or (B) 2 weeks after femoral arterial injury or sham surgery in either SB- or vehicle (Veh)-treated mice. Results are mean ± SD, $n = 3$ mice per group per time point, *, $p < .001$ versus respective Veh+ injury groups. **(C, D):** TβRI blocker inhibited neointima formation after wire injury of femoral artery in mice. Left column of (C): H&E staining; Right columns of (C): Triple-immunofluorescence staining for nestin (green), α-SMA (red), and DAPI (blue) in femoral artery of uninjured control, 2 weeks postinjury, and Injury+SB mice. Scale bars = 100 μm. Left column of (D): H&E staining; right columns of (D): Triple-immunofluorescence staining for nestin (green), α-SMA (red), and DAPI (blue) in femoral artery of uninjured control, 2 weeks postinjury, and Injury+SB mice. Scale bars = 50 μm. **(E):** Calculation of I/M ratio of uninjured (Sham) or injured femoral arteries of mice treated with SB or Veh at 2 weeks postinjury. Results are mean ± SD, $n = 4$ mice per group, *, $p < .001$ versus Veh+Injury group. **(F):** Quantification of the percentage of nestin$^+$ cells out of the total cells in the layer close to luminal side of the vessels. Results mean ± SD, $n = 5$ mice per group, *, $p < .001$ versus Veh+ Injury group. Abbreviations: A, adventitia layer; I/M ratio, ratios of intima/media area; I, intima layer; M, media smooth muscle layer; αSMA, α smooth muscle actin. DAPI, 4′,6-diamidino-2-phenylindole, dihydrochloride.

