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IFATS Series: FGF-2-induced HGF Secretion By Adipose-Derived Stromal Cells Inhibits Post-Injury Fibrogenesis Through A JNK-Dependent Mechanism

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ABSTRACT

Background: Adipose-derived stem/stromal cells (ASCs) not only function as tissue-specific progenitor cells, but are also multipotent and secrete angiogenic growth factors such as hepatocyte growth factor (HGF) under certain circumstances. However, the biological role and regulatory mechanism of this secretion have not been well studied.

Methods and Results: We focused on the role of ASCs in the process of adipose tissue injury and repair, and found that among injury-associated growth factors, fibroblast growth factor-2 (FGF-2) strongly promoted ASC proliferation and HGF secretion through a c-Jun N-terminal kinase (JNK) signaling pathway. In a mouse model of ischemia-reperfusion injury of adipose tissue, regenerative changes following necrotic and apoptotic changes were seen for 2 weeks. Acute release of FGF-2 by injured adipose tissue was followed by upregulation of HGF. During the adipose tissue remodeling process, adipose-derived BrdU-positive cells were shown to be ASCs (CD31–CD34+). Inhibition of JNK signaling inhibited the activation of ASCs and delayed the remodeling process. In addition, inhibition of FGF-2 or JNK signaling prevented post-injury upregulation of HGF and led to increased fibrogenesis in the injured adipose tissue. Increased fibrogenesis also followed the administration of a neutralizing antibody against HGF.

Conclusions: FGF-2 released from injured tissue acts through a JNK signaling pathway to stimulate ASCs to proliferate and secrete HGF, contributing to the regeneration of adipose tissue and suppression of fibrogenesis after injury. This study revealed a functional role for ASCs in the response to injury and provides new insight into the therapeutic potential of ASCs.

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INTRODUCTION

Adipose-derived stem/stromal cells (ASCs) function as tissue-specific progenitor cells, can differentiate into cells of various lineages [1], and secrete many potent growth factors and cytokines such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) [2-4]. Paracrine effects of the secreted factors account for improved vascularity of ischemic hind limbs treated with ASCs [2,4]; however, ASC differentiation into endothelial cells could contribute to the improved vascularity as well [5,6]. The use of ASCs for promoting angiogenesis and tissue repair has gained interest as a potential therapeutic strategy [7-9] and clinical trials involving ASC-mediated enhancement of bone and adipose regeneration and angiogenesis are underway [10-12].

ASCs not only secrete potent growth factors and cytokines but they are also affected by them. Fibroblast growth factor-2 (FGF-2) stimulates the growth of ASCs [13, 14] and has been shown to promote adipogenic [15] and chondrogenic [13] differentiation of ASCs; however, FGF-2 inhibits their osteogenic differentiation [14]. Platelet-derived growth factor (PDGF) induces proliferation and migration of ASCs [16]. A culture medium containing VEGF, FGF-2, epidermal growth factor (EGF), and insulin-like growth factor-1 (IGF-1) markedly accelerated ASC proliferation and preserved their multipotency [17], suggesting synergism between these growth factors on ASC growth.

Mitogen-activated protein kinases (MAPKs) are important signal transducing enzymes that are involved in many facets of cellular regulation by growth factors [18]. Among MAPKs, c-Jun N-terminal kinases (JNK) play key roles in PDGF-induced proliferation and migration of ASCs [16]. There is also a study demonstrating that ASCs produce VEGF, HGF, and IGF-1 in response to tumor necrosis factor-α by a p38 MAPK-dependent mechanism [19].

In this study, we evaluated the influence of injury-associated growth factors on ASCs. In the process of injury and subsequent wound healing, various growth factors and inflammatory cytokines regulate regenerative cellular activities. We previously analyzed wound fluids after liposuction surgery and reported the sequential expression of growth factors: FGF-2 and PDGF were released in the early stage of wound healing, while VEGF and HGF were expressed in the later stage [20]. Administration of growth factors has been reported to enhance the regeneration of injured tissues, such as FGF-2 for burn [21] and HGF for myocardial infarction [22]. However, few studies have focused on the injury and repair process in adipose tissue and the potential role of ASCs, presumably because there is no good model of adipose tissue injury.

We hypothesized that ASCs stimulated by factors released from the injured tissue play an important role in the repair process of adipose tissue, perhaps by secreting regeneration-associated growth factors or by differentiating into adipocytes, endothelial cells, or other cell types. We examined the influence and mechanisms of injury-related factors on human and murine ASCs in vitro. Furthermore, using an original mouse model of ischemia-reperfusion injury to adipose tissue, we assessed the expression of injury-related growth factors at the cellular and molecular level in order to elucidate the role of ASCs during the regeneration process.

MATERIALS AND METHODS

Cell isolation and culture

Liposuction aspirates were obtained from healthy female donors (mean age, 35.4 ± 3.3; mean BMI, 22.1 ± 1.2, n = 9) undergoing liposuction of the abdomen or thighs. Each patient provided their informed consent using an institutional review board-approved protocol prior to the procedure. ASCs were isolated from the aspirated fat as described previously [23]. Briefly, the aspirated fat was washed with phosphate buffered saline (PBS) and digested on a shaker at 37ºC in PBS

containing 0.075% collagenase for 30 min. Mature adipocytes and connective tissue were separated from pellets by centrifugation (800 × g, 10 min). The cell pellets were resuspended, filtered through 100-μm mesh, plated at a density of 5×10^5 nucleated cells/100-mm dish, and cultured at 37ºC in an atmosphere of 5% CO_2 in humid air. The culture medium was Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Primary cells were cultured for 7 days and were defined as "Passage 0". The medium was replaced every 3 days. Cells were passaged every week by trypsinization. Human ASCs at Passage 1 to 3 were used in the experiments. To separate mouse ASCs, adipose tissues were obtained from inguinal fat pads of 6-week-old male ICR mice. The adipose tissues were minced into 2-3-mm pieces and processed as described above. Mouse ASCs at Passage 1 to 3 were used in the experiments. Human dermal fibroblasts (hDFs) were obtained from explant cultures of skin samples from separate donors; hDFs at Passage 3 to 5 were used in the experiments. Human mesenchymal stem cells from bone marrow (hBM-MSCs), frozen at Passage 2, were purchased from Cambrex (Walkersville, MD); hBM-MSCs at Passage 3 to 5 were used in the experiments.

Proliferation assay

Human ASCs were plated in a 6-well plate at 2×10^4 cells/well. Each injury-associated growth factor (VEGF, FGF-2, HGF, and PDGF; all from Wako Pure Chemicals, Osaka, Japan) was added to the control medium (DMEM and 10% FBS) at concentrations of 0.1, 1, and 10 ng/ml. Cell number was counted after 3, 6, and 9 days in culture using a cell counter (NucleoCounter™, Chemometec, Allerod, Denmark). For other types of cells, 2×10^5 cells were plated in a 100-mm dish and cell numbers were counted after 7 days. 5-bromo-2-deoxyuridine (BrdU) incorporation assays were performed using the BrdU *In-Situ* Detection Kit (BD Biosciences, San Diego, CA). Human ASCs were plated in a 4-well chamber slide at 5×10^4 cells/well. Cells were cultured for 48 hours in control medium with a reduced serum concentration (2% FBS) with or without 10 ng/ml of various growth factors. Afterward, BrdU labeling was performed for 3 hours at a final concentration of 10 μM.

Flow cytometry

Human ASCs cultured with VEGF, FGF-2, HGF, or PDGF at 10 ng/ml for 7 days were examined for surface marker expression using flow cytometry. The following fluorochrome-conjugated monoclonal antibodies were used: anti-CD31-PE, CD34-PE (BD Biosciences), Flk-1-PE, and Tie-2-PE (R&D Systems, Minneapolis, MN). Cells were incubated with each antibody for 30 minutes and then analyzed using an LSR II flow cytometry system (BD Biosciences). Gates were set based on staining with combinations of relevant and irrelevant antibodies so that no more than 0.1% of cells were positive using irrelevant antibodies. Mouse stromal vascular fraction (SVF) cells were separated as described above and multi-color flow cytometric analyses were performed using the following monoclonal antibodies conjugated to fluorochromes: anti-CD31-PE (BD Biosciences), CD34-FITC (eBioscience, San Diego, CA), CD45-PE Cy7 (Beckman Coulter, Fullerton, CA), APC BrdU Flow Kit (BD Biosciences), and Annexin V-FITC Apoptosis Detection Kit (BD Biosciences).

