



Published in final edited form as:

Stem Cells. 2012 November ; 30(11): 2498–2511. doi:10.1002/stem.1208.

Injury-Activated TGF β Controls Mobilization of MSCs for Tissue Remodeling

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Abstract

Upon secretion, transforming growth factor (TGF) β is maintained in a sequestered state in extracellular matrix as a latent form. The latent TGF β is considered as a molecular sensor that releases active TGF β 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 TGF β storage in the matrix and its activation is obscure. Here, using several animal models in which latent TGF β is activated in vascular matrix in response to injury of arteries, we show that active TGF β 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 reendothelialization and myofibroblastic cells to form thick neointima. TGF β were activated in the vascular matrix in both rat and mouse models of mechanical injury of arteries. Importantly, the active TGF β released from the injured vessels is essential to induce the migration of MSCs, and cascade expression of monocyte chemotactic protein-1 (MCP-1) stimulated by TGF β amplifies the signal for migration. Moreover, sustained

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

high levels of active TGF β 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 TGF β 1 in uninjured mice rapidly mobilized MSCs into circulation. Further, inhibitor of TGF β type I receptor (T β RI) blocked the mobilization and recruitment of MSCs to the injured arteries. Thus, TGF β is an injury-activated messenger essential for the mobilization and recruitment of MSCs to participate in tissue repair/remodeling.

Keywords

TGF β activation; mesenchymal stem cells; cell mobilization; vascular remodeling

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 remodeling¹⁻⁵. 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 tissues⁶⁻¹³. It is believed that pro-migratory 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 sites¹⁴⁻¹⁷. 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 (CSFs), stromal cell-derived factor-1 (SDF-1), vascular endothelial growth factor (VEGF), erythropoietin, angiopoietin-2, fibroblast growth factor (FGF), stem cell factor (SCF), and interleukin 8 (IL-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 (TGF β), consists of three isoforms: TGF β 1, 2, and 3, regulates a broad spectrum of biological processes. Excessive TGF β activation is a major contributor to a variety of diseases including cancer, autoimmune diseases, vascular diseases and progressive fibrosis in multiple organs. TGF β s 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 TGF β for its action, a process called latent TGF β activation. Thus, the extracellular TGF β activity is primarily regulated by the conversion of latent TGF β to active TGF β , and latent TGF β is considered as a molecular sensor that response to specific signals by releasing active TGF β . These signals are often perturbations of the ECM that are associated with phenomena such as angiogenesis, wound repair, inflammation, and cell growth²⁰. We previously demonstrated that active TGF β 1 released from bone matrix induces MSCs migration for bone remodeling²¹. The fact that TGF β activation occurs during tissue injury or under other stressful conditions²²⁻²⁶ suggests that active TGF β may act as a pro-migratory 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 TGF β ^{25,27,28}. TGF β s are important modulators of vascular remodeling in

diseases such as atherosclerosis, restenosis and hypertension. Importantly, TGF β 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 TGF β 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 the present study, using a rat model of balloon-induced injury of carotid artery⁴⁴ and a mouse model of wire-induced injury of femoral artery⁴⁵, 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 TGF β 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 TGF β 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. Nestin-cre and C57BL/6J-Gtosa26 tm1EYFP mice were purchased from Jackson Lab. 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, 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 previously⁴⁶. Each segment of injured aorta and the uninjured control were cultured in 24-well tissue culture plates with 600 μ l serum free DMEM at 37°C. Two days later, the conditioned media were collected and stored at –80°C. In some experiments, neutralizing antibodies against TGF β 1, TGF β 2, TGF β 3, SDF-1 or MCP-1 at 500 ng/ml or T β RI inhibitor (SB-505124, 2 μ M) were individually added to the conditioned media. Cell migration was assessed in 96-well Transwells (Corning, Inc.) as described previously²¹. The 8 μ m pore membrane between the upper and lower chambers was pre-coated with 0.5 μ g/ml type I collagen (BD Biosciences). 1×10^4 MSCs in 50 μ l serum free DMEM were plated in the upper chambers and 150 μ l undiluted conditioned media from the cultured aortae were added to the lower chambers. After 10 hrs-incubation, cells were fixed with 10% formaldehyde for 4 hrs, and then the MSCs 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). Five fields at 20X 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 previously⁴⁴. 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) 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 previously⁴⁵. 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) was inserted toward the iliac artery to reach 5-mm distance from the incision. The wire was left in place for 1 min 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 TGF β 1 signaling or MCP-1 signaling gradient *in vivo*, some mice were pretreated with T β RI inhibitor (SB-505124, 5 mg/kg, *i.p.* injection), a MCP-1-specific antagonist (RS 504393, 2 mg/kg, *s.c.*) or vehicle (50% 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 wks post surgery, mice were euthanized by *i.p.* administration of ketamine/xylazine. Five hundred μ l peripheral blood from each mouse were collected by cardiac puncture, the blood or plasma were used for following studies. The femoral artery was excised, fixed with 4% paraformaldehyde, and embedded in paraffin. Thin sections (5 μ m) were used for histological and immunohistochemical analyses.

ELISA of TGF β 1, TGF β 3 and MCP-1 in Conditioned Media and Plasma

Levels of active and total TGF β 1 and TGF β 3 in conditioned media of aorta and plasma of wire-injured mice were determined using a DuoSet ELISA Development kit (R&D Systems). Levels of MCP-1 in conditioned media of aorta and plasma were determined using a Mouse MCP-1 ELISA MAX (Biolegend).

Analysis of Biotinylated TGF β 1-Bound Cells in Bone Marrow

Biotinylated TGF β 1 (60 ng/kg bw, R&D System) was injected into mouse via tail vein. Twenty four hrs later, bone marrow was collected by flushing 1 ml mesencult[®] MSC medium (Stem Cell Technologies) 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 TGF β 1 and Texas red for antibodies against nestin, Sca1, CD29, and CD11b.