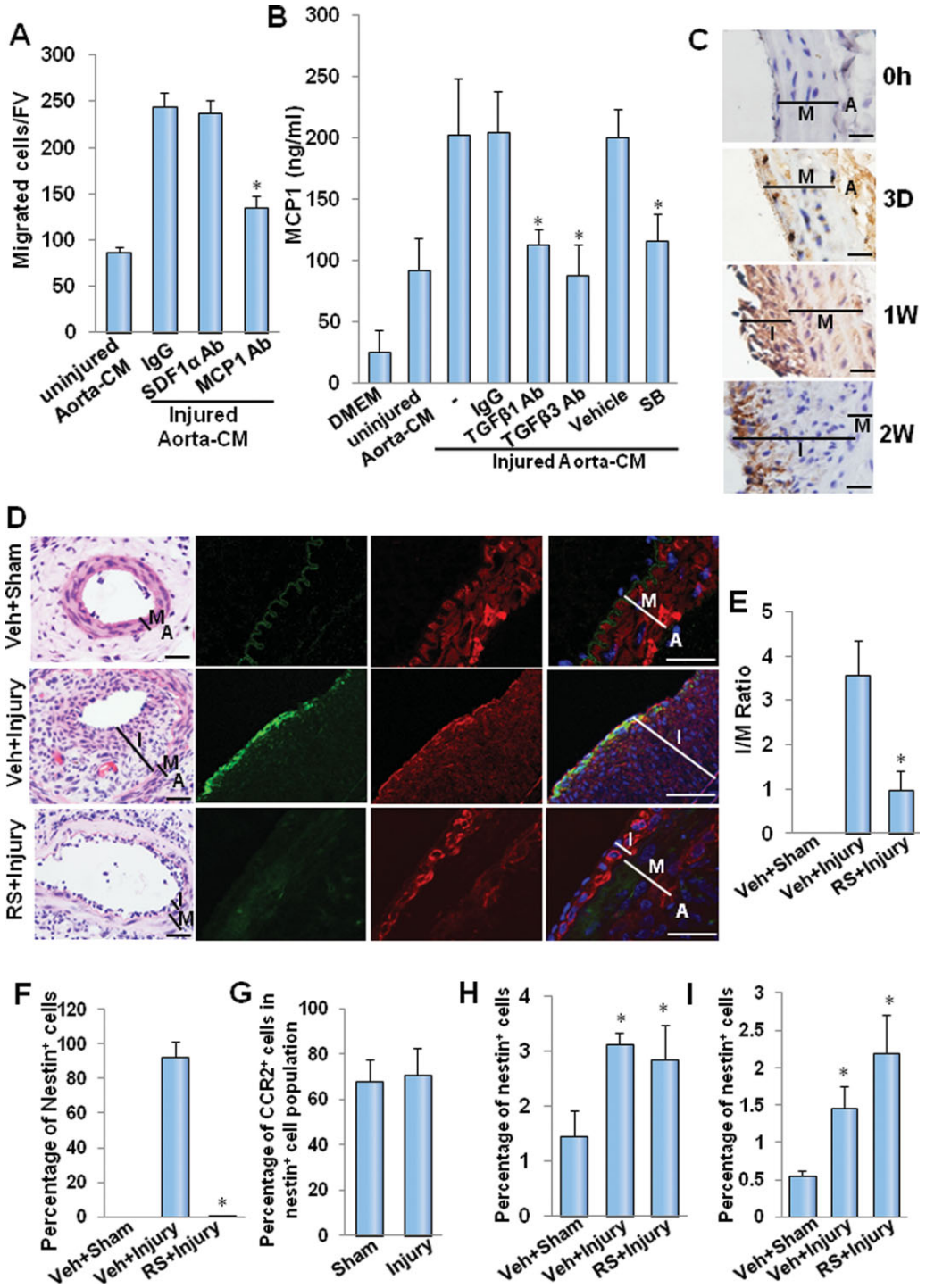

Figure 7. MCP-1-mediated TGFβ-induced migration and homing of MSCs to the injured arteries without affecting the mobilization of the cells into circulating blood. **(A):** Neutralizing MCP-1 inhibited TGFβ-induced MSCs migration. Transwell assay for migration of MSCs using injured Aorta-CM with addition of neutralizing antibodies (Ab) against SDF1α or MCP-1. CM added with IgG-treated cells served as control. Results are mean $\pm$ SD, $n = 4$, *, $p < .01$ versus uninjured Aorta-CM control. **(B):** MCP-1 levels in Aorta-CM measured by ELISA. CM was prepared by incubating the injured aorta in the culture media with addition of control IgG, anti-TGFβ1, or anti-TGFβ3 neutralizing antibodies or TβRI inhibitor SB505124 (SB), or vehicle for 48 hours. Results are the mean $\pm$ SD, $n = 4$, *, $p < .01$ versus IgG or vehicle controls. **(C):** Increased MCP1 expression in balloon-injured carotid arteries in rats. Immunohistochemical staining of MCP1 (brown) in rat carotid arteries at 3 days, 1 week, and 3 weeks postinjury. 0-hour sample was uninjured control. Scale bars = 50 μm. **(D, E):** Selective MCP-1 antagonist RS504393 (RS) inhibited neointima formation in injured femoral arteries in mice. Left column in (D): H&E staining of injured or uninjured (Sham) femoral arteries of mice treated with RS or vehicle 2 weeks postinjury. RS (2 mg/kg-b.wt.) or vehicle was injected subcutaneously once a day, starting 3 days before vascular injury and continued for 2 weeks when arteries were harvested. Scale bars = 100 μm. Right columns in (D): Triple-immunofluorescence staining was performed for nestin (green), α-SMA (red), and DAPI (blue) in cross-sections of femoral arteries 2 weeks postinjury. Scale bars: 50 μm. (E): The I/M ratios of (D). Results are mean $\pm$ SD, $n = 4$ mice per group, *, $p < .001$ versus Veh+Injury group. **(F):** Quantification of the number of nestin$^+$ cells in (D). Results are mean $\pm$ SD, $n = 4$ mice per group, *, $p < .001$ versus Veh+Injury group. **(G):** Calculation of the percentage of CCR2$^+$ cells out of nestin$^+$ cells in peripheral blood from sham control and arterial injured mice. Results mean $\pm$ SD, $n = 4$ mice per group per time point. **(H, I):** Flow cytometrical analysis of the number of nestin$^+$ cells in peripheral blood at (H) 3 days or (I) 2 weeks after femoral arterial injury or sham surgery in RS- or vehicle-treated mice. Results are mean $\pm$ SD, $n = 4$ mice per group per time point, *, $p < .01$ versus Veh+Sham groups. Abbreviations: A, adventitia layer; Aorta-CM, aorta-conditioned medium; FV, field of view; I, intima layer; M, media smooth muscle layer; I/M ratio, ratios of intima/media area; MCP1, monocyte chemotactic protein; SDF, stromal cell-derived factor. DAPI, 4′,6-diamidino-2-phenylindole, dihydrochloride.