Quantitative real-time reverse-transcriptase polymerase chain reaction

We isolated RNA from human ASCs cultured with VEGF, FGF-2, HGF, or PDGF at 10 ng/ml. For other types of cells, RNA was isolated from cells cultured with or without FGF-2 (10 ng/ml). We also isolated RNA from the inguinal adipose tissue of mouse models (see below) after homogenizing. Two micrograms of total RNA was isolated using an RNeasy™ Mini Kit (QIAGEN, Hilden, Germany), followed by reverse transcription. We amplified cDNA for 40 cycles with the ABI 7700 sequence detection system, a TaqMan™ Universal PCR Master Mix, and the following pre-designed primers and fluorescein-labeled probes: human VEGF (Hs00900054_m1); FGF-2 (Hs00266645_m1); HGF (Hs00300159_m1); PDGF (Hs00234042_m1); GAPDH

(Hs99999905_m1); mouse VEGF (Mm00437304_m1); FGF-2 (Mm00433287_m1); HGF (Mm01135182_m1); and GAPDH (Mm99999915_m1; all primers from Applied Biosystems, Foster City, CA). We calculated expression levels by the comparative CT method using GAPDH as an endogenous reference gene.

Quantifying HGF protein by enzyme-linked immunosorbent assay

Conditioned media of human ASCs cultured with or without FGF-2 (10 ng/ml) for 72 hours were analyzed by enzyme-linked immunosorbent assay (ELISA) using an ELISA kit for human HGF (Quantikine™, R&D Systems). Data were expressed as the secreted factor per 10^6 cells at the time of harvest.

Inhibition of MAP kinase signaling pathways

One selective inhibitor for each of three signaling pathways (ERK inhibitor U0126, p38 proteins inhibitor SB202190, and JNK inhibitor SP600125; all from Calbiochem, La Jolla, CA) was added at 10 μM with FGF-2 (10 ng/ml), and the effects on proliferation and gene expression were examined by proliferation assay and real-time reverse transcriptase-polymerase chain reaction (RT-PCR).

JNK activity assay

JNK activity was measured by the extent of c-Jun phosphorylation using a cell-based ELISA kit (CASE™ Kit, SuperArray, Frederick, MD) according to manufacturer's instructions. In brief, human ASCs were seeded at 2×10^4 per well in a 96-well plate and allowed to sit overnight. Cells were then starved in serum-free medium for 24 hours. Cells were pretreated with vehicle (0.1% DMSO), U0126, SB202190, or SP600125 for 15 min, then they were exposed to FGF-2 (10 ng/ml) or HGF (10 or 100 ng/ml) for 15 min. The amounts of activated (phosphorylated) c-Jun protein and total c-Jun protein were measured using anti-phospho-c-Jun (Serine 73) antibody and anti-pan-c-Jun antibody.

Mouse model for ischemia-reperfusion injury to adipose tissue

Care of animals was in accordance with institutional guidelines. Six-week-old ICR mice were anesthetized with pentobarbital (50 mg/kg weight) and a 2-cm incision was made in the inguinal region. The subcutaneous inguinal fat pad was elevated with the main nutrient vessels arising from the femoral vessels intact. Small communicating vessels to the skin from the fat pad were electro-coagulated. The main vessels were clamped with a vessel micro-clip for three hours and then released to allow reperfusion. The adipose tissue samples were harvested at various intervals (at days 1, 3, 7, and 14) after ischemia-reperfusion injury, and examined by flow cytometry, real-time RT-PCR, western blotting, and histology (hematoxylin-eosin staining, Azan staining and others; see below). Fat samples from sham-operated animals (without ischemia-reperfusion injury) were used as controls. At each time point we confirmed that contralateral pads from experimental animals showed no pathological changes as compared to the sham-operated animal samples ("day 0" sample).

In vivo inhibition assays

Inhibitor solutions were infused continuously using an osmotic pump (Model 1007D, ALZET, Cupertino, CA) that released liquid at a rate of 0.5 μl/h for about 7 days. The pump, containing 100 μl of a JNK inhibitor (SP600125; 10 μM), goat anti-FGF-2 antibody (R&D Systems; 50 μg/ml), goat anti-mouse HGF antibody (R&D Systems; 50 μg/ml), or control PBS was implanted subcutaneously at the time of reperfusion.

Histological detection of apoptosis and proliferation

To detect apoptosis after ischemia-reperfusion injury in adipose tissue, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining was performed using an *In Situ* Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany). To detect proliferating cells, 1 mg of BrdU was administered intraperitoneally 6 and 3 hours before tissue harvest. BrdU-

positive cells were detected using a BrdU *In-Situ* Detection Kit (BD Biosciences). The number of TUNEL or BrdU positive cells was counted at a 200× magnification using three randomly selected fields per section.

Immunohistochemistry

Harvested adipose tissue samples were zinc-fixed (Zinc Fixative, BD Biosciences) and paraffin-embedded. We prepared 6-μm-thick sections and performed immunostaining using the following primary antibodies: goat anti-FGF-2 (R&D Systems), goat anti-mouse HGF (R&D Systems), goat anti-mouse CD68 (Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-mouse CD34 (Santa Cruz Biotechnology), rat anti-mouse CD31 (BD Biosciences), and biotinylated mouse anti-BrdU (BD Biosciences). For visualization with diaminobenzidine (DAB), peroxidase-conjugated secondary antibodies appropriate for each primary antibody (Nichirei Biosciences, Tokyo, Japan) or a streptavidin-peroxidase complex (BD Biosciences) were used. For a double fluorescence staining, the following secondary antibodies or reagent were used: Alexa Fluor™ 488-conjugated rabbit anti-rat IgG, Alexa Fluor™ 488 or 568-conjugated donkey anti-goat IgG, and Alexa Fluor™ 568-conjugated streptavidin (Molecular Probes, Eugene, OR). Isotypic antibody was used to serve as a negative control for each staining.

Western blotting

Adipose tissue specimens were homogenized in 1ml of lysis buffer (Santa Cruz Biotechnology) and centrifuged at 15,000 rpm for 2 minutes. The aqueous layer was collected and the protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of protein (10 μg) were loaded into each lane of an SDS-PAGE gel. The resolved proteins were transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA) and immunostaining was performed using goat anti-mouse FGF-2 antibody (R&D Systems), goat anti-mouse HGF antibody (R&D Systems), and goat anti-mouse GAPDH antibody (Santa Cruz Biotechnology). The GAPDH signal served as an internal control. Protein bands were quantified by volume summation of image pixels using Photoshop 7.0 (Adobe Systems Inc., San Jose, CA).

Glycerol-3-phosphate dehydrogenase (GPDH) assay

A GPDH Assay Kit (Cell Garage, Tokyo, Japan) was used according to the manufacturer's instructions, as previously described [24]. In brief, each adipose tissue sample was mixed with a 0.25 M sucrose solution to a total of 5 ml, homogenized, and centrifuged. The supernatants were diluted 10 times with an enzyme-extracting reagent and the optical absorption was measured at 340 nm for 10 minutes in a 96-well plate after addition of twice the volume of a substrate reagent. GPDH activity was calculated based on the following formula: GPDH activity (U/ml) = $\Delta OD \times 0.482 \times 10$ (ΔOD: change in optical density per minute).

Living tissue imaging

Visualization of living adipose tissue was performed using the procedure of Nishimura et al [25]. Briefly, the adipose tissue was minced into 3-mm pieces and incubated with the following reagents for 30 min: BODIPY™ 558/568 or BODIPY™-FL (both from Molecular Probes) to stain adipocytes, Alexa Fluor™ 488-conjugated isolectin GS-IB_4 (Molecular Probes) to stain endothelial cells, Hoechst 33342 (Dojindo, Kumamoto, Japan) to stain all nulei, or propidium iodide (PI; Sigma-Aldrich, St. Louis, MO) to stain nuclei of necrotic cells. The sample was then washed and directly observed with a confocal microscope system (Leica TCS SP2).

Statistical analysis

Results were expressed as mean ± standard error of means (SEM). Comparisons between two groups were performed with Welch's t-test. Comparisons of multiple groups were done by analysis of variance with corrections for multiple comparisons. A value of $p < 0.05$ was considered significant.

RESULTS

Effects of injury-associated growth factors on human ASC proliferation

FGF-2 and PDGF promoted proliferation of human ASCs in a dose-dependent manner, while VEGF and HGF did not promote ASC growth (Fig. 1A). Morphologically, ASCs cultured with FGF-2 were smaller than ASCs cultured with other growth factors (Fig. 1B). The proliferative effects of FGF-2 and PDGF were also demonstrated in a BrdU incorporation assay. Cells were cultured in growth factor-containing medium with a reduced serum concentration (2% FBS) for a fixed period (48 hours) prior to assay (Supplemental online Fig. 1A).

Phenotypic effects of injury-associated growth factors on human ASCs

Flow cytometry showed no significant changes in expressions of endothelial surface markers (CD31, CD34, Flk-1, and Tie-2) by any injury-associated growth factors, indicating that supplementation of a single factor is not enough to induce endothelial differentiation of human ASCs, at least when cultured without any extracellular matrices (Fig. 1C and Supplemental online Fig. 1B).

Interactive effects of injury-associated growth factors on their expressions by human ASCs

FGF-2 and PDGF significantly downregulated expression of their own transcripts by human ASCs, and FGF-2 also significantly suppressed PDGF mRNA expression (Fig. 2A). Most interestingly, FGF-2 promoted a striking increase in HGF mRNA expression. Time course evaluation revealed that the upregulation of HGF mRNA was biphasic, with the first peak at 6 hours and a striking increase beginning after 24 hours (Fig. 2B). ELISA of culture media demonstrated that HGF protein secretion by FGF-2-stimulated human ASCs was significantly elevated at day 3 (Fig. 2C).