Characterization of Human MSCs

Green fluorescence protein (GFP)-labeled human MSCs were purchased from Texas A&M, Institute for Regenerative Medicine. Cells were sorted by FACS using antibodies against CD29, Sca-1, CD45 and CD11b (Bio-Legend). The sorted cells were enriched, cultured and further confirmed by flow cytometry. For osteogenic differentiation, cells were seeded at a density of $5 \times 10^3/\text{cm}^2$ with α -MEM supplemented with 10% FBS, 0.1 μ M dexamethasone (Sigma-Aldrich), 10 mM β -glycerol phosphate (Sigma-Aldrich), and 50 μ M ascorbate-2-phosphate (Sigma-Aldrich). Cultures in α -MEM supplemented with 10% FBS served as a negative control. After 3 wks 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 $1 \times 10^4/\text{cm}^2$ with α -MEM supplemented with 10% FBS, 1 μ M dexamethasone, 0.5 μ M IBMX (Sigma-Aldrich), and 10

ng/ml of insulin (Sigma-Aldrich) for 2 wks. 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], 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 μ M dexamethasone, 1% ITS (Sigma-Aldrich), 50 μ M ascorbate-2-phosphate, 1 mM sodium pyruvate (Sigma-Aldrich), 50 μ g/ml of proline (Sigma-Aldrich), and 20 ng/ml of TGF- β 3 (R&D Systems). Culture cells in high-glucose D-MEM supplemented with 10% FBS served as a negative control. After 3 wks in culture, the pellets were fixed in 10% buffered formalin for 2 days and embedded in paraffin. Then 4- μ m-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), cells were washed with 0.1% BSA in PBS and then counted. 1×10^6 /ml cells were permeabilized in 0.1% Triton X-100 prior to blocking in 2.5% BSA for 1 hr, incubated with Alexa Fluor 647-conjugated Nestin antibody (BD, Pharmingen) or isotype control for 1 hr 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-Kit⁺Sca-1⁺Lin⁻ cells were considered as the primitive HSC fraction³³, and VE-cadherin⁺CD133⁺CD31⁺CD11b⁻ cells were considered as the EPC fraction⁴⁸. 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, PE-conjugated anti-Sca1, PE/Cy7-conjugated anti-CD29, PerCP-conjugated anti-CD45, and 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-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).

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 μ m 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 TGF β 1 expression, paraffin-embedded sections (5 μ m) were incubated with LC(1-30), a polyclonal antibody raised against the amino-terminal 30 amino acids of the mature TGF β 1 and specifically reacts with active TGF β 1⁴⁹⁻⁵¹. An HRP-streptavidin detection system (Dako) was used to detect the positive staining followed by counterstaining with hematoxylin (Dako). Double immunofluorescence staining was performed as described previously⁵². After blocking in 0.5% horse serum, sections were incubated with first antibodies (anti- α -SMA, anti-nestin or anti-Sca1, Abcam) followed by incubation with FITC, or Cy3-conjugated secondary antibodies (Jackson

ImmunoResearch). Nuclei were counterstained with DAPI (Sigma). The sections were mounted with the ProLong Antifade Kit (Molecular Probes) 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 artery⁴⁵, 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 post injury, and the elevation lasted for 2 wks (Fig. 1A). Bone marrow-derived nestin⁺ cells are MSC-enriched cell population⁵³. 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 artery⁴⁴ and mouse model of wire injury of femoral artery⁴⁵. Neointimal tissue was observed at 1 wk post injury, became much thicker at 2 wks post injury in rat carotid artery (Fig. 1C–1E) and in mouse femoral artery (Fig. S1A, and S1B). Neointima hyperplasia continued to grow up to 6 wks post injury until the re-endothelialization is completed^{44,45}. We examined the recruitment of the MSCs at 1 wk and 2 wks 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 (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 (α SMA)⁺ 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 wk and 2 wks, respectively, following injury (Fig. 2A and 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 reendothelialization of the injured vessels.

Approximately 19.0%±3.4% of nestin⁺ cells expressed CD31 at 2 wks following injury (Fig. 2C and 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-Gtosa26 tm1EYFP (R26-stop-EYFP) mice in which both nestin⁺ cells and their descendants at the neointimal tissue can be marked (Fig. S2). Approximately 57.0% and 44.6% of cells in all layers of neointimal tissue were EYFP⁺ cells at 1 wk and 2 wks, respectively, post injury, and almost none of the media smooth muscle cells were EYFP⁺ (Fig. 2D and 2E). Consistent with Fig. 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 reendothelialization 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 remodeling²¹. 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 wk and 2 wks post injury in both rats (Fig. S3A) and mice (Fig. S3B). In contrast, active TGFβ1 was undetectable in uninjured arteries. Active TGFβ1 levels in injury arteries detected by ELISA were elevated at 8 hr post injury, 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 injured arterial sites. We developed an aorta-conditioned medium (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 and 3E) compare to CM prepared from uninjured aorta. 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 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 (JNK inhibitor) in the injured Aorta-CM significantly suppressed the migration of MSCs, whereas U0126 (ERK inhibitor), SB202190 (p38 inhibitor) or Y27632 (RhoA inhibitor) had marginal effect (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 8-fold at 1 wk and 10-fold at 2 wks post injury (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 and 4C). In contrast, the numbers of HSCs and EPCs in peripheral blood remained unchanged post rhTGF β 1 injection (Fig. 4D and 4E). To examine the origin(s) of the mobilized MSCs, biotinylated rhTGF β 1 was injected i.v. and its binding to MSCs at different tissue was analyzed by measuring streptavidin-FITC-bound cells. Biotinylated rhTGF β 1 binds to bone marrow nestin⁺Sca1⁺CD29⁺CD11b⁻ cells (Fig. 5A and 5B). No specific biotinylated TGF β 1-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-negative (Fig. S5). The results suggest that the biotinylated TGF β 1 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 rhTGF β injection were nestin⁺ (Fig. 5C and 5D), indicating that 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). Further, 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 TGF β 1 is sufficient to mobilize MSCs from bone marrow to the peripheral blood.

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

To further validate the role of TGF β in MSCs mobilization, T β RI inhibitor SB-505124 (SB) was injected i.v. of mice with injury of femoral arteries. Blocking of T β RI almost abolished the increase in nestin⁺ cells in peripheral blood at 3 day and 2 wks post arterial injury (Fig. 6A and 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 TGF β is required for the recruitment of MSCs during vascular remodeling.