derived from nestin$^+$ cells, indicating that other cell types are also involved in neointima formation. It was reported that local stem cells in surrounding tissues such as vascular adventitia may also migrate to the injured sites [43, 70, 71]. Thus, it is likely that promigratory factors other than TGFβ1 stimulate the migration of local resident stem cells and/or smooth muscle cells from the media layer to the injured sites to participate in vascular remodeling. The identity of these cell types and their origins remain to be defined.

MCP-1, a local chemoattractant stimulated by TGFβ in the vasculature, acts downstream in the cascade of TGFβ signaling to enhance the recruitment of MSCs (Supporting Information Fig. S6). However, the antagonist of CCR2 did not change the increased number of MSCs in blood circulation, indicating that MCP1 is not involved in mobilizing MSCs from their bone marrow niche to peripheral blood. As the spectrum of promigratory factors downstream of TGFβ in different tissue context may vary, deeper insight is needed into the downstream events by which TGFβ mobilizes MSCs from their original niches such as bone marrow at various biological conditions. Interaction between SDF-1α/CXCL12 and its receptor CCR4 plays a key role in the mobilization of vascular stem/progenitor cells including HSCs and EPCs during the process of tissue repair or regeneration [14, 15]. Even though we found that SDF-1α/CXCL12 was not involved in the migration and homing of MSCs to the injured vessels, whether it mediates TGFβ1-induced MSC mobilization from bone marrow to peripheral blood is an interesting topic of future study. The results demonstrated in this study reveal a new mechanism for TGFβ1-involved vascular repair/remodeling. TGFβs play a crucial role in the vascular remodeling in many cardiovascular diseases. Whether TGFβ-recruited MSCs is a major contributor to the pathogenesis of these vascular disorders remain to be investigated. Furthermore, transplanted MSCs participate in tissue regeneration upon injury events such as myocardial infarction and vascular injuries in animal studies, and injection of MSCs has therapeutic benefits in the treatment of ischemic heart diseases and postmyocardial infarction clinically. It is interesting to determine whether TGFβ-recruited MSCs is a key step for tissue regeneration.

Conclusion

We demonstrate that TGFβ, activated and released from vascular matrix in response to injury, is essential for the recruitment of MSCs to the injured sites to participate in vascular remodeling by inducing MCP1 production. We also reveal that blood active TGFβ1 acts as a systemic mobilizer for MSCs from bone marrow to the peripheral blood, where sufficient MSCs are available for the subsequent migration and homing to the remodeling sites. The finding provides novel mechanisms for TGFβ-involved pathological conditions such as vascular remodeling, fibrosis and wound healing and may shed light on the improvement of cell mobilization and homing in MSC-based therapy.

Acknowledgments

We gratefully thank Dr. Kathleen Flanders for providing us the active TGFβ1 antibody LC (1-30). This work was supported by the National Institutes of Health DK083350 (to M.W.) and AR053973 (to X. C).