Intracellular signaling pathways of FGF-2-induced effects on human ASCs

The proliferative effects of FGF-2 on human ASCs were significantly inhibited by a JNK inhibitor (SP600125) but not by an ERK inhibitor (U0126) or a p38 inhibitor (SB202190; Fig. 3A). Upregulation of HGF mRNA by FGF-2 was also significantly inhibited by the JNK inhibitor (Fig. 3B). Administration of the ERK and p38 inhibitors slightly decreased the expression level of HGF mRNA although the changes were not statistically significant, suggesting crosstalk between JNK and other signaling pathways. Phosphorylation of c-Jun, the prototypical nuclear effector of the JNK signal transduction pathway, increased with FGF-2 treatment, and the FGF-2-induced upregulation of c-Jun phosphorylation was completely prevented by pretreatment with the JNK inhibitor (Fig. 3C). On the other hand, HGF did not increase phosphorylation of c-Jun in human ASCs (Fig. 3C). These results indicate that FGF-2 promotes proliferation and HGF mRNA expression predominantly through the JNK signaling pathway.

FGF-2-induced effects in human ASCs and other cell types

Cell growth was promoted by FGF-2 in all four cell types studied (human ASCs, human DFs, human BM-MSCs, and murine ASCs), although the basal proliferative capacity differed among cell types. The FGF-2-enhanced cell proliferation was significantly inhibited by a JNK inhibitor (SP600125) in all cell types examined (Fig. 3D). HGF mRNA expression was also promoted by FGF-2 in all cell types, and the upregulation of HGF mRNA was significantly inhibited by the JNK inhibitor except in human BM-MSCs (Fig. 3E), suggesting that HGF mRNA upregulation by FGF-2 was mediated through signaling pathways other than JNK in human BM-MSCs.

Ischemia-reperfusion injury and subsequent repair in the inguinal adipose tissue of mice

The murine inguinal fat pad consistently had a dominant feeding artery and vein without anatomical anomaly, which enabled reproducible ischemia-reperfusion injury

experiments (Fig. 4A). The weight of the adipose tissue was increased on day 1, suggesting tissue edema (Supplemental online Fig 2A), while GPDH activity, which correlates with the total number and size of mature adipocytes, did not change significantly during the experimental period (Supplemental online Fig. 2B). Histologically, interstitial infiltration of blood cells was observed as early as day 1 and most prominent on day 3. Small-sized adipocytes appeared on day 1 and increased in number on day 3, suggesting that lipolysis or adipogenesis took place throughout the adipose tissue. On day 7, infiltrated erythrocytes disappeared and some adipocytes increased in size while a substantial number of nucleated cells remained in the interstitial space between adipocytes (Fig. 4B). Flow cytometric analysis of the SVF showed that the total number of SVF cells as well as the number of CD45+ cells was increased by day 7, while the number of CD34+/CD31– cells peaked on day 3 (Fig. 4C). The number of BrdU-positive proliferating cells increased from day 1 and peaked on day 3 (Fig. 4D and Supplemental online Fig. 2C), and most BrdU+/CD45– cells (88.2 ± 4.2%, n = 3) were shown to be CD34+/CD31– (Fig. 4E). The frequency of TUNEL-positive apoptotic cells was significantly high (6-9% of nucleated cells) on day 1 and decreased thereafter (Fig. 4F and Supplemental online Fig. 2D). Flow cytometry demonstrated that most Annexin V-positive apoptotic cells on day 1 (90.1 ± 3.0%, n = 3) were CD45+ (Fig. 4G). In addition, an increase in PI-positive necrotizing cells was observed on day 3; some of these cells (17.2 ± 5.5%, n = 3) were lectin-positive, suggesting that some capillary endothelial cells were necrotizing and that capillary remodeling was underway (Fig. 4H). These results indicate that the adipose tissue was impaired soon after reperfusion, apoptosis and necrosis were involved in the process of adipose degeneration, and regenerative changes occurred thereafter.

FGF-2 protein was detected in the interstitial tissue by immunohistochemistry as early as day 1, with a peak of detection on day 3, although not much FGF-2 was expressed on day 7 (Fig. 5A). Western blotting also showed an increase in FGF-2 protein on days 1 and 3 (Fig. 5B). On the other hand, little HGF protein was detected on day 1, but HGF expression was elevated from day 3 until at least day 7 (Fig. 5C and 5D). Interestingly, FGF-2 mRNA was downregulated from soon after injury throughout the 2 weeks, suggesting that FGF-2 was released from the storage of the injured adipose tissue, such as extracellular matrix or dying cells, rather than produced by viable cells such as ASCs. The released FGF-2 might downregulate FGF-2 mRNA expression by ASCs in the injured adipose tissue, as was seen in human ASCs in vitro. In contrast, HGF mRNA expression in the tissue was significantly elevated on day 3, but not on days 1 and 7 (Fig. 5E), suggesting that the delayed upregulation of HGF mRNA and secretion of HGF were likely induced by the preceding release of FGF-2 protein. Thus, the data suggest that FGF-2 is provided temporarily only when an event such as injury occurs and triggers FGF-JNK-HGF signaling, thereby promoting repair processes such as angiogenesis.

Cellular events in the repair process of injured adipose tissue

Before injury, CD34+/CD31– cells, which were regarded as ASCs, were found throughout the intact adipose tissue, located between mature adipocytes, and especially abundant around vessels (Supplemental online Fig 3A). The number of CD34+/CD31– cells was apparently much larger than that of CD31+ endothelial cells, which were negative or faintly positive for CD34. Most BrdU-positive proliferating cells, frequently detected 3 days after injury, were also positive for CD34 (Fig. 6A, top) and most CD34+ cells were lectin-negative (Fig. 6A, bottom), which suggests that CD34+/lectin– ASCs play substantial roles in the repair process. Living tissue imaging revealed increase of interstitial space and small-sized adipocytes (less than 50 μm in diameter), an increased number of nucleated cells including lectin-positive round cells (Fig. 6B), and capillaries especially around small-sized adipocytes (Supplemental online Fig. 3B). On day 7, large-sized adipocytes were not seen

in samples treated with a JNK inhibitor, while large-sized adipocytes were seen in untreated models (Fig. 6B and 6C). Furthermore, PI-positive nuclei were frequently observed on days 1 and 3, a small number of which (10.7 ± 5.7% on day 1; 4.4 ± 0.5% on day 3; n = 3) were seen in adipocytes, suggesting that not only capillary endothelial cells (Fig. 4H) but also adipocytes were necrotizing (Fig. 6D). On day 7, the PI-positive nuclei were still observed in samples treated with a JNK inhibitor, although they were rarely detected in untreated models (Fig. 6D and 6E). These results suggest that the adipose tissue remodeling involving adipocytes and capillaries was underway during the first week after injury and the tissue repair process was impaired by treatment with a JNK inhibitor. CD68+ cells, regarded as infiltrating macrophages, were scarcely detected in the intact adipose tissue (Supplemental online Fig. 3C). However, infiltrated macrophages aggregated around small-sized adipocyte-like cells after injury, suggesting phagocytosis. This aggregation of CD68+ cells was most frequently observed 3 days after injury.

Influence of signal inhibition on HGF expression and fibrogenesis after ischemia-reperfusion injury

The increase in BrdU-positive proliferating cells on day 3 was significantly inhibited by treatment with a JNK inhibitor or a neutralizing antibody against FGF-2, but not by a neutralizing antibody against HGF (Fig. 7A). Immunohistochemical analysis demonstrated that the JNK inhibitor also suppressed the number of CD34+/lectin– ASCs on days 3 and 7 (Supplemental online Fig. 3D). Administering a JNK inhibitor or a neutralizing antibody against FGF-2 completely prevented upregulation of HGF mRNA in the injured adipose tissue on day 3 (Fig. 7B) and suppressed HGF secretion on day 7 (Fig. 7C and 7D). Histological measurement of the fibrous area at 2 weeks revealed that reperfusion injury to the adipose tissue caused a significant increase in the fibrous area (from 10% to 23%). Furthermore, inhibition of JNK or FGF-2 signaling caused a further significant increase in the fibrous area as compared to administration of vehicle alone. Significantly increased fibrogenesis was also observed in the group treated with an anti-HGF antibody (Fig. 7E). These results indicated that fibrogenesis seen in the injured adipose tissue was suppressed by HGF in the process of adipose tissue repair and suggested that HGF secretion, mainly from ASCs, was induced by FGF-2 through a JNK signaling pathway.