MCP-1 Mediates TGF β -Induced Migration and homing of MSCs to the Injured Arteries without Affecting the Mobilization of the Cells from Bone Marrow to Circulating Blood

MCP1⁵⁵ and CXCL12/SDF1 α 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 SDF1 α , 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~ 2 weeks following injury (Fig. 7C). TGF β has been shown to stimulate MCP1 expression in the vascular smooth muscle cells⁵⁵. We found that MCP1 production by the injured aorta was inhibited by selective T β RI kinase inhibitor SB505124 or neutralizing antibodies against TGF β 1 or TGF β 3 (Fig. 7B), indicating that TGF β s were primary factors that regulate MCP1 expression via a T β RI-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 CCR2, receptor of MCP1, the percentage of CCR2⁺ cells from sham control mice and injured mice are not significantly different (Fig. 7G). Further, the number of nestin⁺ MSCs in peripheral blood of the injured mice was not reduced with the treatment of MCP1 inhibitor (Fig. 7H and 7I).

These results suggest that MCP1/CCR2, a downstream target of TGF β in injured arteries, functions as a local chemoattractant responsible for the migration and homing of MSCs to the injured vessels.

DISCUSSION

Upon secretion, TGF β s 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 TGF β from binding to its receptor and subsequent action. Latent TGF β is considered as a molecular sensor that releases active TGF β s 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 TGF β 1 is activated in vascular tissue in response to injury, and active TGF β 1 stimulates the migration and homing of MSCs to the injured sites for vascular repair and remodeling. The specific inhibitor of TGF β type I receptor blocked the recruitment of the cells to form neointima at the remodeling sites. Previously, we have shown that TGF β activated from bone matrix during osteoclastic bone resorption induces migration of MSCs for normal bone remodeling²¹. Thus, TGF β may act as an injury/stress-activated messenger to recruit MSCs for tissue repair, regeneration, and pathological remodeling in various organs. Excessive TGF β activation is a major contributor to progressive fibrosis in many organs including lung, liver, kidney, heart, skin and arteries^{63,64}. Sustained active TGF β 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 TGF β leads to fibrotic bone formation^{21,65,66} and skin fibrosis^{67,68}, respectively. Thus, tempo-spatial regulation of TGF β 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 TGF β 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 TGF β 1 in peripheral blood are primarily account for the mobilization of MSCs from bone marrow to peripheral blood. Thus, we consider that TGF β 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 (Fig. S6). One of the sources of active TGF β in blood may be the injured vascular matrix. It is known that the extracellular matrix serves to concentrate latent TGF β at the sites of intended function to influence the bioavailability and/or function of TGF β activators^{18–20}. Indeed, in the injured vessels, we observed rapid and sustained elevation of active TGF β 1, which may diffuse into the blood circulation. Platelets and inflammatory cells are major players in the initial thromboinflammatory response following arterial injury⁶⁹. These cells within circulating blood or adhere to the injured tissue release large amount of active TGF β 1 into blood circulation^{25,54}, and may also contribute to the high active TGF β 1 level in peripheral blood after vascular injury. Our data indicate that bone marrow is a primary origin of MSCs mobilized by TGF β 1, but we could not exclude the possibility of the mobilization of MSCs from other niches even though we did not observe biotinylated TGF β -bound MSCs in several other tissues. MSCs seems a major cell type in bone marrow that is mobilized by TGF β 1 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 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 pro-migratory factors other than TGF β 1 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 TGF β in the vasculature, acts as a downstream in the cascade of TGF β signaling to enhance the recruitment of MSCs (Fig. 6S). 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 pro-migratory factors downstream of TGF β in different tissue context may vary, deeper insight is needed into the downstream events by which TGF β mobilizes MSCs from their original niches such as bone marrow at various biological conditions. Interaction between SDF-1 α /CXCL12 and its receptor CXCR4 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-1 α /CXCL12 was not involved in the migration and homing of MSCs to the injured vessels, whether it mediates TGF β 1-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 TGF β 1-involved vascular repair/remodeling. TGF β s play a crucial role in the vascular remodeling in many cardiovascular diseases. Whether TGF β -recruited MSCs is a major contributor to the pathogenesis of these vascular disorders remain to be investigated. Further, 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 TGF β -recruited MSCs is a key step for tissue regeneration.

CONCLUSION

We demonstrate that TGF β , 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 TGF β 1 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 TGF β -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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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

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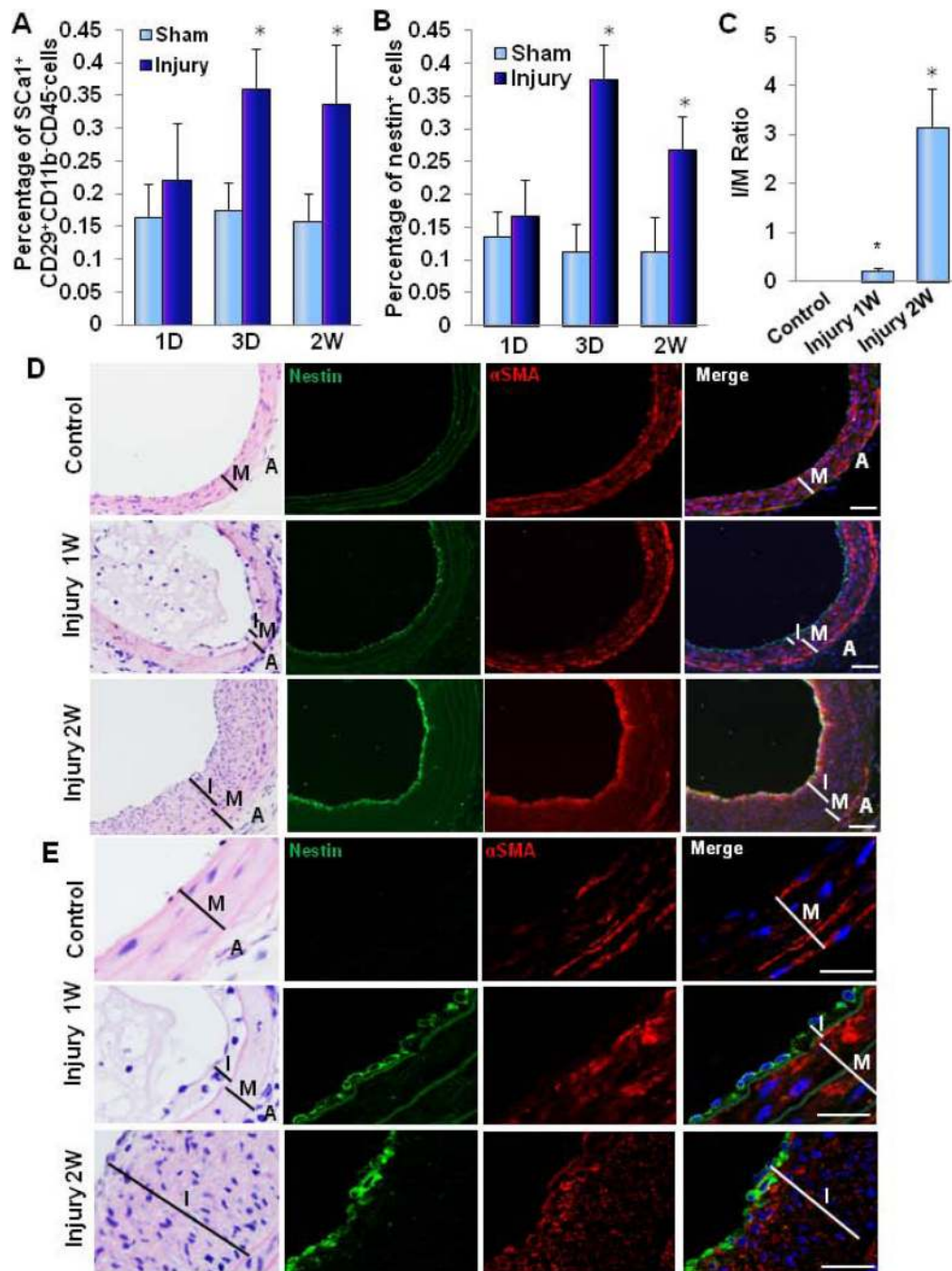