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

References

1 Ferrari G, Cusella-De Angelis G, Coletta M et al. Muscle regeneration by bone marrow-derived myogenic progenitors. Science 1998;279: 1528–1530.
2 Takahashi T, Kalka T, Masuda H et al. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med 1999;5:434–438.
3 Lagasse E, Connors H, Al-Dhalimy M et al. Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. Nat Med 2000;6: 1229–1234.
4 Orlic D, Kajstura J, Chimenti S et al. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Proc Natl Acad Sci USA 2001;98:10344–10349.
5 Kale S, Karihaloo A, Clark PR et al. Bone marrow stem cells contribute to repair of the ischemically injured renal tubule. J Clin Invest 2003;112:42–49.
6 Orlic D et al. Bone marrow cells regenerate infarcted myocardium. Nature 2001;410:701–705.
7 Ortiz LA, Gambelli F, McBride C et al. Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. Proc Natl Acad Sci USA 2003; 100:8407–8411.
8 Sato Y, Araki H, Kato J et al. Human mesenchymal stem cells xenografted directly to rat liver are differentiated into human hepatocytes without fusion. Blood 2005;106:756–763.
9 Rochefort GY, Delorme B, Lopez A et al. Multipotential mesenchymal stem cells are mobilized into peripheral blood by hypoxia. Stem Cells 2006;24:2202–2208.
10 Oh JY, Kim MK, Shin MS et al. The anti-inflammatory and anti-angiogenic role of mesenchymal stem cells in corneal wound healing following chemical injury. Stem Cells 2008;26:1047–1055.
11 Sasaki M, Abe R, Fujita Y et al. Mesenchymal stem cells are recruited into wounded skin and contribute to wound repair by transdifferentiation into multiple skin cell type. J Immunol 2008;180: 2581–2587.
12 Hong HS, Lee J, Lee E et al. A new role of substance P as an injury-inducible messenger for mobilization of CD29(+) stromal-like cells. Nat Med 2009;15:425–435.
13 Quevedo HC, Hatzistergos KE, Oskouei BN et al. Allogeneic mesenchymal stem cells restore cardiac function in chronic ischemic cardiomyopathy via trilineage differentiating capacity. Proc Natl Acad Sci USA 2009;106:14022–14027.
14 Wojakowski W, Landmesser U, Bachowski R et al. Mobilization of stem and progenitor cells in cardiovascular diseases. Leukemia 2012; 36:23–33.
15 Krankel N, Spinetti G, Amadesi S e al. Targeting stem cell niches and trafficking for cardiovascular therapy. Pharmacol Ther 2011;129: 62–81.
16 Schraufstatter IU, Discipio RG, Khaldoyanidi S. Mesenchymal stem cells and their microenvironment. Front Biosci 2011;17:2271–2288.
17 Caplan AI, Correa D. The MSC: An injury drugstore. Cell Stem Cell 2011;9:11–15.
18 Kanzaki T, Shiina R, Saito Y et al. Role of latent TGF-beta 1 binding protein in vascular remodeling. Biochem Biophys Res Commun 1998; 246:26–30.
19 Munger JS, Harpel JG, Gleizes PE et al. Latent transforming growth factor-beta: Structural features and mechanisms of activation. Kidney Int 1997;51:1376–1382.
20 Annes JP, Munger JS, Rifkin DB. Making sense of latent TGFbeta activation. J Cell Sci 2003;116:217–224.
21 Tang Y, Wu X, Lei W et al. TGF-beta1-induced migration of bone mesenchymal stem cells couples bone resorption with formation. Nat Med 2009;15:757–765.
22 Pittet JF, Griffiths MJ, Geiser T et al. TGF-beta is a critical mediator of acute lung injury. J Clin Invest 2001;107:1537–1544.