DISCUSSION

After mechanical injury to human adipose tissue, FGF-2, EGF, transforming growth factor (TGF)-β, and PDGF are first secreted in the early stage of wound healing. Thereafter, as the above growth factors decline, VEGF and HGF secretion gradually increases during the first week post-injury [20]. Our results indicated that, among these injury-associated growth factors, FGF-2 promoted proliferation and HGF secretion by ASCs predominantly through a JNK signaling pathway. FGF-2 is an important endogenous stimulator of angiogenesis [26] and cell proliferation [27] and is known to be released during the early phase of wound healing [28,29]. Cellular FGF-2 is released during the lysis of various cell types, such as fibroblasts [30] and endothelial cells [31], around the wound, while FGF-2 bound up in the extracellular matrix is released by the action of various wound proteases [32,33]. JNK signaling is involved not only in FGF-2-induced ASC proliferation, as revealed in this study, but also in PDGF-induced proliferation and migration of human ASCs [16]. Therefore, it is likely that the JNK inhibitor used in this study inhibited the biological action of both FGF-2 and PDGF on ASCs. PDGF promoted ASC proliferation, but had no significant effect on HGF expression by ASCs. As other researchers have reported, both FGF-2 and EGF promoted HGF secretion by ASCs [3]; therefore, JNK might also be involved in the EGF-induced HGF secretion by ASCs. Although some studies using other cell types found that JNK was involved in the HGF signaling pathway [34,35], our results indicated that HGF, even at high concentrations, did not stimulate the JNK pathway in human ASCs.

It is interesting that the FGF-2-enhanced cell proliferation and HGF production that we observed for ASCs were observed also in other mesenchymal cell types, such as BM-MSCs and DFs, although HGF mRNA upregulation in human BM-MSCs was mediated by signals other than JNK. FGF-2-induced HGF upregulation has been observed in other cell types, such as smooth muscle cells [36], fibroblast-like cells from lung tissue [37], and osteoblasts [38]; however, the intracellular signaling mechanisms were not well investigated. Other factors such as interleukin-1 [39], interferon-γ [40], and ascorbic acid [41] have also been reported to stimulate HGF production. HGF is a key mediator of angiogenesis and wound healing, and its expression seems to be regulated by a number of factors in various cells and tissues.

The injury and repair process in adipose tissue has not been well studied, presumably because there is no standard animal model. Coban et al., using the epigastric adipo-cutaneous flap model, described edema and hemorrhage following ischemia-reperfusion injury of adipose tissue [43]; their results were similar to ours, even though a different model was used. In the injured adipose-tissue, necrotic and apoptotic changes followed edema and hemorrhage in the early phase after injury, and inflammatory and regenerative changes such as phagocytosis, cell infiltration and proliferation were observed throughout the first week. The necrotic changes were seen in capillary endothelial cells and adipocytes, suggesting adipose tissue remodeling, the repair process of which was impaired by a JNK inhibitor. HGF secretion followed release of FGF-2, stored bound or in other forms, from the tissue; the HGF upregulation appears to be derived predominantly from ASCs stimulated by FGF-2 via JNK signaling pathway, because ASCs are known to constitute more than one-half of mesenchymal cells in stromal vascular fractions derived from adipose tissue [23]. The ischemia-reperfusion model used in this study provided reproducible results of injury and regeneration of adipose tissue, all of which were complete within 2 weeks.

In the adipose tissue, CD34+/CD31– cells, which were suggested to be ASCs, were seen in abundance between adipocytes (closely related to capillaries) and around vessel walls (especially in the tunica adventitia) before injury. CD34+ vascular wall resident progenitor cells, which have the capacity to differentiate into vascular endothelial cells and form capillary sprouts, were previously reported [43] and this population might constitute part of ASCs. On the other hand, a periendothelial subpopulation of ASCs with pericytic characteristics has been also characterized [44]. ASCs might consist of heterogenic subpopulations, which may explain the complexity in elucidating ASC characteristics and functions in vivo. As previously suggested, there is a close relationship between adipogenesis and angiogenesis, therefore, ASCs, which are primarily regarded as adipocyte progenitor cells, may function as adipogenic and angiogenic progenitors [5] and manage the interplay between adipocytes, blood cells, and blood vessels in various situations, such as obesity [25], adipose tissue turnover, and post-injury adipose tissue repair. During tissue regeneration, more CD34+/CD31– ASCs were found between adipocytes than in controls, and most BrdU-positive proliferating cells seen on day 3 were CD34+/lectin–, suggesting that ASCs are proliferating and participating in repair processes such as adipogenesis and angiogenesis. The proliferation and migration of ASCs were strongly inhibited by treatment of a JNK inhibitor, resulting in delayed and impaired tissue repair as well as increased fibrogenesis. Macrophages are known to be involved in inflammation in adipose tissue and obesity [25]. In this study, CD68+ macrophages were scarcely detected in controls but were seen in larger numbers on day 3, however, at a much lower level than in ASCs. The role of macrophages in the injured adipose tissue remains to be clarified, but they may be involved in phagocytosis and/or angiogenesis [25].

HGF has been reported to have antifibrogenic effects in various organs including heart [22], liver [45], and kidney [46]. Recent studies

revealed that HGF antagonizes the profibrotic actions of TGF-β by intercepting Smad signal transduction through diverse mechanisms [46,47]. In this study, inhibiting FGF-2 signaling suppressed HGF production by ASCs, and inhibition of HGF led to increased fibrogenesis in the injured adipose tissue. Furthermore, administering a JNK inhibitor or an anti-FGF-2 antibody also resulted in severe post-injury fibrogenesis, which means that FGF-2-induced prevention of fibrogenesis was mediated at least in part by HGF derived from ASCs and that HGF is a key factor in preventing fibrogenesis and preserving adipose volume after injury. FGF-2 is known to promote wound healing by its direct effects on fibroblasts and endothelial cells [48], but this study revealed that FGF-2 could also promote tissue repair and prevent fibrogenesis by inducing HGF from ASCs and/or DFs. Because adipose tissue exists in or around most organs, FGF-2-induced HGF secretion from ASCs might be important in the repair processes following organ injuries such as myocardial infarction, hepatitis, and nephritis.

The results of this study provide new insights into the therapeutic potential of ASCs. Cytotherapies with ASCs, especially when pretreated or combined with FGF-2, might accelerate tissue repair, reduce fibrosis, and improve the function of organs impaired by acute or chronic inflammation through HGF action. It has been suggested that HGF secreted by ASCs might play an important role in autologous fat grafting [9,12]; promoting adipose graft survival and preventing scar formation. HGF is not only antifibrogenic but it is also angiogenic [49], and has been shown to contribute to angiogenesis in adipose tissue [50] and to the improvement of ischemic limb [51]. FGF-2-induced HGF secretion could also promote angiogenesis during tissue repair. The total capillary amount was increased but the vascular density in the injured adipose tissue was not (Supplemental online Fig. 3E), though the measurement of vascular density may have limitation because it can be affected by the size and density of adipocytes. In ASCs, VEGF secretion appears to be induced by hypoxia [2]. The model we used in this study was an injury model rather than an ischemia model, and VEGF expression was downregulated soon after injury (data not shown). To study angiogenesis in adipose tissue and establish a therapeutic strategy for enhancing angiogenesis, studies using specific models of chronic ischemia in adipose tissue are needed..

CONCLUSIONS

FGF-2 promotes proliferation of and HGF secretion by ASCs through a JNK signaling pathway. In injured adipose tissue, ASCs stimulated by FGF-2 released from injured tissue are suggested to be the main proliferating cell population in the adipose repair process. This JNK-mediated signal plays an important role in preventing fibrogenesis and preserving adipose volume after injury. This study also revealed a new role for ASCs in the injury response and provides insights into future strategies for ASC-based therapies.

ACKNOWLEDGMENTS

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Figure 1. Effects of injury-associated growth factors on proliferation and surface marker expression of human ASCs.

(A) ASC counts after 3, 6, or 9 days culture with VEGF, FGF-2, HGF, or PDGF. FGF-2 and PDGF promoted proliferation of human ASCs in a dose-dependent manner, while VEGF and HGF showed no proliferative effects ($n = 4$; $*p < 0.05$). **(B)** Light microscopy photographs of human ASCs cultured with each growth factor for 6 days. Cells cultured with FGF-2 (10 ng/ml) were smaller than those cultured with other growth factors. Scale bars = 200 μm. **(C)** Flow cytometric analyses of human ASCs cultured with each growth factor for 7 days. No significant changes in the expression of vascular endothelial markers (CD31, CD34, Flk-1, and Tie-2) were observed.