Figure 1.

MSCs were mobilized to peripheral blood and recruited to the remodeling arteries in response to vascular injury. (A and B) Percentages of Sca1⁺CD29⁺CD11b⁻CD45⁻ cells or nestin⁺ cells, respectively in peripheral blood at 1 day (1D), 3 days (3D) and 2 wks (2W) after femoral arterial injury or sham surgery in mice. Results are the means±SD, n=4 mice per group per time point. * p<0.05 vs respective Sham groups. (C) Calculation of the ratio of intima/media areas (I/M ratio) of rat carotid artery from uninjured artery (Control), and 1 wk (1W) or 2 wks (2W) after balloon injury. Results are the means±SD, n=4 mice per group per time point. * p<0.001 vs uninjured control group. (D and E) Representative H&E and

immunofluorescence images of tissue sections of rat carotid artery from uninjured artery (Control), and 1 wk (1W) or 2 wks (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, 1W or 2W post injury. Scale bars: 100 μ m. Left column of **(E)**: H&E staining; Right columns of **(E)**: Triple-immunofluorescence staining for nestin (green), α -SMA (red), and DAPI (blue) in carotid artery of uninjured control, 1W or 2W post injury. Scale bars: 50 μ m. I, intima layer; M, media smooth muscle layer; A, adventitia layer.

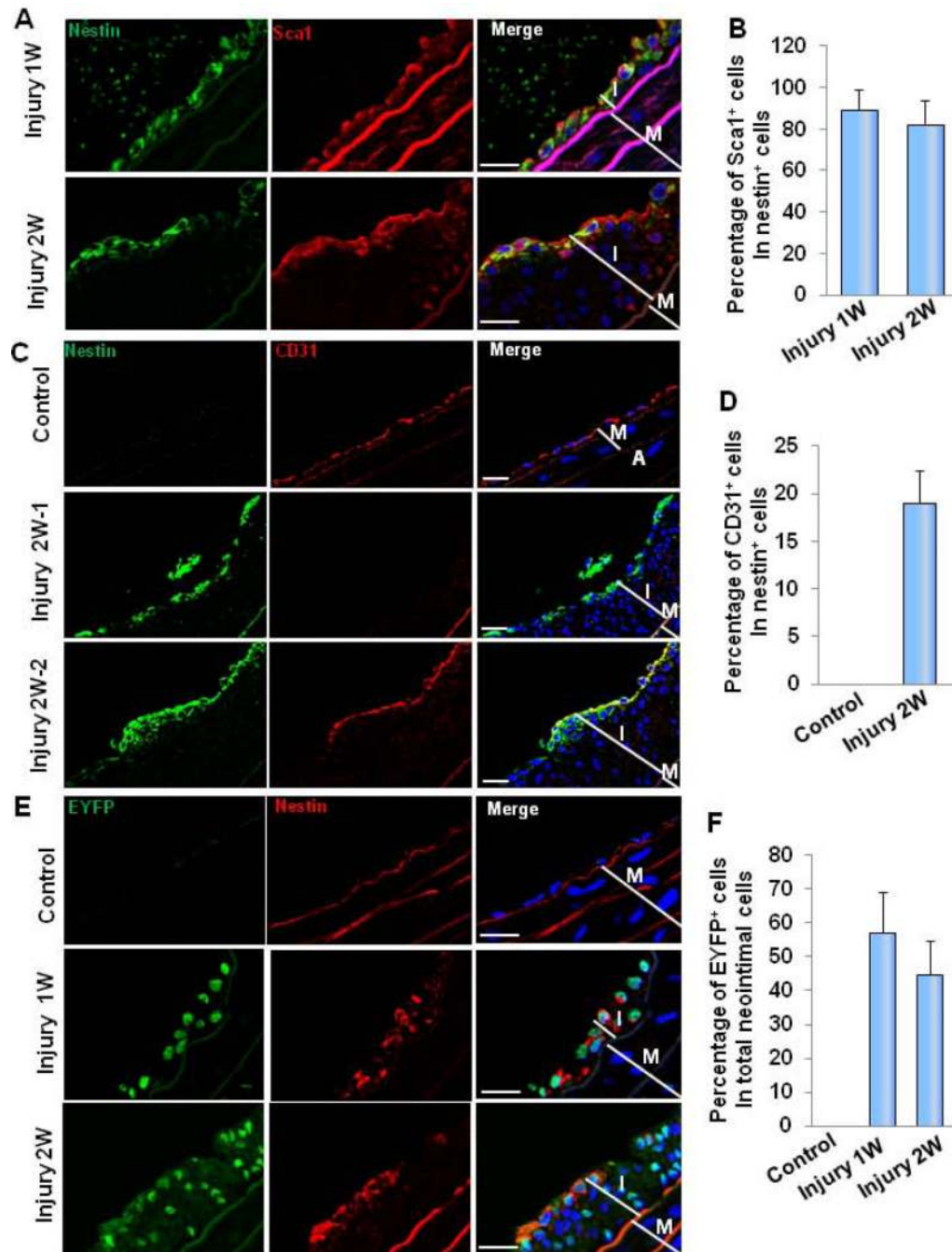


Figure 2.