23 Sinha S, Heagerty AM, Shuttleworth CA et al. Expression of latent TGF-beta binding proteins and association with TGF-beta 1 and fibrillin-1 following arterial injury. Cardiovasc Res 2002;53:971–983.
24 Kim KK, Kugler MC, Wolters PJ et al. Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix. Proc Natl Acad Sci USA 2006; 103:13180–13185.
25 Ahamed J, Burg N, Yoshinaga K et al. In vitro and in vivo evidence for shear-induced activation of latent transforming growth factor-beta1. Blood 2008;112:3650–3660.
26 Brunner G, Blakytny R. Extracellular regulation of TGF-beta activity in wound repair: Growth factor latency as a sensor mechanism for injury. Thromb Haemost 2004;92:253–261.
27 Qi YX, Jiang J, Jiang XH et al. PDGF-BB and TGF-β1 on cross-talk between endothelial and smooth muscle cells in vascular remodeling induced by low shear stress. Proc Natl Acad Sci USA 2011;108: 1908–1913.
28 Ohno M, Cooke JP, Dzau VJ et al. Fluid shear stress induces endothelial transforming growth factor beta-1 transcription and production. Modulation by potassium channel blockade. J Clin Invest 1995;95: 1363–1369.
29 Grainger DJ. Transforming growth factor beta and atherosclerosis: So far, so good for the protective cytokine hypothesis. Arterioscler Thromb Vasc Biol 2004;24:399–404.
30 Ryan ST, Koteliansky VE, Gotwals PJ et al. Transforming growth factor-beta-dependent events in vascular remodeling following arterial injury. J Vasc Res 2003;40:37–46.
31 Asahara T, Murohara T, Sullivan A et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997;275:964–967.
32 Shi Q, Rafii S, Wu MH et al. Evidence for circulating bone marrow-derived endothelial cells. Blood 1998;92:362–367.
33 Sata M, Saiura A, Kunisato A et al. Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. Nat Med 2002;8:403–409.
34 Sata M, Fukuda D, Tanaka K et al. The role of circulating precursors in vascular repair and lesion formation. J Cell Mol Med 2005;9:557–568.
35 Massberg S, Konrad I, Schürzinger K et al. Platelets secrete stromal cell-derived factor 1alpha and recruit bone marrow-derived progenitor cells to arterial thrombi in vivo. J Exp Med 2006;203:1221–1233.
36 Wang CH, Anderson N, Li SH et al. Stem cell factor deficiency is vasculoprotective: Unraveling a new therapeutic potential of imatinib mesylate. Circ Res 2006;99:617–625.
37 Simper D, Stalboerger PG, Panetta CJ et al. Smooth muscle progenitor cells in human blood. Circulation 2002;106:1199–1204.
38 Sartore S, Chiavegato A, Faggin E et al. Contribution of adventitial fibroblasts to neointima formation and vascular remodeling: From innocent bystander to active participant. Circ Res 2001;89:1111–1121.
39 Li G, Chen SJ, Oparil S et al. Direct in vivo evidence demonstrating neointimal migration of adventitial fibroblasts after balloon injury of rat carotid arteries. Circulation 2000;101:1362–1365.
40 Hu Y, Davison F, Ludewig B et al. Smooth muscle cells in transplant atherosclerotic lesions are originated from recipients, but not bone marrow progenitor cells. Circulation 2002;106:1834–1839.
41 Hu Y, Zhang Z, Torsney E et al. Abundant progenitor cells in the adventitia contribute to atherosclerosis of vein grafts in ApoE-deficient mice. J Clin Invest 2004;113:1258–1265.
42 Passman JN, Dong XR, Wu SP et al. A sonic hedgehog signaling domain in the arterial adventitia supports resident Sca1+ smooth muscle progenitor cells. Proc Natl Acad Sci USA 2008;105:9349–9354.
43 Klein D, Hohn HP, Kleff V et al. Vascular wall-resident stem cells. Histol Histopathol 2010;25:681–689.
44 Xing D, Feng W, Nöt LG et al. Increased protein O-GlcNAc modification inhibits inflammatory and neointimal responses to acute endoluminal arterial injury. Am J Physiol Heart Circ Physiol2058;295: H335–H342.
45 Sata M, Maejima Y, Adachi F et al. A mouse model of vascular injury that induces rapid onset of medial cell apoptosis followed by reproducible neointimal hyperplasia. J Mol Cell Cardiol 2000;32: 2097–2104.
46 Kimura M, Sudhir K, Jones M et al. Impaired acetylcholine-induced release of nitric oxide in the aorta of male aromatase-knockout mice: Regulation of nitric oxide production by endogenous sex hormones in males. Circ Res 2003;93:1267–1271.
47 Belema-Bedada F, Uchida S, Martire A et al. Efficient homing of multipotent adult mesenchymal stem cells depends on FROUNT-mediated clustering of CCR2. Cell Stem Cell 2008;2:566–575.