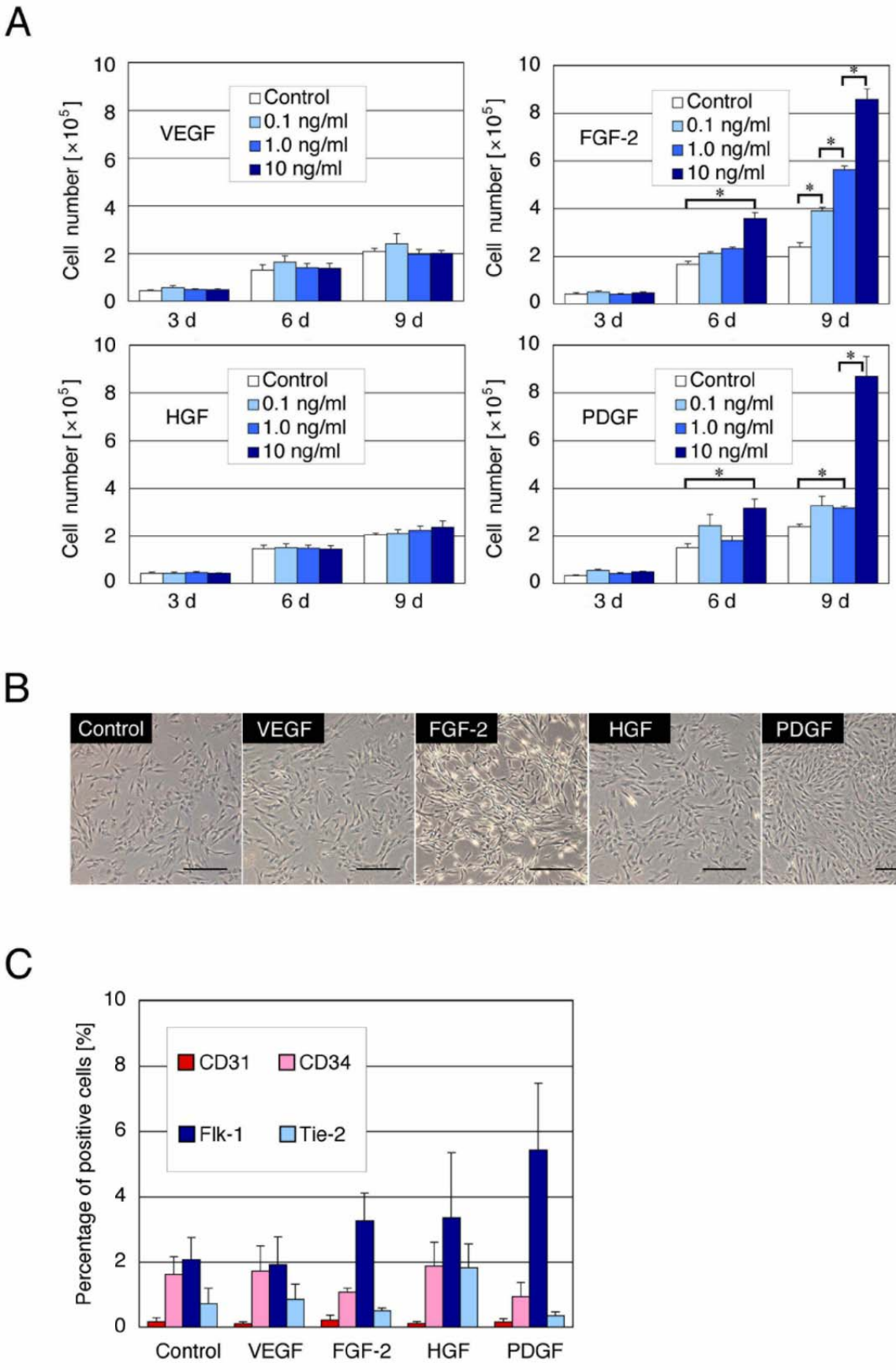


Figure 2. Interactive effects of injury-associated growth factors on their own mRNA expression by human ASCs.

(A) Injury-associated growth factor mRNA expression by human ASCs. FGF-2 (10 ng/ml) induced striking upregulation of HGF mRNA expression by human ASCs, while FGF-2 and PDGF downregulated their own expression ($n = 7$, $^*p < 0.05$). **(B)** Time course of HGF mRNA expression induced by FGF-2. FGF-2 (10 ng/ml) promoted biphasic HGF mRNA expression by human ASCs, with the first peak occurring at 6 h and the second increase beginning after 24 h ($n = 6$, $^*p < 0.05$). **(C)** FGF-2-induced HGF protein secretion by human ASCs. ELISA of cultured media on day 7 showed that FGF-2 (10 ng/ml) promoted secretion of HGF protein from human ASCs ($n = 4$, $^*p < 0.05$).

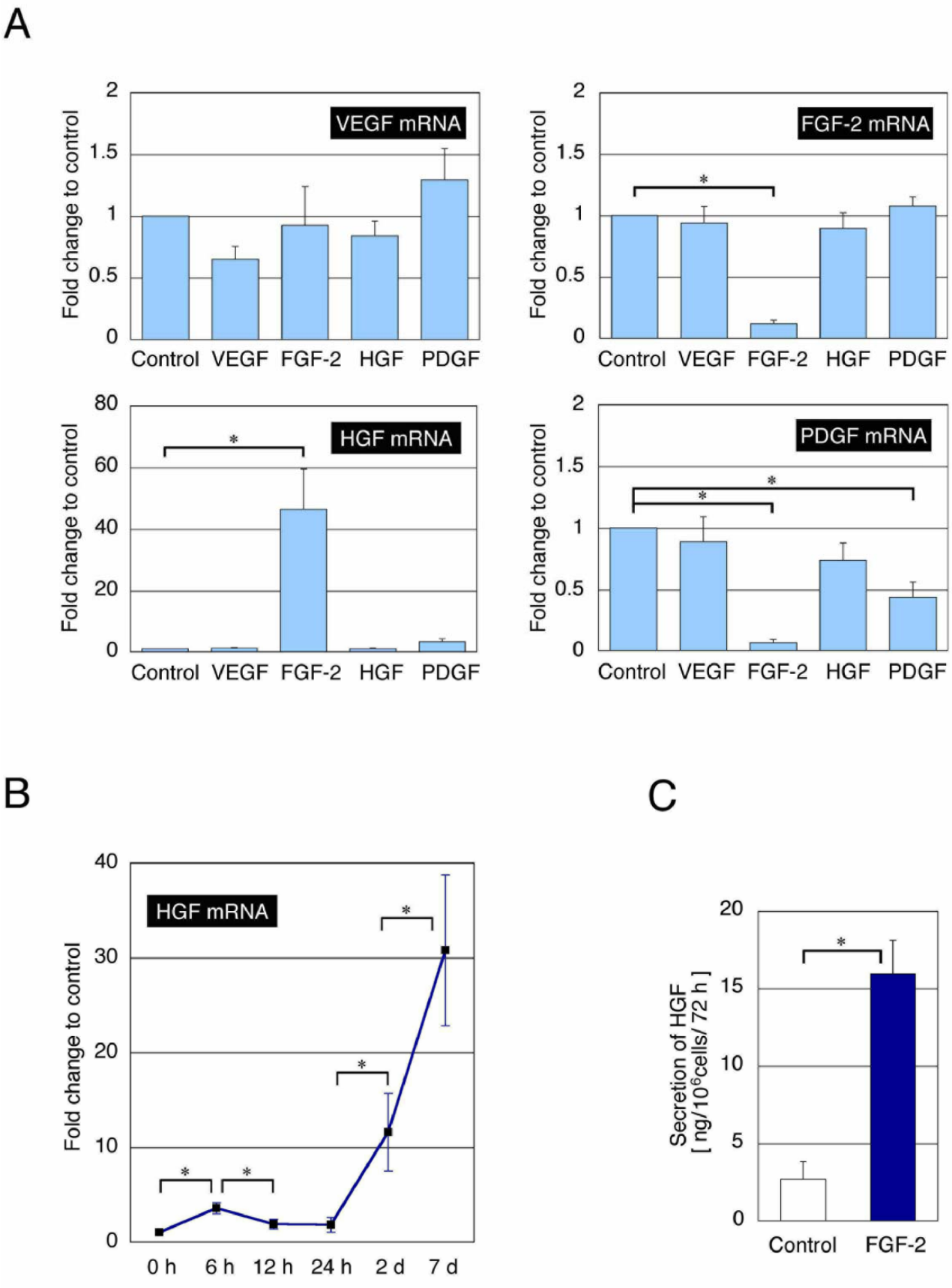


Figure 3. Inhibition of FGF-2 intracellular signaling pathways.

(A) Effects of downstream signal inhibitors on ASC counts after 7 days in culture with or without FGF-2 (10 ng/ml). The JNK inhibitor SP600125 (SP) significantly inhibited the proliferative effect of FGF-2 on human ASCs, while U1026 (an ERK inhibitor) and SB202190 (SB, a p38 protein inhibitor) showed no or limited inhibitory effect on ASC proliferation ($n = 5$, $^*p < 0.05$). **(B)** HGF mRNA expression by human ASCs cultured with or without FGF-2 (10 ng/ml) on day 7. FGF-2-induced upregulation of HGF mRNA was significantly inhibited by SP ($n = 5$, $^*p < 0.05$), while U1026 and SB had limited inhibitory effects. **(C)** Inhibitor specificity. *Left*: Phosphorylation of c-Jun increased following 15-minute treatment with FGF-2 (10 ng/ml). FGF-2-induced phosphorylation of c-Jun was completely prevented by pretreatment with SP ($n = 4$, $^*p < 0.05$). *Right*: Phosphorylation of c-Jun increased with HGF treatment (10 or 100 ng/ml). HGF, even at a high concentration (100 ng/ml), did not increase c-Jun phosphorylation in human ASCs ($n = 3$). **(D)** Cell counts after 7 days in culture. FGF-2-induced cell proliferation was significantly inhibited by the JNK inhibitor SP in all cell types examined (hASC, human adipose-derived stem/stromal cells; hBM-MSC, bone marrow-derived mesenchymal stem cells; hDF, human dermal fibroblasts; mASC, mouse adipose-derived stem/stromal cells; $n = 5$, $^*p < 0.05$). **(E)** Real-time PCR assays for HGF mRNA expression on day 7. HGF mRNA expression was promoted by FGF-2 in all cell types, while the FGF-2-induced upregulation of HGF mRNA was significantly inhibited by a JNK inhibitor except in human BM-MSCs ($n = 5$, $^*p < 0.05$).