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 1W or 2W post injury. Scale bars: 50 μ m. (B) Calculation of the percentages of Sca1⁺ cells out of nestin⁺ cells in the layer near to luminal side. Results are the means \pm 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 post injury (Injury 2W-1 & Injury 2W-2 indicate two different artery samples). Scale

bars: 50 μ m. **(D)** Calculation of the percentages of CD31⁺ cells out of nestin⁺ cells in the layer near to luminal side. Results are the means \pm 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 1W or 2W post injury in Nestin-cre and R26-stop-EYFP mice. Scale bars: 50 μ m. I, intima layer; M, media smooth muscle layer; A, adventitia layer. **(F)** Calculation of the percentages of EYFP⁺ cells out of total neointimal cells. Results are the means \pm SD, n=4 mice per group per time point.

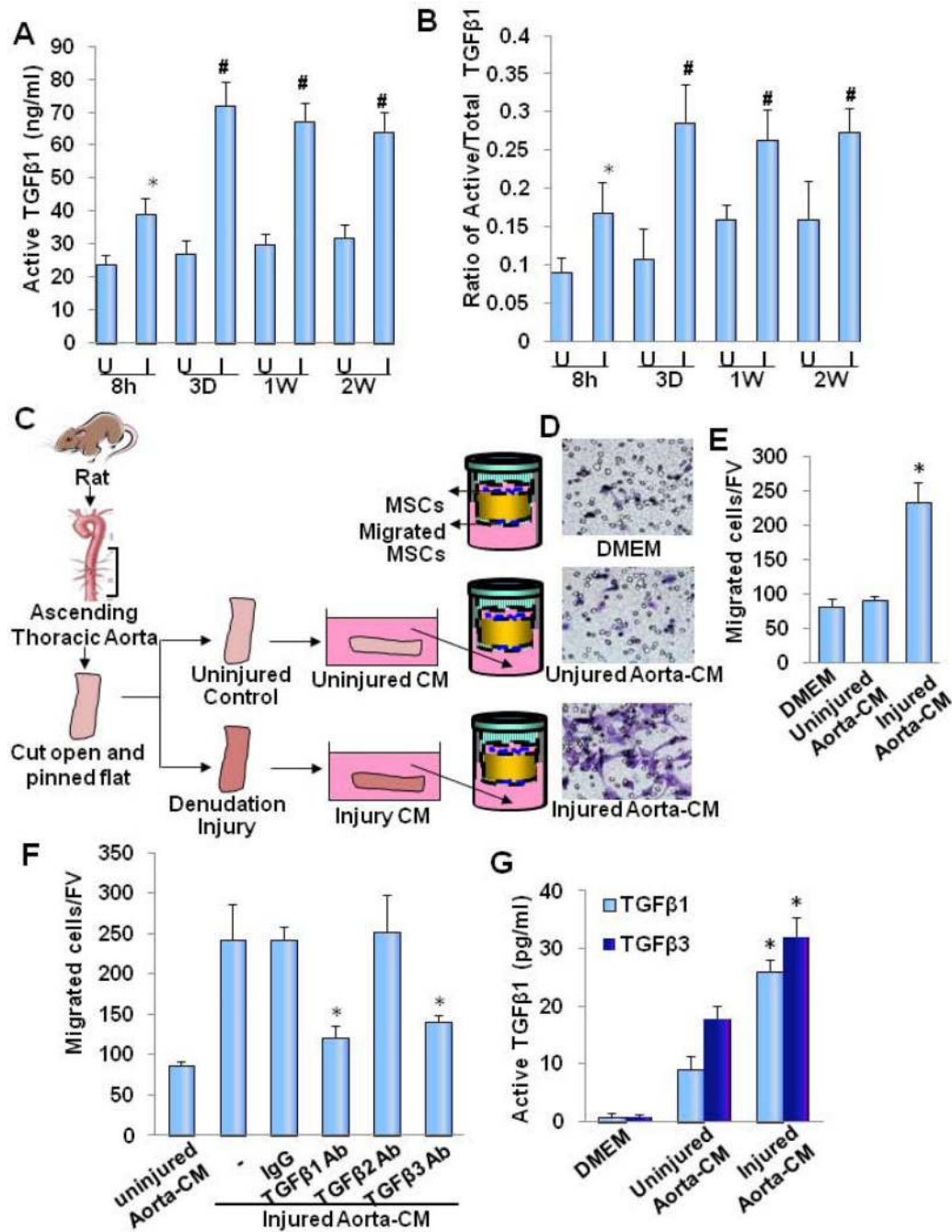


Figure 3.

Active TGFβ1 is released from injured arteries to recruit MSCs to the injured sites. (A) (A and B) ELISA analysis of active TGFβ1 levels and ratios of active/total TGFβ1, respectively, in wire-injured mouse femoral arteries at 8 hrs, 3 days, 1 wks and 3 wks post injury (I). 0 hr sample was uninjured control (U). Results are the means± SD, n=5 mice per group, * $p < 0.05$, # $p < 0.01$, vs 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-conditioned medium (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 hrs, 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 field of view (FV, magnification X20). Results are means±SD, n=4 preparation per group, *p<0.001 vs 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 20X). Results means±SD, n=4 per group, *p<0.01 vs. IgG control. **(G)** Active TGFβ1 and β3 levels measured by ELISA in CM prepared in **(D)**. Results means±SD, n=4 per group, *p<0.01 vs Uninjured Aorta-CM.