48 Gao D, Nolan DJ, Mellick AS et al. Endothelial progenitor cells control the angiogenic switch in mouse lung metastasis. Science 2008; 319:195–198.
49 Flanders KC, Thompson NL, Cissel DS et al. Transforming growth factor-beta 1: Histochemical localization with antibodies to different epitopes. J Cell Biol 1989;108:653–660.
50 Williams AO, Flanders KC, Saffiotti U. Immunohistochemical localization of transforming growth factor-beta 1 in rats with experimental silicosis, alveolar type II hyperplasia, and lung cancer. Am J Pathol 1993;142:1831–1840.
51 Barcellos-Hoff MH, Ehrhart EJ, Kalia M et al. Immunohistochemical detection of active transforming growth factor-beta in situ using engineered tissue. Am J Pathol 1995;147:1228–1237.
52 Wan M, Yang C, Li J et al. Parathyroid hormone signaling through low-density lipoprotein-related protein 6. Genes Dev 2008;22:2968–2979.
53 Mendez-Ferrer S, Michurina TV, Ferraro F et al. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature 2010;466:829–834.
54 Li MO, Flavell RA. Contextual regulation of inflammation: A duet by transforming growth factor-beta and interleukin-10. Immunity28;2008: 468–476.
55 Zhang F, Tsai S, Kato K et al. Transforming growth factor-beta promotes recruitment of bone marrow cells and bone marrow-derived mesenchymal stem cells through stimulation of MCP-1 production in vascular smooth muscle cells. J Biol Chem 2009;284:17564–17574.
56 Schober A, Knarren S, Lietz M et al. Crucial role of stromal cell-derived factor-1alpha in neointima formation after vascular injury in apolipoprotein E-deficient mice. Circulation 2003;108:2491–2497.
57 Schober A, Zernecke A, Liehn EA et al. Crucial role of the CCL2/CCR2 axis in neointimal hyperplasia after arterial injury in hyperlipidemic mice involves early monocyte recruitment and CCL2 presentation on platelets. Circ Res 2004;95:1125–1133.
58 Subramanian P, Karshovska E, Reinhard P et al. Lysophosphatidic acid receptors LPA1 and LPA3 promote CXCL12-mediated smooth muscle progenitor cell recruitment in neointima formation. Circ Res 2010;107:96–105.
59 Nemenoff RA, Simpson PA, Furgeson SB et al. Targeted deletion of PTEN in smooth muscle cells results in vascular remodeling and recruitment of progenitor cells through induction of stromal cell-derived factor-1alpha. Circ Res 2008;102:1036–1045.
60 Wipff PJ, Hinz B. Integrins and the activation of latent transforming growth factor beta1—An intimate relationship. Eur J Cell Biol 2008; 87:601–615.
61 Tenney RM, Discher DE. Stem cells, microenvironment mechanics, and growth factor activation. Curr Opin Cell Biol 2009;21:630–635.
62 Worthington JJ, Klementowicz JE, Travis MA. TGFbeta: A sleeping giant awoken by integrins. Trends Biochem Sci 2011;36:47–54.
63 Nishimura SL. Integrin-mediated transforming growth factor-beta activation, a potential therapeutic target in fibrogenic disorders. Am J Pathol 2009;175:1362–1370.
64 Hinz B, Gabbiani G. Fibrosis: Recent advances in myofibroblast biology and new therapeutic perspectives. F1000. Biol Rep 2010;2:78.
65 Janssens K, Gershoni–Baruch R, Guañabens N et al. Mutations in the gene encoding the latency-associated peptide of TGF-beta 1 cause Camurati-Engelmann disease. Nat Genet 2000;26:273–275.
66 Janssens K, ten DP, Ralston SH et al. Transforming growth factor-beta 1 mutations in Camurati-Engelmann disease lead to increased signaling by altering either activation or secretion of the mutant protein. J Biol Chem 2003;278:7718–7724.
67 Neptune ER, Frischmeyer PA, Arking DE et al. Dysregulation of TGF-beta activation contributes to pathogenesis in Marfan syndrome. Nat Genet 2003;33:407–411.
68 Avvedimento EV, Gabrielli A. Stiff and tight skin: A rear window into fibrosis without inflammation. Sci Transl Med 2010;2:23ps13.
69 Nacher M, Hidalgo A. Fire within the vessels: Interactions between blood cells and inflammatory vascular injury. Front Biosci (Schol Ed) 2011;3:1089–1100.
70 Psaltis PJ, Harbuzariu A, Delacroix S et al. Resident vascular progenitor cells—Diverse origins, phenotype, and function. J Cardiovasc Transl Res 2011;4:161–176.
71 Zengin E, Chalajour F, Gehling UM et al. Vascular wall resident progenitor cells: A source for postnatal vasculogenesis. Development 2006;133:1543–1551.

See www.StemCells.com for supporting information available online.