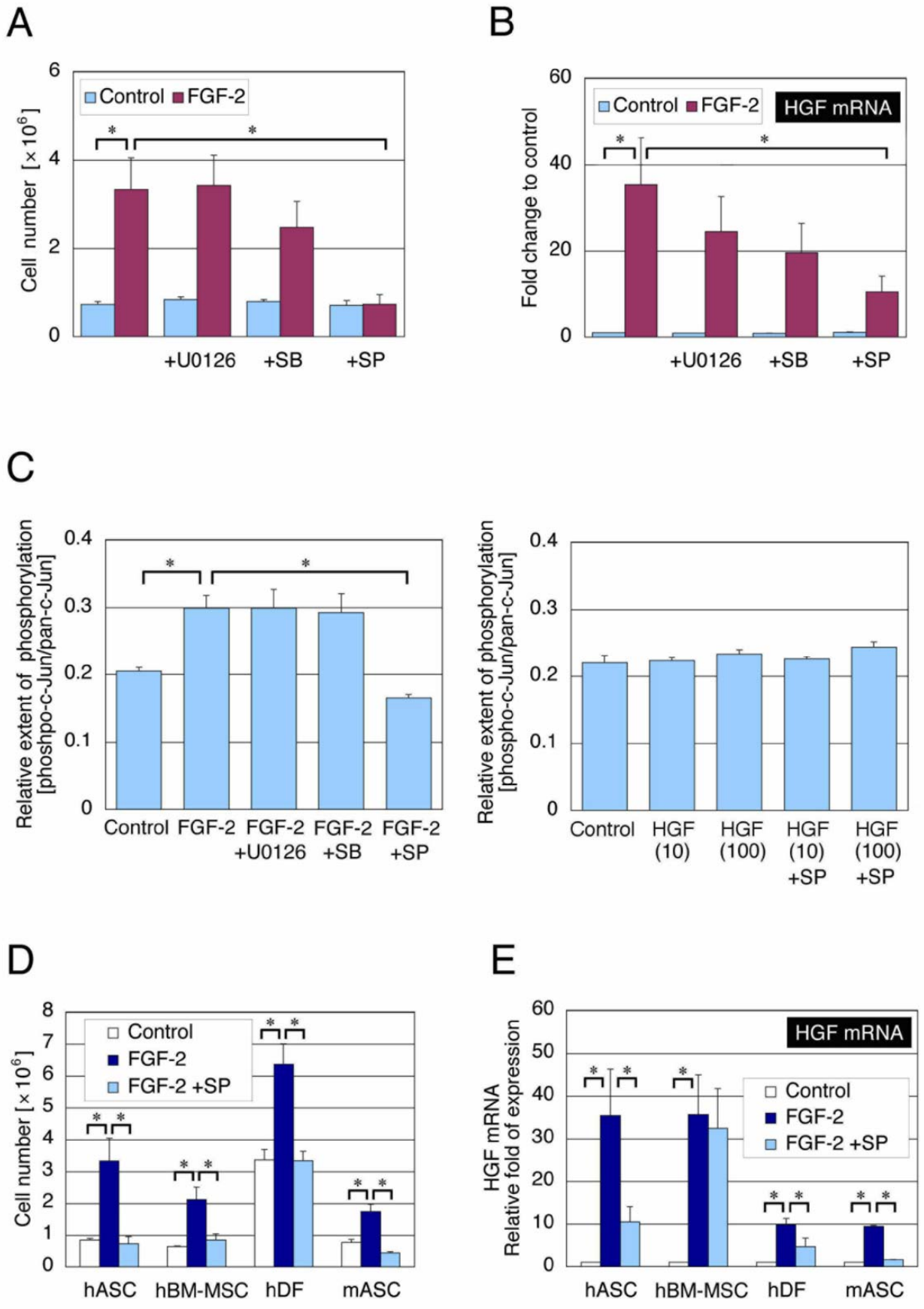


Figure 4. Ischemia-reperfusion injury and subsequent regenerative changes in the inguinal adipose tissue of mice.

(A) Ischemia-reperfusion injury model. *Top*: The murine inguinal fat pad. The arrow indicates the dominant nutrient vessels arising from the femoral vessels. Arrowheads indicate communicating branches to the skin. *Bottom*: The communicating branches were severed and the dominant vessels were clamped for 3 hours with a vessel clip. **(B)** Hematoxylin-eosin staining revealed that interstitial infiltration of blood cells occurred as early as day 1 and diminished by day 7. Regenerative changes were seen in the injured adipose tissue throughout the first week. Scale bars = 100 μm. **(C)** Analysis of SVF by flow cytometry. *Left:* Total SVF cells and CD45+ cells, *Right:* CD31–/CD34+ cells. Total SVF cells increased by day 7, with the similar increase of CD45+ cells, while the number of CD34+/CD31– cells peaked on day 3 ($n = 3$, $^*p < 0.05$). **(D)** Quantification of BrdU-positive cells by immunohistochemistry (Supplemental online Fig. 2C). BrdU-positive proliferating cells increased after injury, peaking on day 3 ($n = 4$, $^*p < 0.05$). **(E)** Flow cytometric analysis of SVF cells on day 3. BrdU-positive proliferating cells included both CD45+ and CD45– cells; most BrdU+/CD45– cells (88.2 ± 4.2%, $n = 3$) were CD34+/CD31–. **(F)** Quantification of TUNEL-positive cells by immunohistochemistry (Supplemental online Fig. 2D). TUNEL-positive apoptotic cells were observed most frequently 1day after injury ($n = 4$, $^*p < 0.05$). **(G)** Flow cytometric analysis of SVF cells for expression of Annexin V and CD45 on day 1. Most Annexin V-positive apoptotic cells (90.1 ± 3.0%, $n = 3$) were CD45+. **(H)** Living tissue stained with Hoechst 33342 (blue), lectin (endothelial cells; red), or PI (necrotic cells; green), imaged and merged with interference contrast images. PI-positive necrotizing cells increased on day 3; some of these cells (17.2 ± 5.5%, $n = 3$) appeared to be lectin-positive capillary endothelial cells. Scale bars = 100 μm.