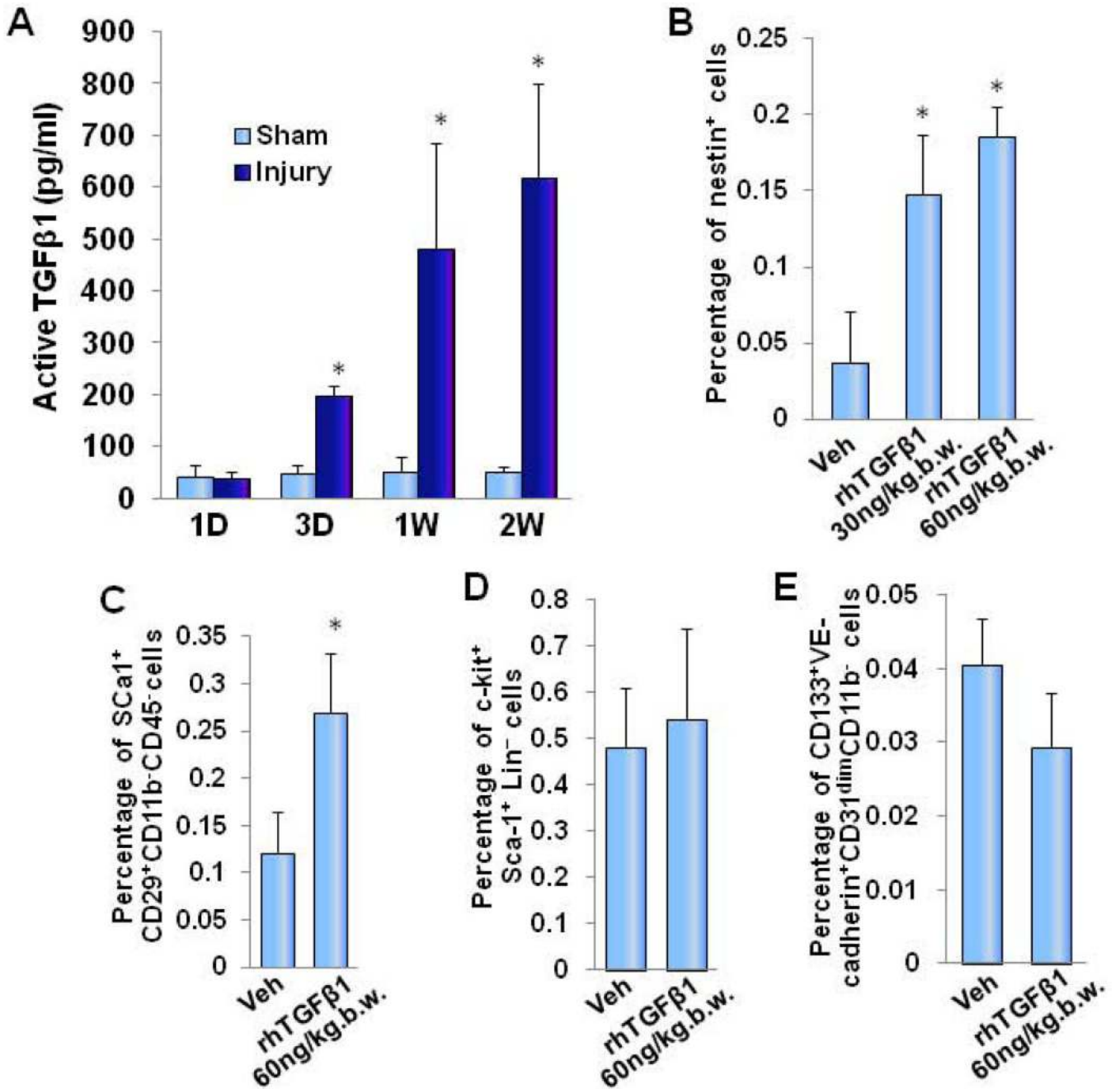


Figure 4.

Active TGFβ1 is sufficient to mobilize MSCs into peripheral blood. (A) Active TGFβ1 levels in peripheral blood of mouse 1 day, 3 days, 1 wk and 2 wks post femoral artery injury. Sham controls were mouse without injury. Results are the means±SD, n=5 mice per group, * p<0.01 vs respective Sham controls. (B–E) Mobilization of stem cells into peripheral blood by recombinant human TGFβ1 (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 means±SD, n= 4 mice per group, * p<0.01 vs. respective Veh groups.

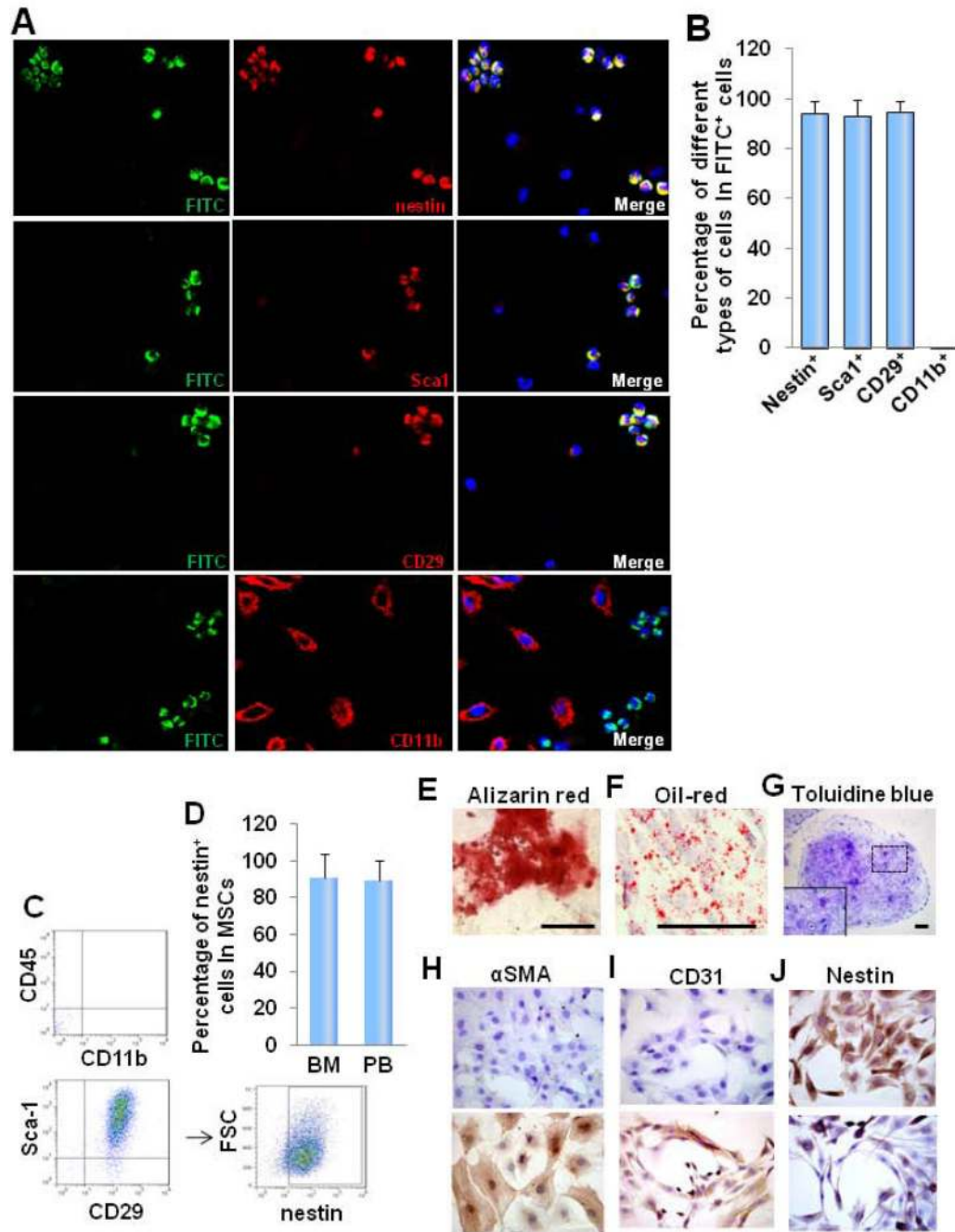


Figure 5.

TGFβ-mobilized cells are a subset of MSC lineage. (**A** and **B**) TGFβ1-bound cells in flushed mouse bone marrow at 24 hrs 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 means±SD calculated from 3 preparations of each mouse, n= 3 mice per group. (**C**) Sca-1⁺CD29⁺CD45⁻CD11b⁻ cells were sorted from murine bone marrow 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⁻CD11b⁻ cells in the bone marrow (BM) and peripheral blood (PB). Results are the means±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: 100µm. **(H and 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.

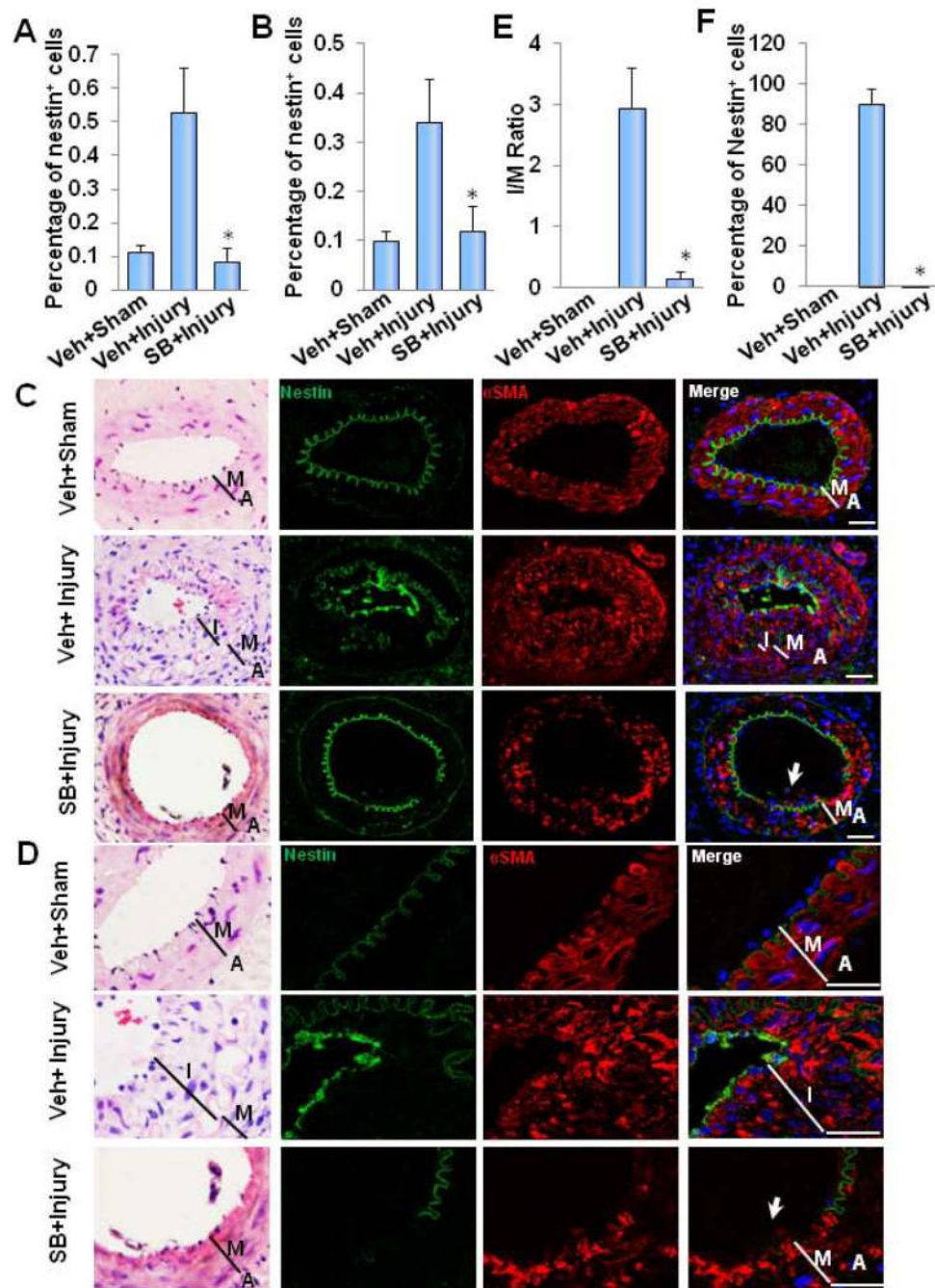


Figure 6. TGF β was required for the mobilization and recruitment of MSCs to the remodeling arteries in response to injury. (**A** and **B**) Injection of T β RI blocker SB-505124 (SB, 5 mg/kg-bw, i.p. daily) blocked mobilization of MSCs following arterial injury. Flow cytometrical quantitation of the numbers of nestin⁺ cells (% of total cell counted) in blood at (**A**) 3 days or (**B**) 2 wks after femoral arterial injury or sham surgery in either SB- or vehicle (Veh)-treated mice. Results are means \pm SD, n= 3 mice per group per time point, * p<0.001 vs. respective Veh+injury groups. (**C** and **D**) T β RI 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, 2W post injury, and Injury+SB mice. Scale bars: 100 μ m. 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, 2W post injury, and Injury+SB mice. Scale bars: 50 μ m. I, intima layer; M, media smooth muscle layer; A, adventitia layer. (E) Calculation the ratios of intima/media area (I/M Ratio) of uninjured (Sham) or injured femoral arteries of mice treated with SB or Veh at 2 wks post injury. Results are means \pm SD, n= 4 mice per group, *p<0.001 vs 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 means \pm SD, n=5 mice per group, *p<0.001 vs. Veh+Injury group.