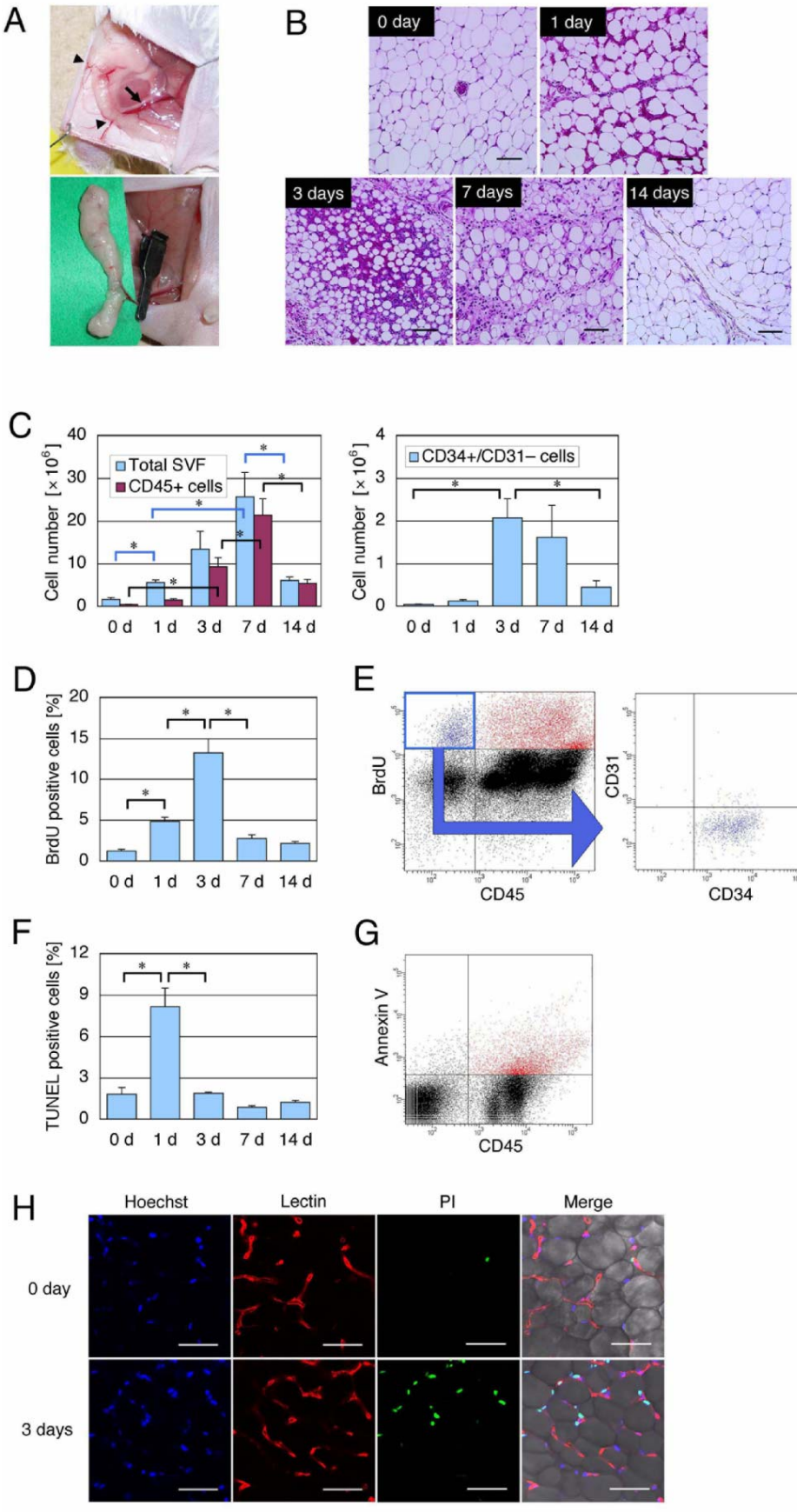


Figure 5. Expression of FGF-2 and HGF after ischemia-reperfusion injury to the adipose tissue.

(A) Immunostaining for FGF-2. FGF-2 protein was detected as early as 1 day post-injury with the peak of detection on day 3. Scale bars = 100 μm. **(B)** Western blot analysis for FGF-2. FGF-2 protein increased significantly on days 1 and 3 ($n = 3$, $*p < 0.05$). **(C)** Immunostaining for HGF. Little HGF protein was detected 1 day after injury, but it was detected as late as 3 days post-injury, peaking 7 days after injury. Scale bars = 100 μm. **(D)** Western blot analysis for HGF. HGF protein increased significantly on day 7 ($n = 3$, $*p < 0.05$). **(E)** Real-time PCR assay of FGF-2 mRNA and HGF mRNA expressions in the injured adipose tissue. FGF-2 mRNA was downregulated soon after injury ($n = 5$, $*p < 0.05$), while HGF mRNA was significantly upregulated 3 days after injury ($n = 5$, $*p < 0.05$).

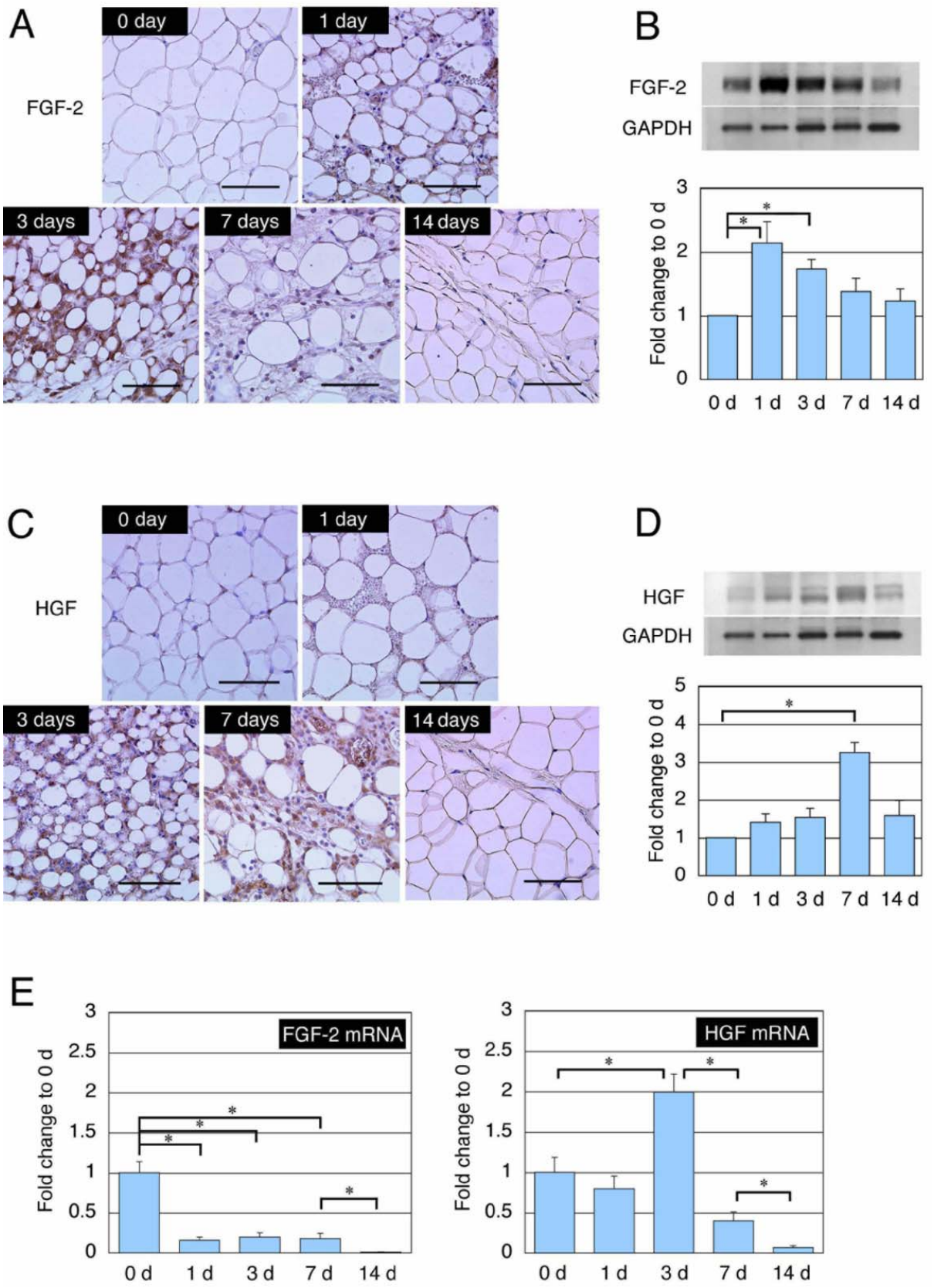


Figure 6. Cellular events in the repair process of injured adipose tissue.

(A) The injured tissue on day 3. *Top*: Staining for CD34 (green), BrdU (red) or Hoechst 33342 (blue). Most BrdU-positive cells, frequently observed 3 days after injury, were also positive for CD34. Scale bars = 20 μm. *Bottom*: Living tissue image of the tissue on day 3 stained with CD34 (green), lectin (endothelial cells; red), or Hoechst 33342 (nuclei; blue).On day 3, most of CD34+ cells were lectin-negative, suggesting that they were ASCs. Scale bars = 20 μm. **(B)** Living tissue image stained with BODIPY (adipocytes; yellow), lectin (endothelial cells; red), or Hoechst 33342 (nuclei; blue). Increased number of nucleated cells and small-sized adipocytes were observed on days 1 and 3. Lectin+ small round cells, which may be infiltrating blood-derived cells, were seen on day 1, though they remained to be characterized. Large-sized adipocytes increased on day 7, but not when treated with a JNK inhibitor. Blue scale bars = 200 μm, and white scale bars = 50 μm. **(C)** Quantification of small adipocytes (less than 50 μm in diameter). In the group treated with a JNK inhibitor, the percentages of small adipocytes were significantly higher on days 7 and 14 (n = 3, $*p < 0.05$). **(D)** Three-dimensional images of living tissue stained with BODIPY (adipocytes; yellow), PI (nuclei of necrotizing cells; red), or Hoechst 33342 (all nuclei; blue); images dissected horizontally and longitudinally at levels of white broken lines are also shown. PI-positive cells were frequently observed on days 1 and 3; some of these cells (10.7 ± 5.7% on day 1; 4.4 ± 0.5% on day 3; n = 3) were proven to be adipocytes. Treatment with a JNK inhibitor increased the number of PI-positive cells on day 7. Scale bars = 50 μm. **(E)** Quantification of PI-positive cells. In the group treated with a JNK inhibitor, the percentage of PI-positive cells was significantly higher on day 7 (n = 3, $*p < 0.05$).