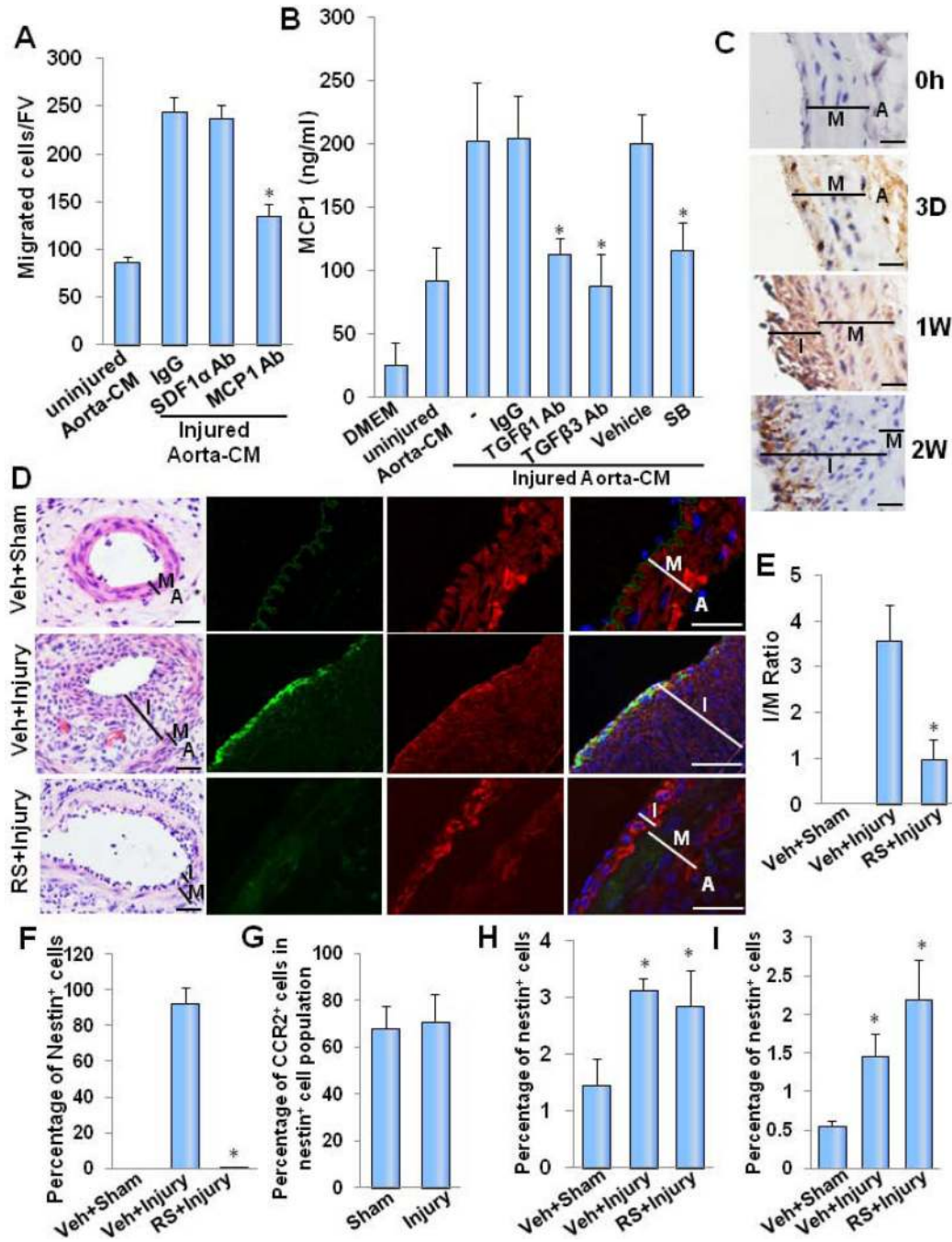


Figure 7. MCP-1 mediated TGFβ-induced migration and homing of MSCs to the injured arteries without affecting the mobilization of the cells into circulating blood. **(A)** Neutralizing MCP-1 inhibited TGFβ-induced MSCs migration. Transwell assay for migration of MSCs using injured Aorta-CM with addition of neutralizing antibodies (Ab) against SDF1α or MCP-1. CM added with IgG-treated cells served as control. Results are means±SD, n=4, * p<0.01 vs 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 hrs. Results are the means \pm SD, n=4, * p<0.01 vs. 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 wks and 3 wks post injury. 0 hr sample was uninjured control. Scale bars: 50 μ m. I, intima layer; M, media smooth muscle layer; A, adventitia layer. **(D and 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 wks post injury. RS (2 mg/kg-bw) or vehicle were injected subcutaneously once a day, starting 3 days before vascular injury and continued for 2 wks when arteries were harvested. Scale bars: 100 μ m. 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 wks post injury. Scale bars: 50 μ m. **(E)**: The I/M ratios of **(D)**. Results are means \pm SD, n=4 mice per group, * p<0.001 vs Veh+Injury group. **(F)** Quantification of the number of nestin⁺ cells in **(D)**. Results are means \pm SD, n=4 mice per group, * p<0.001 vs 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 means \pm SD, n=4 mice per group per time point. **(H and I)** Flow cytometrical analysis of the number of nestin⁺ cells in peripheral blood at **(H)** 3 days or **(I)** 2 wks after femoral arterial injury or sham surgery in RS- or vehicle- treated mice. Results means \pm SD, n=4 mice per group per time point, * p<0.01 vs Veh+Sham groups.