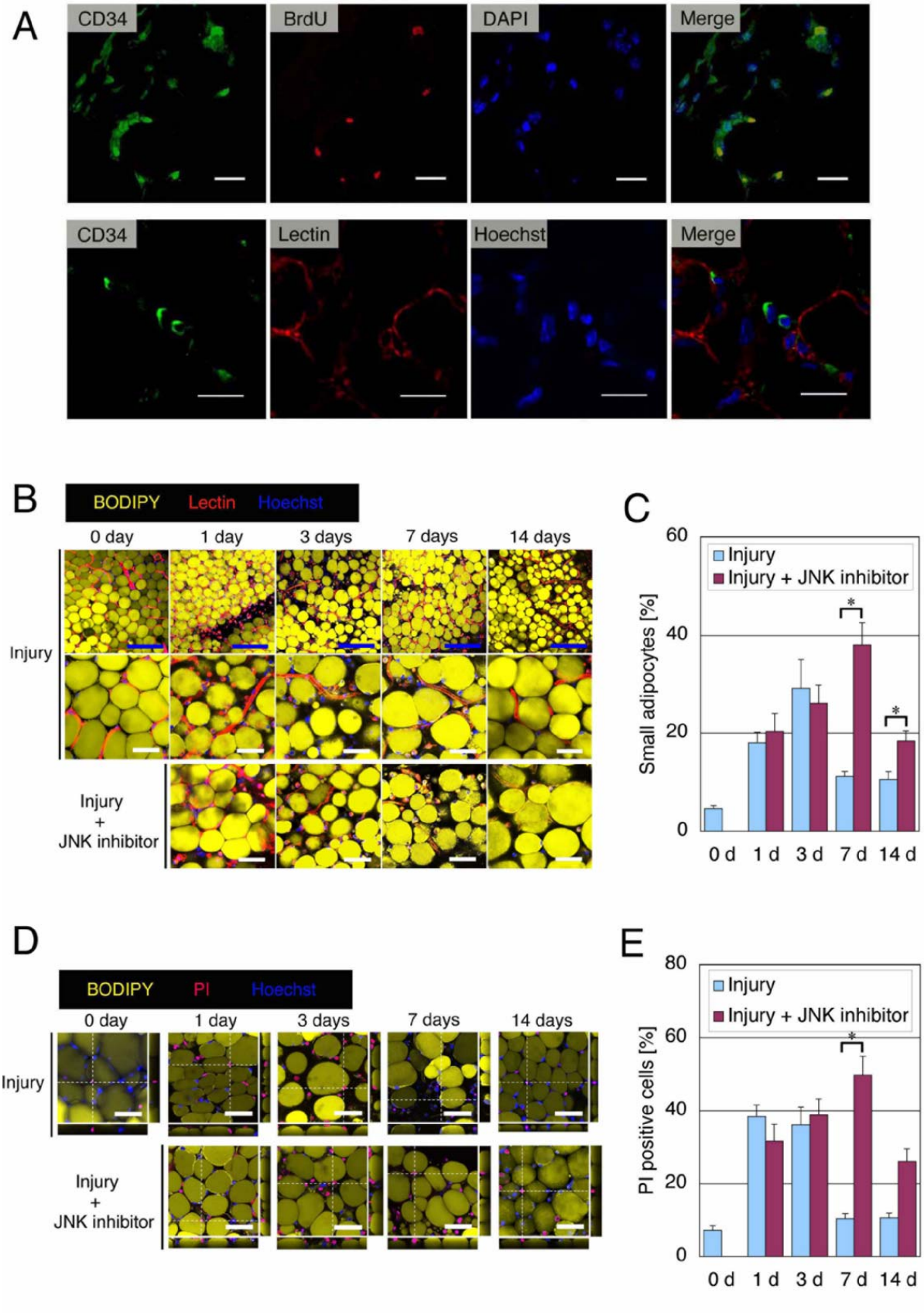
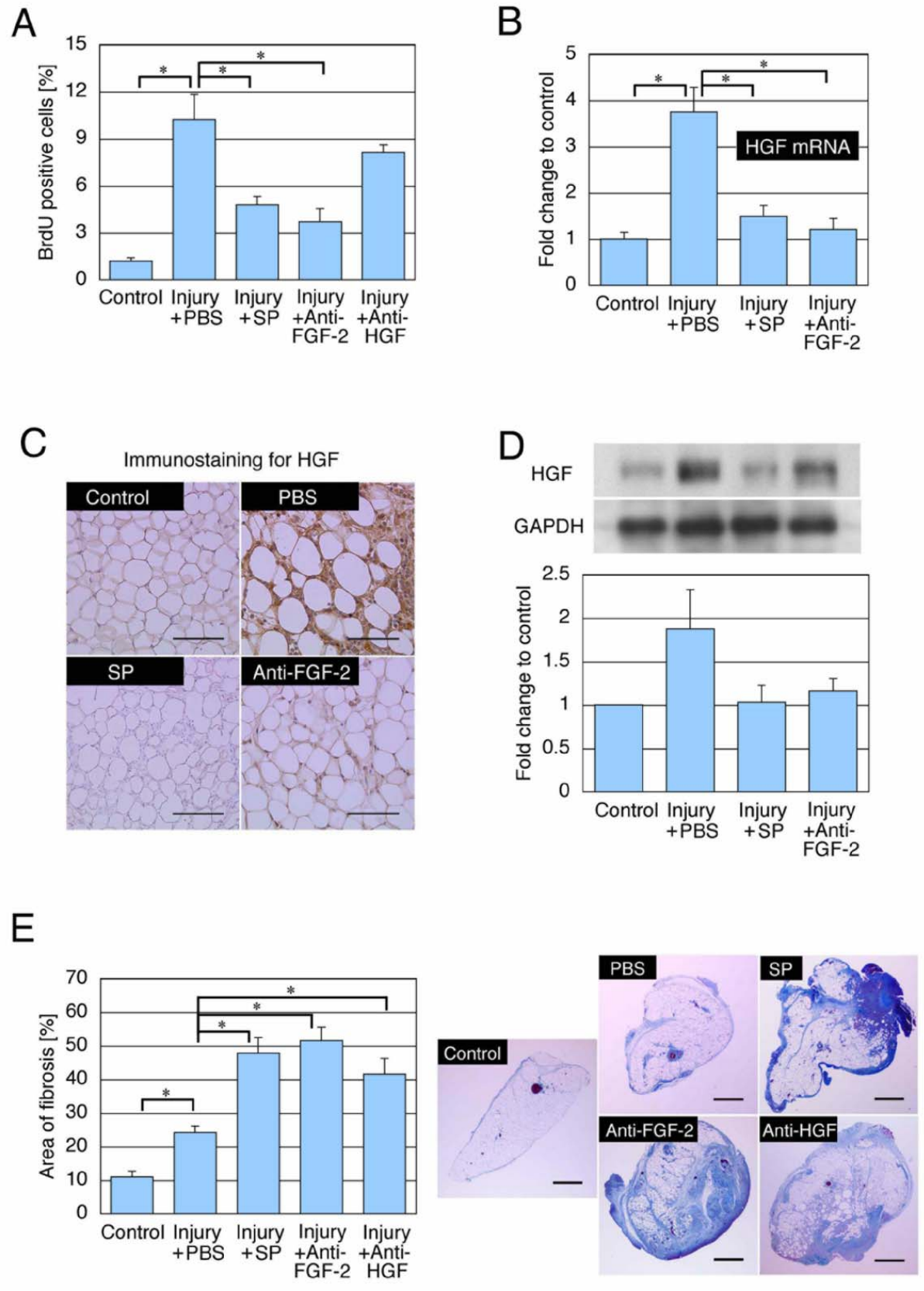


Figure 7. Influences of signal inhibition after ischemia-reperfusion injury to adipose tissue.

(A) Proliferating cell counts according to immunohistology on day 3. The injury-induced increase in BrdU-positive proliferating cells was significantly inhibited by a JNK inhibitor (SP) or an anti-FGF-2 antibody ($n = 4$, $*p < 0.05$), but not by an anti-HGF antibody. **(B)** HGF mRNA expression by adipose tissue on day 3. Continuous administration of SP or anti-FGF-2 antibody prevented upregulation of HGF mRNA on day 3 ($n = 5$, $*p < 0.05$). **(C)** Immunostaining for HGF on day 7. Continuous administration of SP or anti-FGF-2 antibody inhibited HGF secretion in the injured adipose tissue on day 7. Scale bars = 100 μm. **(D)** Western blot analysis for HGF on day 7. Treatment with SP and anti-FGF-2 antibody inhibited the expression of HGF protein. **(E)** Fibrogenesis in the injured adipose tissue at 2 weeks after injury. Fibrotic area was stained with Azan staining. Scale bar = 1 mm. Ischemia-reperfusion injury induced significant fibrogenesis; the area of fibrosis was twice as large as that in the uninjured animal. Continuous administration of SP, anti-FGF-2, or anti-HGF antibody significantly increased fibrosis ($n = 6$, $*p < 0.05$).



IFATS Series: FGF-2-induced HGF Secretion By Adipose-Derived Stromal Cells Inhibits Post-Injury Fibrogenesis Through A JNK-Dependent Mechanism

Hirotaka Suga, Hitomi Eto, Tomokuni Shigeura, Keita Inoue, Noriyuki Aoi, Harunosuke Kato, Satoshi Nishimura, Ichiro Manabe, Koichi Gonda and Kotaro Yoshimura

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